

4 DEVELOPMENT OF A LATEX SLIDE  
AGGLUTINATION TEST AND ITS USE FOR  
SERODIAGNOSIS OF HEARTWATER  
( *Cowdria ruminantium* infection ) IN SMALL

RUMINANTS

BY

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A THESIS SUBMITTED IN PARTIAL FULFILMENT FOR THE DEGREE OF  
MASTER OF SCIENCE IN VETERINARY PATHOLOGY AND  
MICROBIOLOGY

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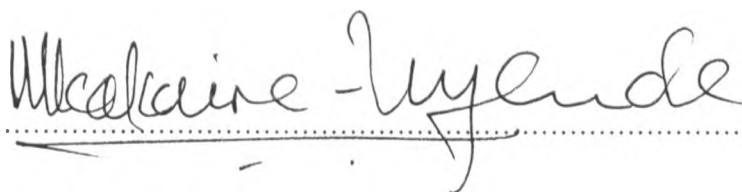
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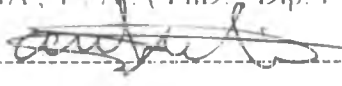
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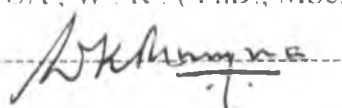
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## DEDICATION

Dedicated to my parents, Mr. and Mrs. Z . K . Nyende, and my brother, Kakaire Tampa, for their unwavering support in so many ways .

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## LIST OF ABBREVIATIONS

- $\mu$  - Micron.  
 $\mu\text{m}$  - Millimicron.  
iu - international units.  
 $\mu\text{g}$  - Microgram.  
mg - Milligrams.  
 $\mu\text{l}$  - Microlitre.  
ml - Millilitre.  
mM - Millimole.  
g - Gravitational force .  
 $\text{cm}^2$  - Square centimetre.  
 $^{\circ}\text{C}$  - Degrees centigrade.  
BAR - Bovine abortion rickettsia.  
BPA - Bovine palmonary artery endothelial cells.  
CPE - Cytopathic effect.  
 $\text{CO}_2$  - Caborn dioxide.  
DAB - 3,3,-diaminobenzidine.  
DNA - Deoxyribonuclic acid.  
EBs - Elementary bodies.  
ELISA - Enzyme linked immunosorbent serum assay.  
cELISA - Competitive Enzyme linked immunosorbent assay.  
FBS - Fetal bovine serum.  
FCA - Freund's complete adjuvant.  
FIA - Freund's incomplete adjuvant.  
GMEM - Glasgow modified essential medium.  
HCl - Hydrochloric acid .  
H & E - Haematoxylin and Eosin stain.  
IFAT - Indirect fluorescent antibody test.  
kDa - Kilodalton.

MAb - Monoclonal antibodies.

MAP2 - Major antigenic protein 2.

aMAP2 - Rabbit anti-MAP2 serum.

mins. - Minutes.

NaCl - Sodium chloride.

PBS - Phosphate buffered saline.

RNA - Ribonucleic acid.

SDS-PAGE - Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Temp. - Temperature.

TPB - Tryptose phosphate broth.

## ABSTRACT

Heartwater is a febrile tick-borne disease of domestic and wild ruminants. The serological tests, for example, IFAT, ELISA and nucleic acid based tests which are currently used for diagnosis of heartwater require sophisticated laboratory equipment and trained personnel to be performed and are therefore not suitable for routine field use. This means that there is need for a simple rapid and specific test that can be used in the field for routine diagnosis of heartwater.

The possibility of detecting *C. ruminantium* antibodies and antigens circulating in sera and plasma of experimentally infected goats, using latex beads coated with crude *Cowdria* antigen (latex slide agglutination antibody test) and latex beads coated with anti-MAP2 immunoglobulin fraction (latex slide agglutination antigen test) was investigated. The crude *Cowdria* antigen was prepared from *C. ruminantium* organisms cultivated in bovine pulmonary artery endothelial cells (BPA). The anti-MAP2 immunoglobulin fraction was precipitated by use of 50% saturated ammonium sulphate solution from sera obtained from rabbits which had been immunised with recombinant *Cowdria* MAP2 antigen. Sera and plasma collected from experimentally infected goats were tested for *Cowdria* antibodies and antigen respectively. Four sera from goats that suffered an acute and fatal heartwater disease tested negative for *Cowdria* antibodies, using latex agglutination antibody test based on whole *Cowdria* antigen, while 2 sera from goats that suffered a non-acute and non-fatal disease started testing positive for *Cowdria* antibodies during the 6<sup>th</sup> week after inoculation. In addition latex beads were coated with recombinant *Cowdria* MAP2 antigen and used to test 81 random field goat serum samples for *Cowdria* antibodies. Of the 81 random field goat serum samples tested, 19 (23.4 %) were positive, 11 (13.6 %) weak positive (border line) and 51 were negative. It was not possible to detect *Cowdria* antigen in plasma of the infected goats with the Latex slide agglutination antigen test. The latex slide agglutination antigen test failed to detect *Cowdria* antigen in the plasma of the sick goats probably because of either low rickettsemia, low levels of MAP2 from lysed elementary bodies (EBs) in circulation, coating of circulating EBs with host proteins or

the Latex slide agglutination antigen test was not sensitive enough. In addition, failure to detect elementary bodies could be attributed to the fact that MAP2 is an internal and not an integral membrane protein as was demonstrated by the phase partitioning of MAP2 in the aqueous phase. Therefore, MAP2 in the intact elementary bodies was inaccessible to the monospecific rabbit anti-MAP2 antibodies. To test this hypothesis, intact and solubilized elementary bodies were examined by use of latex beads coated with rabbit anti-MAP2 antibodies. A weak agglutination reaction was observed with the solubilized elementary bodies and no agglutination was observed with the intact elementary bodies, thus indicating that solubilization of elementary bodies exposed epitopes recognised by the rabbit anti-MAP2 antibodies.

In the present study, sensitivity and specificity parameters of the latex slide agglutination antibody test were not investigated, but it was demonstrated that the latex slide agglutination antibody test is a potential diagnostic tool for detection of *C. ruminantium* antibodies in sera of animals that suffer a non-acute and non-fatal heartwater disease. However, there is need to identify a specific *Cowdria* surface protein to which antibodies can be raised and coated on latex beads to be able to detect *Cowdria* antigen in the plasma of sick animals.

## 1. INTRODUCTION

Heartwater is a tick-borne rickettsial disease of domestic and wild ruminants caused by *Cowdria ruminantium*. The disease is characterised by fever, nervous signs, excessive amounts of fluid in various body cavities and infrequently diarrhoea. It is an important disease throughout most of the sub-saharan Africa and islands off Africa, Guadeloupe in the Caribbean and possibly the southern part of the Arabian peninsula (Uilenberg, 1983; Provost and Bezuidenhout, 1987).

Tentative diagnosis of heartwater can readily be made on the basis of clinical signs. The disease can be confirmed at necropsy by detection of the organisms in histological sections of the cerebral or cerebellar cortex tissues (Purchase, 1945). Unfortunately, heartwater commonly presents itself as a febrile condition which can easily be confused with other diseases such as the nervous form of malignant catarrhal fever, cerebral babesiosis, meningitis, and encephalitis due to bacterial infections (Njenga and Mugera, 1989). Animals which have developed nervous signs stand limited chances of survival, in spite of proper treatment.

The serological tests so far available for the diagnosis of heartwater include the Indirect fluorescent antibody (IFAT) test and the Enzyme-linked immunosorbent assay (ELISA). These tests require sophisticated laboratory equipment and well trained personnel and hence are not suitable for routine use in the field. Instead they are more of research tools for epidemiological studies and disease surveillance.

Other diagnostic methods which include subinoculation of infected blood into susceptible animals, use of electro-encephalography, and / or microscopic examination of brain biopsy tissue (Van Amstel, 1987) are also not suitable for use in the field.

This means that there is need for a simple, specific and rapid serological test for demonstrating *Cowdria* organisms and therefore confirmation of diagnosis of heartwater in the sick animal (Uilenberg, 1983; Jongejan, 1991b). Availability of such a test that can detect antibodies to *C. ruminantium* or *Cowdria* antigens in whole blood of suspected cases would make it possible to differentiate heartwater from other febrile conditions early enough, so that correct and prompt treatment can be given.

Since the infection can be transmitted mechanically by inoculating infected blood into a susceptible animal (Camus and Barre, 1987a), this suggests that the causative agent is present in blood. On this basis, it appears that a test to detect the organisms in blood is feasible (Uilenberg, 1983).

*C. ruminantium* can be propagated *in vitro* (Bezuidenhout, Paterson and Barnad, 1985, Bezuidenhout, 1988); the extracellular elementary bodies of *Cowdria* can be harvested from endothelial cell culture supernatant (Yunker, Byrom and Semu, 1988; Martinez, Swinkels, Camus, and Jongejan, 1990). The organisms can be purified (Neitz and Vermeulen, 1987), and used as *Cowdria* antigen for serological assays (Yunker, *et al.* 1988 ; Martinez , *et al.*,1990). Buscher, Draelants, Magnu, Vervoot and Van Meirveune (1991) used latex beads coated with Trypanosome antigen and detected antibodies in sera of human patients suffering from African trypanosomiasis (sleeping sickness), and Nantulya (1994), using latex beads coated with monoclonal antibodies against *Trypanosoma evansi*, detected *T. evansi* antigen in sera of infected camels . Theoretically, it is possible to coat latex beads with either *Cowdria* antigen or antibody against *Cowdria*. The coated latex beads agglutinate in the presence of specific antibody or antigen, respectively. Such an agglutination test, when done on a slide, is rapid and can be performed using whole blood , plasma or serum (Tizard, 1987). It is therefore hypothesized that latex agglutination test may be a potential diagnostic test for rapid detection of *C. ruminantium* antibody or antigen in blood of experimentally infected animals.

## 1.1. OBJECTIVES OF THE STUDY

The objectives of this study were:

1. To detect *C. ruminantium* antibodies in serum of experimentally infected goats using the latex agglutination antibody test, based on whole *C. ruminantium* antigen.
2. To detect *C. ruminantium* antigens in plasma of experimentally infected goats using the latex agglutination antigen test, based on antibodies raised against a purified antigen.

3. To study the pathology of heartwater in goats experimentally infected with *C. ruminantium* and hence assess the reliability of the latex agglutination test in diagnosis of heartwater.

## 2 LITERATURE REVIEW

### 2.1 DEFINITION OF THE DISEASE

Heartwater is a tick-borne rickettsial disease of domestic and wild ruminants caused by *C. ruminantium* and is characterised by fever, nervous signs, excessive fluid in various body cavities (Alexander, 1931; Neitz, 1968) and occasionally diarrhoea.

### 2.2 AETIOLOGY

The causative agent of heartwater is a rickettsia, belonging to Order Rickettsiales, Family Rickettsiaceae, Tribe Ehrlichieae, Genus *Cowdria* and Species *Cowdria ruminantium* (After Ristic and Huxsoll, 1984).

In animal and tick tissues the organisms usually occur in coccoid and ellipsoidal forms, and occasionally in short bacillary forms. Irregular pleomorphic forms also occur sometimes in densely packed masses. In the vascular endothelial cells of animals, the cocci measure 0.2-0.5  $\mu\text{m}$  in diameter while in tick tissues they measure 0.2-0.3  $\mu\text{m}$ . Bacillary forms are 0.2-0.3 by 0.4-0.5  $\mu\text{m}$  and pairs are 0.2 by 0.8  $\mu\text{m}$ . The organisms stain dark blue with Giemsa stain, they can also be stained with methylene blue and other basic aniline dyes (Ristic and Huxsoll, 1984; Prozesky, 1987a).

#### 2.2.1. Life cycle

Adequate information is not available to completely describe the life cycle of *C. ruminantium*. Prozesky and Du Plessis (1987) suggested the following preliminary life cycle:

*C. ruminantium* is transmitted trans-stadially by certain *Amblyomma* tick species and trans-ovarial transmission only occurs infrequently (Bezuidenhout and Jacobsz, 1986). It appears that organisms initially develop in the gut epithelial cells and the subsequent stages invade and develop in the salivary gland acini cells of the vector. The development of the infective stages of the organisms seems to be co-ordinated with the



feeding cycle of the tick. The vertebrate host is infected when the infected tick feeds on it (Kocan, Bezuidenhout and Alet, 1987).

In the vertebrate host the spread of organisms from the site of infection to the rest of the body is poorly understood. Du Plessis (1970, 1975a) proposed that following infection, initial development of the organisms appears to be mainly, but not exclusively, confined to the reticulo-endothelial cells. Apparently the parasitized reticulo-endothelial cells eventually rupture and the organisms are released into the general circulation to invade the vascular endothelial cells (Du Plessis, 1975b).

Depending on the host and strain of the organism, the organisms appear to have a predilection for vascular endothelial cells in certain organs. In ruminants, the highest concentration of organisms is found in the brain, followed by kidneys, whereas in mice infected with the Welgevonden strain the highest concentration is in the lungs (Prozesky and Du Plessis, 1985b).

The role that leukocytes play in the life cycle of *C. ruminantium* is poorly understood. It has been postulated that in naturally infected animals, infective organisms are drained from the site of infection by the afferent lymphatics (Du Plessis, 1975a) or phagocytosed by leukocytes. Parasitized leukocytes are either drained by the afferent lymphatics to the regional lymph nodes or directly into the general circulation. After multiplication in the leukocytes, organisms are released into the general circulation where they parasitize the vascular endothelial cells.

Infected animals serve as a source of infection for ticks. Alexander (1931) reported that blood from sheep was infective 24 hours before the onset of the febrile response, and remained infective for up to 60 days. According to Oberem and Bezuidenhout (1987), certain animals such as the crowned guinea fowl (*Numida meleagris*) and leopard tortoise (*Geochelone pardalis*) can serve as subclinical carriers of *C. ruminantium* and act as a source of infection for ticks.

### 2.2.2. Cultivation

*In vitro* cultured *Cowdria* organisms or infected cells have been used as antigen in serological tests such as the direct and indirect fluorescent antibody test (Bezuidenhout *et al.*, 1985; Yunker *et al.*, 1988; Martinez *et al.* 1990), in an enzyme linked immunosorbent assay (ELISA) (Viljoen, Vermeulen, Oberem, Prozesky, Verschoor, Bezuidenhout, Putterill, Visser and Neitz, 1985) and in the production of monoclonal antibodies used in the competitive ELISA (cELISA) (Jongejan, Theilemans, Van Kooten, De Groot and Van der Zeiojst, 1991c). The first successful *in vitro* cultivation of *Cowdria* was in bovine umbilical cord endothelial cells (E5) (Bezuidenhout *et al.*, 1985). Yunker *et al.*, (1988) tested eleven cell lines other than E5 for their susceptibility to *Cowdria*. Three of the cell lines established from bovine arterial endothelium following the conditions established by Hirumi and Hirumi (1984) supported the growth of *Cowdria* on a continual basis. Bovine umbilical endothelial cells (BUE) (Van de Wiel, Pieters, Van de Pijl and Bloksman, 1989) were used by a group of workers in Utrecht during studies on *Cowdria* in culture (Jongejan, 1990; Jongejan, Zandbergen, Van de Wiel, De Groot and Uilenberg, 1991b). An ovine endothelial cell line (SBE) was recently established at the Veterinary Research Institute (VRI), Onderstepoort (Philips, Kumar Pat, Kumar and Waghe, 1979). Another ovine cell line, isolated from pulmonary artery endothelium, was found to be susceptible to *Cowdria* ( Byrom, Yunker, Donovan and Smith, 1991). The Glasgow modification of Eagle's minimum essential medium (GMEM, Gibco version containing L-Glutamine 292 µg per ml) produced at VRI Onderstepoort , with the addition of tryptose phosphate broth (TPB), bovine serum and antibiotics, was used during the first successful *Cowdria* cultivation experiments. The medium is still in use with excellent results in yielding of highly infected *Cowdria* cultures (Bezuidenhout *et al.*,1985; Bezuidenhout, 1987). The GMEM (commercially available) supplemented with TPB, L-Glutamine, foetal calf serum (FCS) at 10% (sometimes even as low as 3%) and antibiotics, is used widely (Yunker *et al.*, 1988; Martinez *et al.*,1990; Jongejan *et al.*, 1991b). Byrom and Yunker (1990) obtained better results by using Leibovitz L-15

medium supplemented with 0.45% glucose, 5% foetal bovine serum, 292 µg per ml L-Glutamine and antibiotics. The pH of GMEM culture medium is adjusted to 6.0-6.5 by gassing with CO<sub>2</sub> and Leibovitz L-15 by adding HCl.

Irradiation and cyclohexamide were used in early culture studies to inhibit the growth of cells in order to facilitate initial infection with *Cowdria* (Bezuidenhout *et al.*, 1985; Yunker *et al.*, 1988). It is now generally accepted that the irradiation of cells is not a prerequisite for successful initial infection of cultures (Bezuidenhout, 1987, 1988; Byrom and Yunker, 1990; Jongejan *et al.*, 1991b). Very little or no success has been reported after treatment of cultures with cyclohexamide (Bezuidenhout, 1987; Yunker *et al.*, 1988). A very simple and practical method to facilitate closer contact between the cells and inoculum is to place the *Cowdria* cultures on a slow rocking platform (2-4 rocks per min.) or, for mass production, to use a roller bottle system (Brett, S. unpublished results, 1997). Cultures are incubated at 37°C.

Sheep or goat blood collected in heparin (50 units per ml) during the febrile reaction is mostly used for the inoculation of cultures (Bezuidenhout *et al.*, 1985; Yunker *et al.*, 1988; Martinez *et al.*, 1990; Jongejan *et al.*, 1991b). It appears that fresh blood gives better results than frozen blood. A variety of blood components such as leukocytes, neutrophils and monocytes have been used as inocula without success (Byrom and Yunker, 1990; Byrom *et al.*, 1991) but blood plasma gives better results. In case of mice infected with murinotropic stocks, pooled samples of infected organs such as spleen, liver, heart, kidney and blood have been used successfully as inocula, and lungs alone have been used (Brett, S., unpublished results, 1997). Inocula of tick stabilates prepared from *C. ruminantium* infected ticks have also been successfully cultivated *in vitro* (Bezuidenhout *et al.*, 1985; Bezuidenhout, 1987). Due to their toxicity to the cultured cells and the fact that they often contain bacterial contamination, tick stabilates are, however, not regarded as a very effective inoculum. After *C. ruminantium* is taken up by endothelial cells in culture, it divides by binary fission within intracytoplasmic vacuoles, forming colonies of reticulate bodies. After

three to four days in culture, the reticulate bodies develop into smaller intermediate bodies characterised by an electron-dense core. The intermediate bodies condense further into electron-dense elementary bodies which rupture from the cells and are released into the culture medium. From here they invade other endothelial cells thus initiating a new infectious cycle which lasts between four and six days (Jongejan *et al.*, 1991b).

Reports of detecting *Cowdria* colonies only 11-14 days after inoculation (Bezuidenhout, 1987; Yunker *et al.*, 1988) were probably due to low initial infection rate allowing detection only after the organisms had multiplied through one or more cycles (Bezuidenhout and Brett, 1991). After gaining more experience and information on the *in vitro* cultivation of *Cowdria*, colonies are now detectable as early as two days after inoculation (Byrom *et al.*, 1991; Jongejan *et al.*, 1991b; Brett, S., unpublished observation, 1997). Depending on the level of infection of the cultures, a cytopathic effect (CPE) is observed usually from day 5 after infection, but sometimes only after 11-14 days or even later. Cytopathic effect is especially noticeable if the cultures are kept in constant movement on a rocker or in roller bottles. Under such conditions CPE is seen as clear streaks in the monolayer where affected cells are in the process of detaching or already detached (Yunker *et al.*, 1988; Byrom *et al.*, 1991; Brett, S., unpublished observation, 1997).

### 2.2.3. Other properties

*Cowdria ruminantium* is gram-negative and non motile. It has never been cultivated in cell-free media (Ristic and Huxsoll, 1984). The organisms persist in recovered cattle, sheep and goats for up to 60 days. Microscopic evidence suggests that the organisms persist up to 90 days as inapparent infection in laboratory mice and rats, but cannot be serially passaged in them (Ristic and Huxsoll, 1984).

Two methods are useful for preservation of the infectious organisms .

- (i) preservation of whole blood collected from animals during acute infection mixed with 10 % dimethyl sulfoxide (DMSO) and stored in liquid nitrogen;

- (ii) tissue homogenates from infected ticks mixed with 10 % DMSO and stored in liquid nitrogen (Ristic and Huxsoll, 1984).

### 2.3. EPIDEMIOLOGY

Heartwater affects imported breeds of cattle, sheep, and goats in southern Africa and is regarded as one of the most important tick-borne disease in the region (Bezuidenhout, 1985). It is also found in many wild ruminants many of which die of the disease, while others become asymptomatic carriers. Young animals have an innate resistance which is not due to passive immunity derived from their dams. Goats are more susceptible than cattle (Gruss, 1983).

The disease is limited in its occurrence to Africa, Madagascar, Guadeloupe in the Caribbean and some islands of the West Indies (Uilenberg, 1983; Birnie, Burrige, Camus and Barre, 1985). It is transmitted by many ticks of the genus *Amblyomma*, especially *A. variegatum* (Uilenberg, 1983). Because of the large number of *Amblyomma* species that are capable of acting as vectors, it is feared that the disease will spread much further than it has (Uilenberg, 1983). In the tick vectors, the infection is transmitted trans-stadially. The most serious losses caused by heartwater are in exotic susceptible cattle, sheep and goats that are imported into areas where the disease is enzootic and at times when the vector population is high (Blood and Radostits, 1989).

### 2.4 PATHOGENESIS

The pathogenesis of heartwater is one of several of the very important aspects of the disease that is poorly understood (Du Plessis, Malan and Kowlski, 1987a). There seems to be general agreement that increased permeability of smaller blood vessels plays an important role in the pathogenesis of the disease (Clark, 1962; Owen, Littlejohn, Johanna and Erasmus, 1973; Du Plessis, 1975a; Prozesky and Du Plessis, 1984). Opinions differ, however, on how the increased permeability is caused. Most researchers ascribe the vascular defect to a toxin (Steck, 1928; Piennar, Basson and

Van der Merwe, 1966; Neitz, 1968; Piennar, 1970; Ilemobade, 1976), but such a toxin has not been demonstrated and no experimental evidence supports this theory. Although vascular lesions in the brain were at first ascribed to the direct necrotizing effect of the heartwater agent (Piennar *et al.*, 1966), Piennar (1970) subsequently conceded that cytopathic effects were rarely seen in brain endothelial cells that were themselves parasitized by *C. ruminantium* and consequently favoured the toxin hypothesis. The absence of a direct link between the presence of colonies of the heartwater agent and cytopathic changes of the cells harbouring them was later confirmed by Prozesky and Du Plessis (1985b).

Another hypothesis is that vasoactive substances liberated by mast cells are responsible for the increased permeability (Du Plessis, 1975a). Further observations suggest that the mast cells become degranulated upon contact with the heartwater agent and the degranulation is not necessarily IgE antibody mediated. Preliminary findings suggest that a hypersensitive type of reaction triggered by the release of pharmacologically active substances may possibly be basic to the pathogenesis of heartwater. Complement and the products of arachidonic acid metabolism possibly play a role in the release of the vasoactive substances (Du Plessis *et al.*, 1987a). As a result of increased permeability of smaller blood vessels there is (i) oedema of the lungs, hence the signs of respiratory distress seen in heartwater (Alexander, 1931, Neitz, 1968; Van de Pypekamp and Prozesky, 1987); (ii) oedema of the brain, hence the nervous signs; and (iii) effusion into body cavities, leading to hydrothorax and hydropericardium (Clark, 1962; Owen *et al.*, 1973, Du Plessis, 1975a; Prozesky and Du Plessis, 1984). Petechial haemorrhages on mucous membranes and serosal surfaces plus lesions observed in the kidneys are due to damage of blood capillaries (Uilenberg, 1983; Prozesky, 1987b).

## 2.5. CLINICAL SIGNS

Clinical signs of heartwater differ slightly in cattle, sheep and goats (Van de Pypekamp and Prozesky, 1987). In experimental infection the incubation period is

influenced by species of the animal, route of inoculation, virulence of the organism and the dose (Neitz, 1968; Uilenberg, 1983). In cattle, fever develops 12 days after intravenous inoculation (Van der Merwe, 1979) and 18 days after natural infection (Alexander, 1931). In sheep and goats it develops within 9-10 days after intravenous inoculation and 14 days following natural infection (Alexander, 1931; Uilenberg, 1983). The course of the disease may vary from peracute to mild (Neitz, 1968; Uilenberg, 1983). Peracute disease is mainly seen in exotic breeds of cattle. Pyrexia develops suddenly and animals die within a few hours without overt clinical signs; or may show paroxysmal convulsions and death follows within 36-48 hours. There is respiratory distress due to lung oedema (Alexander, 1931; Neitz, 1968). The acute disease is the most common in endemic areas, affecting mainly cattle of 3-18 months of age. There is fever (40°C or more) which remains high, then drops subnormally shortly before death. In some animals, the fever may persist for upto 9 days without other clinical signs (Alexander, 1931; Uilenberg, 1983). Anorexia then follows and petechiae are visible on the mucous membranes of the conjunctiva in most animals (Van de Pypekamp and Prozesky, 1987). Nervous signs vary from mild incoordination to pronounced convulsions (Alexander, 1931). There is hypersensitivity to handling, sudden noise and bright light, some animals remain standing for long while others push against objects. Often there is high stepping gait. Animals may suddenly fall in lateral recumbency, with opisthotonus and legs extended or paddling. There is weakness and death follows soon after convulsive attacks (Van de Pypekamp and Prozesky, 1987).

The mild form of the disease is difficult to detect because it is asymptomatic. It develops in calves under three weeks of age (Uilenberg, 1981), animals infected with organisms of low virulence (Neitz, 1968) and immune animals that are reinfected (Alexander, 1931). The affected animals appear normal, although they have a fever, apathy and slight tachypnoea, and most cases recover within a few days (Camus and Barre, 1982). In small ruminants, the peracute disease is more common in goats than sheep. Most animals collapse and die after a few paroxysmal convulsions (Uilenberg,

1983). Sometimes animals appear dull and inappetent before death (Karrar, 1960), while others retain their appetite until they die (Njenga, 1987). In goats there is bleating, continuous twitching of the tail, respiratory distress, frequent micturation and defecation, nystagmus, and there may be paddling and chewing movements (Van de Pypekamp and Prozesky, 1987). Sheep mostly suffer the acute form of the disease. Affected sheep show nervous signs which are less pronounced than in cattle and goats (Alexander, 1931). Initially there is a progressive unsteady gait, and often victims stand with their legs apart, head down, ears drooping, and appear restless. They eventually go down in lateral recumbency and show continuous galloping and chewing movements, licking of the lips and nystagmus (Alexander, 1931). Sheep and goats with high natural resistance suffer the mild form of the disease. Apart from fever, animals are asymptomatic (Alexander, 1931; Uilenberg, 1983).

#### **2.5.1. Complications of heartwater**

Non-fatal cases may develop complications, recumbent animals develop hypostatic pneumonia and ruminal stasis. A few animals become blind, sheep may shed their fleece, calves and kids may develop torticollis (Alexander, 1931; Karrar, 1960; Van der Merwe, 1979; Van de Pypekamp and Prozesky, 1987).

#### **2.6. CLINICAL PATHOLOGY**

The main clinical pathological changes measured include a progressive anaemia, fluctuations in total and differential white cell count, of which an eosinopenia and a lymphocytosis are most marked. Increases in total bilirubin which coincide with darkening of plasma colour, and a drop in total serum proteins mostly shown in the drop in albumin levels (Van Amstel *et al.*, 1987).



## 2.7. NECROPSY FINDINGS

### 2.7.1. Gross lesions

In cattle, sheep and goats gross lesions are fairly similar, although quite variable in extent and some changes are more common in certain species than in others (Steck, 1928; Uilenberg, 1981). Fluid accumulation in body cavities is a very common change in most fatal cases of heartwater. The transparent or slightly turbid light yellow fluid observed in body cavities often coagulates on exposure to air. Hydrothorax may amount to several litres in bovines, about half a litre in sheep, and rarely exceeds 20 ml in goats (Steck, 1928). Hydropericardium is a striking feature in most animals that die of the disease and is usually more pronounced in sheep and goats than in cattle (Henning, 1956). Oedema of the lungs is a regular finding in animals infected with heartwater and appears to be more severe in most animals that die of the peracute form of the disease (Van de Pypekamp and Prozesky, 1987). The interlobular septae of the lungs, mediastinum and associated lymph nodes are oedematous; and serous frothy fluid oozes from the cut surface of the lung. The trachea and bronchi are often congested and contain a serofibrinous exudate. Splenomegaly is present in the majority of animals. In sheep and goats the spleen is often only slightly enlarged (Uilenberg, 1971; Andreasen, 1974; Ilemobade, 1976). The cut surface is dark red in colour and has a pulpy consistency. Splenomegaly, epicardial and endocardial haemorrhages are sometimes the only significant changes observable in animals that die of the peracute form of the disease (Alexander, 1931).

In some animals, the kidneys are markedly swollen and pale, and there is edema of the perirenal tissue (Steck, 1928; Prozesky and Du Plessis, 1985a). Congestion and edema of the mucosa of the abomasum are regularly seen in cattle but are less common in sheep and goats. Enterorrhagia (affecting the small and large intestine) is present in a small percentage of domestic ruminants. The lymph nodes are moderately swollen in most animals. The cut surface is moist and petechiae are often present especially in the retropharyngeal, submaxillary, cervical, bronchial and mediastinal lymph nodes

(Alexander, 1931). Petechiae are frequently visible on mucus membranes of tissues including those of the urinary bladder, vagina and the conjunctiva (Uilenberg, 1983; Prozesky, 1987b).

There is cerebral edema (Van de Pypekamp and Prozesky, 1987). The gyri of the cerebrum may be strikingly swollen and severe oedema of the brain may even result in a partial prolapse of the cerebellum through the foramen magnum. Most animals that die of heartwater show congestion of the meninges, accumulation of excessive fluid in the subarachnoid space and thickening of the choroid plexus. In some animals petechiae, ecchymoses and sometimes sanguinations are evident in the mid brain, brain stem and cerebellum (Piennar *et al.*, 1966). Hepatic lesions in animals with heartwater are not striking. Apart from a mild hepatomegaly the gall bladder is slightly distended in most animals (Prozesky, 1987b).

### 2.7.2. Histopathology

Comprehensive studies on the histopathological changes of heartwater have been carried out (Steck, 1928; Alexander, 1931; Piennar *et al.*, 1966); but the changes in most organs are not striking (Steck, 1928; Alexander, 1931).

#### **Lungs:**

An alveolar and interstitial edema occurs in most animals but is not always discernible histopathologically because the fluid in the alveolar spaces is washed out during the routine processing of the tissue.

#### **Kidneys:**

Nephrosis is a common change in domestic ruminants that die of heartwater (Steck, 1928). *Cowdria* organisms are frequently observed in endothelial cells of glomerular capillaries (Cowdry, 1926; Steck, 1928). In Angora goats, the proximal and distal convoluted tubules and collecting ducts are dilated. Epithelial cells of non-dilated tubules are swollen and many of the tubules contain hyaline, granular and cellular casts. The Bowman's capsule spaces are dilated and contain variable amounts of eosinophilic

material. This irreversible kidney damage is most probably the cause of death (Prozesky and Du Plessis, 1985a).

**Brain :**

Microscopic lesions in the brain of cattle, sheep and goats naturally and experimentally infected with *C. ruminantium* have been described by Piennar *et al.*, (1966). Swollen astrocytes which are often necrotic are present in most bovines and in some sheep and goats. Other lesions include swollen axons, multifocal microcavitations and haemorrhages affecting mainly the mid brain, brain stem, cerebral white matter and cerebral peducles. Perivascular infiltration with cells (mainly macrophages and a few neutrophils) and occasionally vasculitis are observed in all bovines and in some sheep. Diffuse meningitis (mainly with macrophage infiltration) is present in a few bovine cases. Brain lesions in recumbent animals often comprise different degrees of status spongiosus and in severe cases the white matter of the entire brain may be affected (Van de Pypekamp and Prozesky, 1987).

**Other organs :**

In most animals that die of heartwater, the hepatic changes are inconspicuous; the lymph nodes are congested and oedematous, and congestion is the only splenic change.

## 2 8. DIAGNOSIS

Diagnosis of heartwater can be based on epidemiology (Yunker, 1991), clinical signs (Camus and Barre, 1987a), subinoculation of blood or homogenised ticks collected from suspected animals into susceptible sheep or goat (Camus and Barre, 1987a), stained brain biopsies (Synge, 1978) and serology (Camus and Barre, 1987b). Yunker (1990) described a microscopical method for diagnosis of heartwater in living animals which uses clear excised membranous ' windows ' of omental tissue. Electro-encephalography has been tried in diagnosing heartwater, but results may be difficult to interpret (Van Amstel, 1987).

Heartwater can further be suspected in the field by observing the pathological changes in the dead animals (Steck, 1928). The lesions seen most frequently are pulmonary edema, hydrothorax and hydropericardium (Logan, 1987); subendocardial haemorrhages are also common (Neitz, 1968; Logan, 1987). Diagnosis of heartwater can be confirmed at histology by demonstration of the causative agent (*C. ruminantium*) in brain squash smears (Purchase, 1945). Fixed or unfixed smears are still suitable for diagnostic purpose for at least one month (Prozesky, 1987c). The causative organisms can also be demonstrated in brain smears from animals in advanced state of putrefaction (Prozesky, 1987c). Demonstration of *C. ruminantium* organisms in histopathological sections has also been used to confirm heartwater (Cowdry, 1925; 1926), but it is often difficult to demonstrate the organisms in histopathological tissue sections (Prozesky, 1987c).

### 2.8.1. Staining methods

It is possible to demonstrate *Cowdria ruminantium* organisms in impression smears of various visceral organs and brain squash smears using Giemsa stain (Cowdry, 1926, Piennar, 1970; Njenga, 1987; Parklea, 1991) Piennar (1970) demonstrated *C. ruminantium* organisms in brain squash smears using May-Grunwald-Giemsa stain. Camus described a rapid method of staining brain smears using acridine orange (*Cowdria* Newsleter, March 1988). Different staining methods have been used to demonstrate *Cowdria* organisms in histopathological tissue sections (Burdin, 1962), but toluidine blue or Giemsa are the most preferred (Prozesky, 1987a) Logan, Whyard, Quintero and Mebus (1987), using Diff Quik stain, Giemsa stain, direct immunofluorescent microscopy and transmission electron microscopy, demonstrated one to several small cocci, large ring forms and rods in neutrophils in blood smears and cytopreparations of neutrophil fractions of experimentally infected goats during the febrile response. Njenga (1987) and Parklea (1991) using Haematoxylin and Eosin (H & E) staining technique demonstrated *C. ruminantium* organisms in kidney, liver, brain and lung sections of experimentally infected goats and mice.

### 2.8.2. Differential diagnosis

In cattle, heartwater has to be differentiated from rabies (Bruckner, Hurter and Boshoff, 1978; Barnard, 1979; Kaplan, Turner and Warrel, 1986), the nervous form of malignant catarrhal fever (Plowright, 1964), cerebral babesiosis (De Vos, 1982), meningitis and encephalitis due to bacterial infections (Jubb, Kennedy and Palmer, 1985). Pesticide poisoning (Terblache and Minne, 1968) and heavy metal poisoning (Clarke and Clarke, 1970) could also cause confusion in diagnosis. In sheep and goats, heartwater may be confused with abscess of the hypophysis, meningo-encephalitis, plant and chemical poisoning (Van de Pypekamp and Prozesky, 1987).

### 2.8.3. Serodiagnosis

Serological assays for detecting antibodies to *Cowdria ruminantium* were, until recently, of limited value because of lack of sufficient quantities of an effective antigen (Jongejan, 1991b). Several tests have been developed based on antigen preparations obtained from infected host tissues. Such tests include :-

- ( i ) Capillary flocculation test (Ilemobade and Blotkamp, 1976) where an antigen extracted by acetone from infected brain was used.
- ( ii ) Complement fixation test (Du Plessis, 1982a; Musisi and Hussein, 1985) where a sucrose acetone extract antigen from infected brain was used.
- ( iii ) Indirect fluorescent antibody (IFAT) test (Du Plessis, 1981) where infected peritoneal mouse macrophages were used as antigen. The Kumm strain which is highly pathogenic for mice was used to infect mice (Du Plessis and Kumm, 1971). The test has been used to monitor infected experimental animals in South Africa (Du Plessis and Malan, 1987) and Guadeloupe (Camus, 1987). The test could also detect colostrum derived antibodies in calves and lambs (Du Plessis, 1982b).
- ( iv ) Enzyme-linked immunosorbent assays (ELISA), using antigen isolated from infected sheep brain or *Amblyomma hebraeum* ticks by wheat germ lectin affinity chromatography (Viljoen *et al.*, 1985; Neitz, Viljoen, Bezuidenhout,

Oberem, Van Wyngaard and Vermeulen, 1986a; 1986c) or Percoll density gradient centrifugation (Neitz *et al.*, 1986b), have been reported. Viljoen, Vermeulen and Neitz (1987) reviewed the theoretical aspects of ELISA and its use in the detection of *C. ruminantium* antigens and antibodies in various heartwater infected hosts.

*Cowdria* antigens have also been produced using short term granulocytic neutrophil cell cultures. Such antigens were used by Logan (1987) to develop an IFAT test. Jongejan, Wassink, Thielemans, Parie and Uilenberg (1989) modified the test slightly and found it suitable for some *Cowdria* isolates; but not all strains could develop equally well in neutrophil cell cultures. The test was therefore inadequate. The other problem associated with the test was the non-specific fluorescence (Jongejan *et al.*, 1989). The non-specific fluorescence could however be absorbed out using acid glycine buffer as described for serodiagnosis of anaplasmosis (Montenegro-James, James and Ristic, 1985). Using the IFAT test Jongejan, Uilenberg, Franssen, Gueye and Nienwenhuijs (1988; 1991d) demonstrated the presence of serotypes within the genus *Cowdria*.

For large scale production of *Cowdria* antigens, infected endothelial cell cultures have been shown to be far superior (Bezuidenhout, 1987). This followed the successful long term *in vitro* propagation of *Cowdria*, first achieved by Bezuidenhout and co-workers (Bezuidenhout *et al.*, 1985). Using antigens generated in culture, Martinez *et al.* (1990) compared three antigens for the serodiagnosis of heartwater by IFAT. Antigens were obtained from infected bovine endothelial cell lines (E 5 cell line), mouse peritoneal macrophages infected with the Kumm isolate, and primary cultures of infected caprine neutrophils. The use of endothelial cell cultures as a source of *Cowdria* antigen proved superior in all respects. However no serotypes within the genus *Cowdria* were detected by IFAT which utilises antigen obtained from endothelial cell cultures (Yunker, 1990). Indirect ELISA using unpurified *Cowdria* antigen harvested from infected endothelial cell cultures does not give satisfactory results due to high non specific background reaction with pre-immune sera

(Thielemans and Jongejan, unpublished observations, 1997). The non specific background reactions associated with crude antigen could be avoided by using monoclonal antibodies (Hewitt, Coates, Mitchison and Ivanyi, 1982). For instance, competitive ELISA mediated by monoclonal antibody gave better results when used for serodiagnosis of *Ehrlichia risticii* (Shankarappa, Dutta, Sanusi and Mattingly, 1989).

*Cowdria* contains an antigenically conserved immunodominant protein of relative molecular weight 32,000 Daltons (Cr 32) (Jongejan and Thielemans, 1989), now called Major Antigenic Protein 1 (MAP1) (Barbet, Semu, Chigagure, Kelly, Jongejan and Mahan, 1994, Van Vliet, Jongejan, Mirinda van Kleef and Ben va der Zeijst, 1994). Monoclonal antibodies (MAb) raised against Cr32 recognized a 40 kDa *Cowdria* protein, as was demonstrated by Western blotting. Four anti Cr32 MAb were found reactive in an IFAT test based on extracellular elementary bodies. Using methods originally designed by Faulk and Taylor (1971) and adapted for *in vitro* labelling of *Chlamydia* elementary bodies by Kuo and Chi (1987); and methods for transmission electron microscopy (Ito and Rikihisa, 1981), it was shown that Cr32 has surface exposed antigenic determinants (Jongejan *et al.*, 1991c). A competitive ELISA based on MAb against Cr32 was developed and detected *Cowdria* specific antibodies in goats, sheep and cattle sera (Jongejan *et al.*, 1991c).

#### 2.8.4. Cross-reactions

Serodiagnostic tests developed for diagnosis of heartwater cross react with *Ehrlichia* and *Chlamydia* species (Du Plessis, Camus, Oberem and Malan, 1987b, Jongejan, 1991b). Indirect fluorescent antibody (IFAT) test based on infected neutrophil cell cultures is limited due to cross-reactions between *Cowdria* and *Ehrlichia* ( Logan, Holland, Mebus and Ristic, 1986; Camus, 1987; Jongejan *et al.*, 1989). Similar cross-reactions have been reported using IFA tests based on infected mouse peritoneal macrophages (Camus, 1987; Du Plessis *et al.*, 1987b). The cross-reactivity is not linked to cross-immunity between the organisms (Jongejan and

Wassink, 1991) *Cowdria* and *Chlamydia* are to a certain extent antigenically related (Jongejan, Bax, Meddens and Quint, 1991a). Molecular phylogenetic analysis of 16s ribosomal DNA sequences has revealed that *Cowdria* is closely related to several species of *Ehrlichia* (Van Vliet, Jongejan and Van der Zeijst, 1992). An obligate intracellular rickettsial organism, Bovine Abortion Rickettsia (BAR), was recently isolated from tissues of an aborted bovine fetus in the USA (Dilbeck, Everman, Crawford, Ward, Holland, Mebus, Logan, Rurangirwa and McGuire, 1990; Kocan, Crawford, Dilbeck, Everman and McGuire, 1990). High cross-reactivity was found between BAR and *Cowdria*. In collaboration with Washington State University and the University of Florida working group in Zimbabwe, the antigenic relationship between BAR and *Cowdria* was re-examined using tests; namely IFAT, competitive ELISA and Western blotting. The results obtained did not confirm the previously reported cross-reactivity between BAR and *Cowdria* (Jongejan, 1991b). The use of recombinant MAPI for serodiagnosis has been investigated, and two immunogenic regions on the MAPI protein, designated MAPI-A and MAPI-B were identified. An indirect ELISA based on MAPI-B was developed and validated using sera from animals experimentally infected with *C. ruminantium* and several *Ehrlichia* species. Cross reactivity with MAPI-B was limited to *E. canis* and *E. chaffensis* (two rickettsias which do not infect domestic ruminants). Antibodies to *Ehrlichia* species which infect domestic ruminants (*E. bovis*, *E. ovina* and *E. phagocytophila*) did not react with MAPI-B. Further validation was done with sera obtained from sheep raised in heartwater-free areas in Zimbabwe and several Caribbean islands. One hundred and fifty nine (159) out of 169 samples which were considered to be false positives by Western immunoblots or indirect ELISA, did not react with MAPI-B. Hence recombinant MAPI-B may be a suitable antigen for a sensitive serological test for cowdriosis, with dramatically improved specificity (Van Vliet, Ben van der Zeijst, Camus, Mahan, Martinez and Jongejan, 1995). Although cross-reaction with other organisms interferes with interpretation of sero-epidemiological surveys of heartwater



(Du Plessis *et al.*, 1987b) and limits the value of these tests, they are still useful tools for research work in the laboratory (Jongejan, 1991b).

#### 2.8.5. Nucleic acid based tests

Ribonucleic acid (RNA) and Deoxyribonucleic acid (DNA) probes have been developed to detect rickettsia and other organisms in animal hosts and arthropod vectors by nucleic acid hybridization (Barker, Suesbsaeng, Rooney, Alecrim, Donardo and Wirth, 1987, Goff, Barbet, Stiller, Palmer, Knowles, Kocan, Gorham and McGuire, 1988; Mclaughlin, Breman, Collins, Schwartz, Bennet, Sulzer, Collins, Skinner, Ruth and Andrysiak, 1987; Shompole, Waghela, Rurangirwa and McGuire, 1989) and have been acclaimed to have high specificity. Mahan, Waghela, Rurangirwa, Yunker, Crawford, Barbet, Burrige and McGuire (1991) identified a DNA probe (pCS20) for the diagnosis of heartwater in ticks and animals. The pCS20 has been derived from DNA of the Crystal Springs heartwater isolate of Zimbabwe (Waghela, Rurangirwa, Mahan, Yunker, Crawford, Burrige and McGuire, 1991). It can be used to detect the parasite in different endemic regions of Africa (Mahan *et al.*, 1991) and has been shown to detect *C. ruminantium* organisms in *A. variegatum*. *Cowdria ruminantium* DNA was detectable in elementary bodies (pelleted down by centrifugation) from plasma of five experimentally infected sheep five days after infection (Mahan *et al.*, 1991). To allow routine use of DNA-based diagnostic tests for *Cowdria* in the field, a non-radioactive detection method using chemiluminescence is being developed (Mahan *et al.*, 1991). Polymerase chain reaction (PCR) has also been used to detect *Cowdria* DNA in *Amblyomma* ticks and *C. ruminantium* in antelopes (*Cowdria* Newsletter vol. 8 No. 2 . Sept. 1994).

#### 2.8.6. Purification of *Cowdria* antigen

*Cowdria* organisms to be used as antigen in serological assays can be separated from heterogenous mixtures of host cells and purified by one of the following methods -

- ( i ) density gradient centrifugation (Neitz, Vermeulen and Viljoen, 1987) .
- ( ii ) lectin cellular affinity chromatography (Vermeulen, Neitz and Viljoen, 1987).
- ( iii ) immunosorbent affinity chromatography (Neitz and Vermeulen, 1987).

### 2.8.7. Latex slide agglutination test

Principle : This is a slide test in which latex particles sensitized with antibody or antigen agglutinate in the presence of specific antigen or antibody respectively (Tizard, 1987; Hudson and Hay, 1991).

The latex slide agglutination test is simple and quick to perform. The serum samples are tested without dilution, thus removing the need for buffers. Moreover, anticoagulated whole blood can be used in the test (Rurangirwa, McGuire, Kibor and Chema, 1987); there by removing the need for centrifugation. The sensitised latex suspension is used without reconstitution and is simple to keep. The sensitised latex beads are preserved in 0.1 % sodium azide and can stay for up to 4 weeks at room temperature, for 8 weeks at 37°C, and for one year at 4°C without significant loss of activity (Nantulya, 1994). These characteristics make the Latex test a suitable test for field routine use .

This test has been employed in the serodiagnosis of a number of diseases; for example, contagious caprine pleuropneumonia (Rurangirwa *et al.*, 1987); human African trypanosomosis (Buscher, Draelants, Magnu, Vervoort and Van Meirveune, 1991) and *Trypanosoma evansi* infections in camels (Nantulya, 1994). Rurangirwa *et al.* (1987) sensitised latex beads with a polysaccharide isolated from an F38 *Mycoplasma* organism strain culture supernatant and used them in a slide agglutination test (latex slide agglutination antibody test) to detect serum antibodies in goats with contagious caprine pleuropneumonia. Using the latex slide agglutination antibody test and the complement fixation test, they tested 181 sera from a farm which was free of the disease and 763 sera from two distant farms with outbreaks of classical contagious caprine pleuropneumonia (CCPP). Both tests were negative with the 181 sera from the disease-free farm; 63 % of the 763 sera from the farms with classical CCPP

outbreaks were positive by the latex slide agglutination antibody test and 23 % were positive by the complement fixation test. These findings suggested that the latex slide agglutination antibody test was specific and more sensitive than the complement fixation test. Nantulya (1994) coated latex beads with monoclonal antibody against a *T. evansi* internal antigen and used them in a slide agglutination test (latex slide agglutination antigen test) to detect circulating invariant trypanosomal antigen in camels with *T. evansi* infection. Using the latex slide agglutination antigen test and the buffy coat technique he tested 30 camels from a *T. evansi*-free herd and 32 camels from a herd with *T. evansi* outbreak. All the 30 camels from the *T. evansi*-free herd were negative for both tests, 30 of the camels from the herd with *T. evansi* outbreak were positive by the latex slide agglutination antigen test and 5 were positive by the buffy coat technique. These findings demonstrated that the latex slide agglutination antigen test was specific and more sensitive than the buffy coat technique. Available literature on serodiagnosis of heartwater (Jongejan, 1991b), however, reveals no parallel test having been used in the serodiagnosis of heartwater.

## 2.9. IMMUNITY

There is ample evidence from both field observations and laboratory results that farm animals develop a specific immune response after recovery from infection with *C. ruminantium* (Alexander, 1931), and after vaccination with *in vitro* attenuated rickettsiae (Jongejan, 1991a). The severity of the heartwater reaction does not have any influence upon the degree of immunity, provided the reaction is produced (Alexander, 1931). The duration of the immunity normally varies from 6 months to 4 years (Stewart, 1987). However, this may not apply to Angora goats. In Angora goats, it is difficult to produce a specific immune response with vaccination, probably due to breed related immunodeficiency (Du Plessis, Jansen and Prozesky, 1983). Knowledge about the mechanisms of immunity to heartwater is limited. T-cell mediated immunity seems to play a key role, although antibodies have been shown to neutralise infectivity *in vitro* (Byrom, Mahan and Barbet, 1993). It is thought that T cells, particularly CD4<sup>+</sup> T cells, produce interferons and other cytokines which are

responsible for limiting the infection (Totte, Blankaert, Zilimwabagabo and Werenne, 1993; Totte, Jongejan, de Gee and Werenne, 1994). Calves of all breeds show an age-linked resistance to infection with *C. ruminantium*. The mechanism of this resistance is unknown, and it is of very short duration (2 to 3 weeks) (Uilenberg and Camus, 1993). In older animals (over 2 to 3 weeks) there are pronounced differences in susceptibility between breeds. In general, indigenous breeds from heartwater-endemic areas show medium to high levels of resistance (Camus and Barre, 1987b; Uilenberg and Camus, 1993). Exotic breeds are highly susceptible and mortality can be as high as 100 %.

## 2.10 TREATMENT AND CONTROL

Tetracyclines are the standard treatment and may be used combined with natural or artificial infection as a prophylactic measure. The dosage rate is 10 mg / kg body weight / day intramuscularly for three days (for short acting tetracycline) or 20 mg / kg once (for long acting tetracycline) (Uilenberg, 1983). Natural exposure of calves to heartwater infection induces immunity and the age of 2-4 weeks is considered to be the optimum natural exposure time (Uilenberg, 1983). Contrived exposure can be effected in several ways, the most common of which is the intravenous injection of heartwater infected blood or ground up infected ticks. Vaccination by these methods is very effective in terms of the protection provided, but is hazardous because of the deaths that may occur in spite of treatment (Bezuidenhout and Spickett, 1985; Simpson, Lindsay, Morris, Muirhead, Prichard, Stanley, Thirlwell, Hunter, Bradley and Windsor, 1987). Its use is usually confined to imported cattle which can be kept under close surveillance and treated intensively.

Long acting tetracyclines administered to calves weekly for 4 weeks when they enter an enzootic zone has been an effective preventive strategy against heartwater (Blood and Radostits, 1989). Control by eradication of the tick population is not usually a practical option but regular dipping of cattle and other livestock at 3-7 day intervals is common practice in enzootic areas and serves to keep the disease in check (Uilenberg, 1983).

### 3. MATERIALS AND METHODS

#### 3.1. Experimental Animals

A herd of goats was identified in Olmagogo, out of which thirty three goats were randomly selected, bled, and screened for the presence of antibodies to *C. ruminantium* using the Competitive Enzyme Linked Immunosorbent Assay (cELISA) test (Shompole, unpublished data 1997). Eleven goats were purchased, housed and treated with long-acting oxytetracyclines to clear them of any *Ehrlichia* species organisms, which cross react with *Cowdria ruminantium*, and kept for a period of 30 days to enable reacting antibodies to wane before the goats were infected. In addition to the above treatment; the goats were dewormed, acaricide was applied on them and daily rectal body temperatures were taken for a period of 30 days to establish the base line body temperature range. Two rabbits were purchased from the Veterinary laboratories, Kabete.

#### 3.2. Experimental Design

The experimental goats were divided into groups A , B , and C each with 4 , 2 and 1 goat respectively. The goats in group A were inoculated with a high dose (10 ml.) of *Cowdria* blood stabilate, those in group B received a low dose (2 ml.) while the goat in group C served as the uninfected control. At the onset of the febrile reaction goats in group A were not treated, but those in group B were treated.

#### 3.3. Infective material

*Cowdria ruminantium* blood stabilate of a Kenyan strain field isolate that had been propagated in goats was obtained from the KARI / SR-CRSP Biotechnology Laboratories, Kabete.

#### 3.4. *Cowdria ruminantium* major antigenic protein 2 ( MAP2 )

Recombinant MAP2 *Cowdria* antigen was obtained from the KARI / SR-CRSP Biotechnology Laboratories, Kabete.

### 3.5. Known positive and negative control sera

Known positive serum from a case of heartwater (*C. ruminantium* infection) in sheep that had been infected and recovered ; and known negative serum from sheep in the USA (where heartwater has not been reported), were obtained from the KARI / SR-CRSP Biotechnology Laboratories, Kabete.

### 3.6. Latex beads

10 % aqueous suspension of latex beads (particle diameter 1.16  $\mu$ ) were purchased from Sigma Chemical Company, USA.

### 3.7. Immunization of rabbits

Antibodies against *Cowdria* MAP2 were raised in two rabbits. Before the prime immunization injection, the rabbits were bled by puncture of the ear vein and preimmune serum was harvested from clotted blood and stored at -20°C. Each rabbit was injected intramuscularly (im) with 50 $\mu$ g of recombinant *Cowdria* MAP2 emulsified in Freund's Complete Adjuvant (FCA). After 14 days the rabbits were bled, serum harvested and stored at -20°C. The rabbits were given booster injections subcutaneously (sc) with an equivalent amount of MAP2 emulsified in Freund's Incomplete Adjuvant (FIA). After 14 days, immune serum was collected again from each rabbit and stored at -20°C. A second booster injection of 25 $\mu$ g MAP2 emulsified in FIA was administered at the same time. After monitoring the immune response each rabbit was given a final injection of 30  $\mu$ g MAP2 emulsified in FIA.

### 3.8. Monitoring of the immune response

The immune response was monitored using a preliminary dot blot test (Poumerat, Longchamon and Martel, 1992) and Western immunoblotting (Towbin, Staehelin and Gordon, 1979)

### 3.8.1. Dot blot

The dot blot test was done following the method of Poumerat *et al* ( 1992 ) with slight modifications. Recombinant *Cowdria* MAP2 was solubilized in SDS-PAGE sample buffer (0.5 M Tris-HCl, pH 6.8; 2 % SDS; 10 % glycerol; 5 % 2- $\beta$ -mercaptoethanol and 0.01 % bromophenol blue) boiled and 5 $\mu$ l dot blotted onto each of two nitrocellulose membrane strips. The nitrocellulose strips were air-dried and blocked with 5 % skimmed milk in PBS containing 0.05 % Tween 20 for 30 minutes. The strips were then incubated with preimmune and immune sera respectively (diluted 1:5 in 5 % skimmed milk) at room temperature overnight, washed 3 times in PBS containing 0.05 % Tween 20; horse radish peroxidase conjugated goat anti-rabbit immunoglobulin G (IgG) diluted 1:1000 (in 5 % skimmed milk) was added and the strips were further incubated at room temperature for one hour. The strips were washed 3 times in PBS and the bound antibodies were visualised using 3,3'-diaminobenzidine (DAB) and hydrogen peroxide.

### 3.8.2 SDS-PAGE and Western immunoblotting

SDS-PAGE and Western immunoblotting were done according to the methods of Studier (1973) and Towbin *et al.* (1979) with some modifications. Recombinant *Cowdria* MAP2 was solubilized in SDS-PAGE sample buffer as already mentioned and separated on a 7.5-17.5 % gradient SDS-PAGE gel. The separated polypeptides were electroblotted onto nitrocellulose membrane, air-dried, blocked as mentioned above, air dried and stored at -20°C. until required. For Western immunoblots, strips were cut from the immunoblot and processed as described for the dot blot strips.

### 3.9. Precipitation of Rabbit anti-MAP2 immunoglobulin (aMAP2)

Seven days after the third booster injection, the rabbits were bled twice a week, sera harvested over a period of 30 days and stored at -20°C. A total of 130 ml of sera was pooled and the immunoglobulins precipitated using saturated ammonium sulphate solution before storage at -20°C. To determine the potency of the immunoglobulin

fraction, a dot blot test was carried out using the isolated anti-MAP2 immunoglobulin (aMAP2) diluted 1:100 to probe *Cowdria* elementary bodies (EBs), MAP2 and bovine pulmonary artery endothelial cell (BPA) antigens. The BPA antigen was included to serve as a control for cross reacting antibodies to BPA in which *Cowdria* is propagated

In addition to the dot blot test, a 7.5- 17.5 % gradient preparatory SDS-PAGE gel of EBs and BPA was run and electroblotted on to nitrocellulose membrane. The immunoblot was blocked for 30 mins with 5 % skimmed milk in phosphate buffered saline (PBS) containing 0.05 % Tween 20. A Western blot was done using aMAP2 diluted 1:100 to probe EBs and BPA antigen strips cut from the blocked immunoblot. Strips of EBs and BPA antigen probed with aMAP2 adsorbed with foetal bovine serum (FBS), BPA and *Escherichia coli* were also included to rule out reactions due to presence of cross reacting antibodies to bovine serum albumin (present in FBS added to media for growing *Cowdria*), BPA (in which *Cowdria* propagates) and *E. coli* (in which MAP2 was produced using recombinant DNA technology).

### 3.10. Propagation of Bovine Pulmonary Artery ( BPA ) endothelial cells

Bovine pulmonary artery (BPA) endothelial cells (required for the *in vitro* propagation of *C. ruminantium*) were propagated in Glasgow Modified Essential Medium (GMEM) pH 7.2 to which was added FBS 10%; tryptose phosphate broth (TPB) 10 %, penicillin 100 IU / ml medium, streptomycin 100 µg / ml medium, and L-glutamine 292 µg / ml medium. The initial passage was made from a confluent BPA cell culture flask (a 75 cm<sup>2</sup> tissue culture flask) obtained from a stock of confluent BPA cell culture flasks maintained in the laboratory (Biotechnology Laboratories, Kabete) at 37°C for routine *in vitro* propagation of *C. ruminantium*. Media in the confluent BPA cell culture flask was poured off and the monolayer cell culture was rinsed with PBS / EDTA solution once. Two ml of trypsin / EDTA were added and the flask was incubated at 37°C for 2 mins. Then 13 ml of GMEM were added and the cells were dispersed with a pipette by sucking up and down, after which 10 ml of



the suspended cells were sucked off with a pipette and dispensed in 5ml amounts into two other tissue culture flasks. Ten ml of GMEM were added to each of the three flasks, the flasks were then labelled and incubated at 37°C. Media was changed after 24 hrs, and the cultures were examined daily using an inverted microscope. When the cells became confluent, they were passaged again to generate more flasks of confluent BPA monolayer cell cultures which were used for *in vitro* propagation of *C. ruminantium*.

### 3.11 *In vitro* propagation of *Cowdria ruminantium*

*Cowdria ruminantium* was propagated in monolayer BPA endothelial cell cultures. The BPA cell cultures were infected with frozen *Cowdria ruminantium* culture stabilate (the Crystal Springs strain from Zimbabwe). One ampoule of frozen culture stabilate was thawed rapidly in a water bath at 37°C. The media in the BPA cell culture flasks was poured off; and the monolayer BPA cell cultures were rinsed once with GMEM base media to remove traces of streptomycin which may inhibit growth of *C. ruminantium* (Shompole *et al.*; unpublished observations). Then 2 ml of the thawed *Cowdria* culture stabilate was added to each of the monolayer BPA endothelial cell culture flask and incubated at 37°C on a slow rocking platform for two hours to allow adsorption of the infective material to the BPA endothelial cells. At the end of the two hour incubation the inoculum was removed and GMEM (without streptomycin and adjusted to pH 6.0-6.5 with HCl) was added; the culture flasks were incubated at 37°C on a slow rocking platform; checked for cytopathic effect (CPE) three days post infection and daily thereafter using an inverted light microscope. To detect and thereafter passage *Cowdria ruminantium* into other BPA endothelial cell culture flasks, the infected cell culture flasks showing CPE were sampled by scraping off some of the BPA cell monolayer with a sterile spatula and thin smears made on microscope slides. The slides were air dried, fixed in methanol, stained with Giemsa and examined under X100 oil immersion objective for inclusions and elementary bodies of *Cowdria ruminantium*. The supernatant of the positive infected BPA cell culture

flasks was passaged into other BPA cell culture flasks for massive propagation of *Cowdria ruminantium*.

Media in the confluent BPA cell culture flasks was removed and the monolayer BPA cell cultures were rinsed once with GMEM base media to remove traces of streptomycin. Then 5 mls of the supernatant of positive infected BPA cell cultures were added to each of the 75 cm<sup>2</sup> monolayer BPA cell culture flasks and incubated at 37°C on a slow rocking platform for two hours to allow the infective material to adsorb to the BPA endothelial cell cultures. At the end of the two hours incubation; 10 mls of GMEM (without streptomycin and adjusted to pH 6.0-6.5 with HCl) was added to each flask, the flasks were incubated at 37°C on a slow rocking platform and checked for CPE three days post-infection and daily thereafter using the inverted light microscope.

### 3.12. Harvesting of crude *Cowdria* antigen

Crude *Cowdria* antigen was harvested when 90% of the BPA endothelial cell cultures were infected. The cell cultures were scraped, passed through a 26G needle three times (to lyse the intact infected BPA endothelial cells) and centrifuged at 1000 x g for 5 minutes at 4°C. The supernatant was recovered and centrifuged at 30,000 x g for 15 minutes to pellet the EBs. The supernatant was poured off, the EBs pellet resuspended in PBS, and washed two times in PBS by centrifugation at 30,000 x g for 15 minutes, after which the pellet was resuspended in a minimal amount of PBS and stored at -20°C until required. This constituted the crude *Cowdria* antigen.

### 3.13. Inoculation of goats

During the acclimatization period, four goats died thus leaving only 7 goats to be used in the study. Before inoculation, the remaining 7 goats were bled, preimmune sera harvested and stored at -20°C. The goats were divided into three groups : A , B , and C . Group A contained 4 goats, B contained two goats and C contained one goat. Each of the goats in group A was intravenously inoculated with 10 ml of *Cowdria ruminantium* blood stabilate (high dose) while each of the goats in group B was

inoculated with 2 ml of the blood stabilate (low dose) and the one goat in group C (the control) was not inoculated.

#### 3.14. Monitoring and sampling of goats post inoculation

Every morning the goats were clinically examined, their daily rectal temperatures recorded, bled once every week, sera harvested and stored at -20°C. To demonstrate that *C. ruminantium* was present in the blood of the inoculated animals during the febrile phase (when rectal temperature was 40°C and above), blood was aseptically collected in heparin (50units/ml) and placed in sterile centrifuge tubes. The tubes were centrifuged at 1000 x g for 20 minutes at 4°C, and plasma was removed for inoculation of BPA cell cultures as described by Byrom *et al.* (1991). Smears were made from blood and the buffy coat, air-dried, fixed in methanol, stained with Giemsa and examined under the X100 oil immersion objective. The inoculated animals in group B that developed a febrile reaction (when rectal temperature was 40°C and above) were treated by intramuscular injection with long acting tetracyclines (20 mg / kg im) and challenged again; while those in group A were not treated. Those animals in group A which developed a fatal disease and died were subjected to a thorough postmortem examination following the standard routine necropsy procedures (Jones and Gleiser , 1954). Brain squash smears were made, air-dried, fixed in methanol, stained with Giemsa and examined under the X100 oil immersion objective. Tissue samples were taken from the kidneys, liver, spleen, and lymph nodes; fixed in 10 % formal saline, embedded in paraffin wax; sections cut and stained with H & E, mounted in DPX and examined under the X10 and X40 objectives ( Humanson, 1972; Putt, 1972).

#### 3.15. Coupling of latex beads

Latex beads were coupled according to the method of Buscher *et al* (1991) with crude *Cowdria* antigen (to detect antibodies to *Cowdria rumnantium* in sera of experimentally infected goats) and aMAP2 (to detect *Cowdria* antigen in plasma of infected goats). The harvested *Cowdria* antigen was sonicated; and protein estimation of the *Cowdria* antigen and aMAP2 was done following the UV-light absorption

method (Johnstone and Thorpe, 1982). One hundred and fifty (150)  $\mu$ l of latex beads (diluted 1:5 in PBS) were mixed with 300 $\mu$ l of *Cowdria* antigen (3.1 mg / ml); and another 150  $\mu$ l of latex beads was mixed with 300 $\mu$ l of aMAP2 (79 mg / ml). The two mixtures were incubated at 37<sup>0</sup>C for one hour, with gentle shaking after every 30 minutes. Another two separate 150 $\mu$ l amounts of latex beads were coupled with *Cowdria* antigen solubilized in 2% Nonidet P-40 (N P-40) in Ten buffer (containing 0.02M Tris HCl; 0.005M EDTA , 0.1M NaCl and 0.015M NaN<sub>3</sub> ) and SDS-PAGE sample buffer (without bromophenol blue) respectively; as described above. In addition, another 100  $\mu$ l of latex beads were coated with recombinant *Cowdria* MAP2 following the same procedure and used to test 81 goat serum samples collected from Machakos District ( Kenya ), where heartwater is reported to be endemic.

### 3.16. Latex slide agglutination assays

The latex beads coated with crude *Cowdria* antigen were evaluated against one known positive and one negative sera, while the latex beads coated with aMAP2 were evaluated against sonicated, NP-40 and SDS-PAGE sample buffer solubilized EBs. Testing was done by mixing 10 $\mu$ l of test sera/plasma with 10  $\mu$ l of the appropriate coated latex beads on a glass plate. The glass plate was then rocked gently for 2 mins. and the agglutination read visually. Samples which caused visible agglutination within 2 mins were recorded as positive and samples that showed agglutination after rocking the glass plate for an extra 3 mins were recorded as weak positive while those samples which showed no agglutination after 5 mins were recorded as negative.

### 3.17. Triton X-114 separation of integral membrane proteins

*Cowdria ruminantium* integral membrane proteins were separated in Triton X-114 according to the method of Clement (1981) with slight modifications. One ml of *Cowdria* antigen (0.38 mg / ml), in 10 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.5 % Triton X-114, was incubated at 0<sup>0</sup>C for 3 mins. After dissolution of the surfactant the mixture was overlaid on 1.5 ml. of 6 % sucrose cushion (containing 10

mM Tris-HCl pH 7.4, 150mM NaCl and 0.06 % Triton X-114) in a conical centrifuge tube and incubated at room temperature for 3 mins (during which time clouding of the solution occurred) The tube was centrifuged at 300 x g for 3 mins and the detergent phase was found as an oily droplet at the bottom of the tube. The upper aqueous phase was carefully pipetted off and mixed with fresh surfactant to give 0.5 % Triton X-114 and the mixture incubated at 0°C for 3 mins. After dissolution the mixture was again overlaid on the sucrose cushion used previously, incubated for 3 mins. at room temperature for condensation, and centrifuged on the previous detergent phase. The aqueous phase was pipetted off and the separation repeated 3 more times as described above. At the end of the separation, the aqueous phase was mixed with fresh surfactant to give 2 % Triton X-114 in a separate tube without the sucrose cushion, and was left to stand at room temperature for the detergent and aqueous phases to separate. After this, the aqueous phase was recovered and the detergent phase of this last separation was discarded. After separation, Triton X-114 and Tris-HCl saline buffer pH 7.4 were added to the aqueous and detergent phases respectively in order to obtain equal volumes and approximately the same salt and detergent content for both samples. The samples were then fractionated on a 7.5-17.5 % SDS-PAGE gel. The separated polypeptides were electroblotted on to nitrocellulose membrane and probed with aMAP2 (as described above for the dot blot and Western immunoblotting) to determine phase separation of the 21kDa protein ( MAP2 ).

### 3.18. Statistical Analysis

Descriptive statistical methods were used to analyse the preinfection and postinfection daily rectal temperatures recorded over a period of 30 days . The base line preinfection rectal temperature range, mean and standard deviation (SD) of the preinfection daily rectal temperature for each goat were determined and graphs of post-infection daily rectal temperatures against time in days were plotted to show the pattern of the daily rectal temperatures of the infected goats and the control after infection.

## 4. RESULTS

### 4.1. Preinfection daily rectal temperatures

The preinfection daily rectal temperatures of the experimental goats over a period of 30 days were as indicated in Appendix 1. The base-line preinfection daily rectal temperature range was 37-39.7<sup>0</sup>C. and the mean preinfection daily rectal temperature for each experimental goat was as indicated in Table 1  $\pm$  the standard deviation (SD).

Table 1 :

Mean and standard deviation ( SD ) of the pre-infection daily rectal temperature for each experimental goat.

Tag No	7139	2380	9180	5335	25	8060	6170
N	30	30	30	30	30	30	30
Mean	37.8	38.2	37.9	38.5	38.4	38.3	38.2
SD	0.5	0.7	0.6	0.3	0.3	0.4	0.3

The number of readings taken (N) from each goat were 30, the mean pre-infection temperature for each goat ranged from 37.8 to 38.5, while the standard deviation (SD) for each goat varied from 0.3 to 0.7.

### 4.2. Monitoring of the immune response

#### 4.2.1. Dot blots

The recombinant MAP2 antigen dot blots probed with preimmune and immune rabbit sera diluted 1:5 showed no reaction with preimmune rabbit serum, but strongly reacted with the 2<sup>nd</sup> boost immune rabbit serum (Figure 1). The EBs and MAP2 antigens dot blots probed with aMAP2 diluted 1:100 showed a strong reaction while the BPA antigen dot blot probed with the same aMAP2 showed a weak reaction (Figure 2).

#### 4.2.2. SDS-PAGE and Western Immunoblots

The recombinant MAP2 antigen immunoblot strips probed with pre-immune and immune rabbit sera diluted 1:5 showed no reaction with the pre-immune rabbit serum, but showed a faint reaction band with the 2nd boost immune rabbit serum in the 21 kDa region (Figure 3). The EBs immunoblot strips probed with adsorbed and non adsorbed aMAP2 diluted 1:100 showed two reaction bands: one just above the 66kDa region and another in the 21kDa region, while the EBs immunoblot strip probed with pre-immune rabbit serum diluted 1:5 showed no reaction. The BPA immunoblot strips probed with FBS-, BPA-adsorbed aMAP2 and non adsorbed aMAP2 diluted 1:100 each showed a reaction band approximately mid-way between the 66kDa and 97kDa regions (a reaction band which was not observed in the EBs immunoblot strips) (Figure 4).

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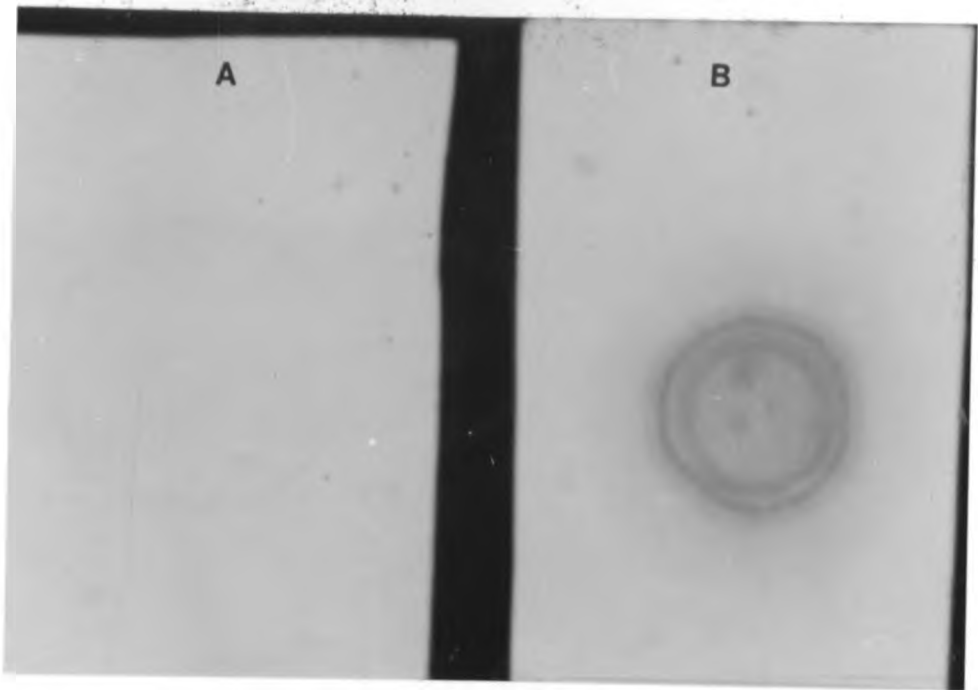


Figure 1 :

Dot blots of recombinant *Cowdria* MAP2 probed with pre-immune and immune rabbit sera diluted 1:5. The pre-immune rabbit serum showed no reaction (A), while the immune rabbit serum showed a strong reaction (B).

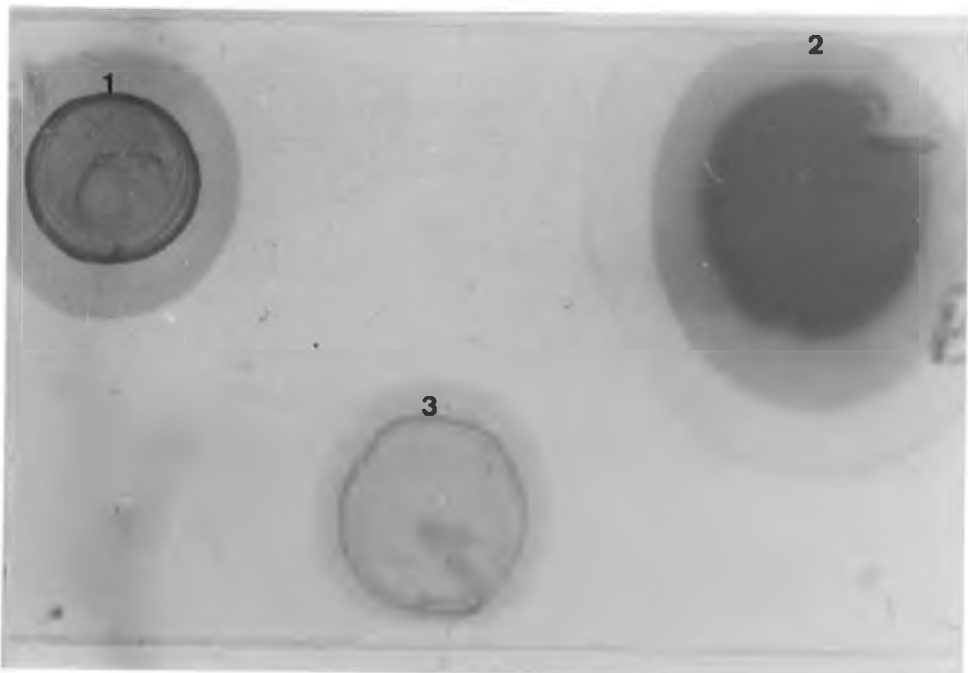


Figure 2 :

Dot blots of EBs, MAP2 and BPA antigens probed with aMAP2 diluted 1:100. The EBs (1) and recombinant MAP2 (2) antigens showed strong reactions, while BPA (3) antigen showed a weak reaction.



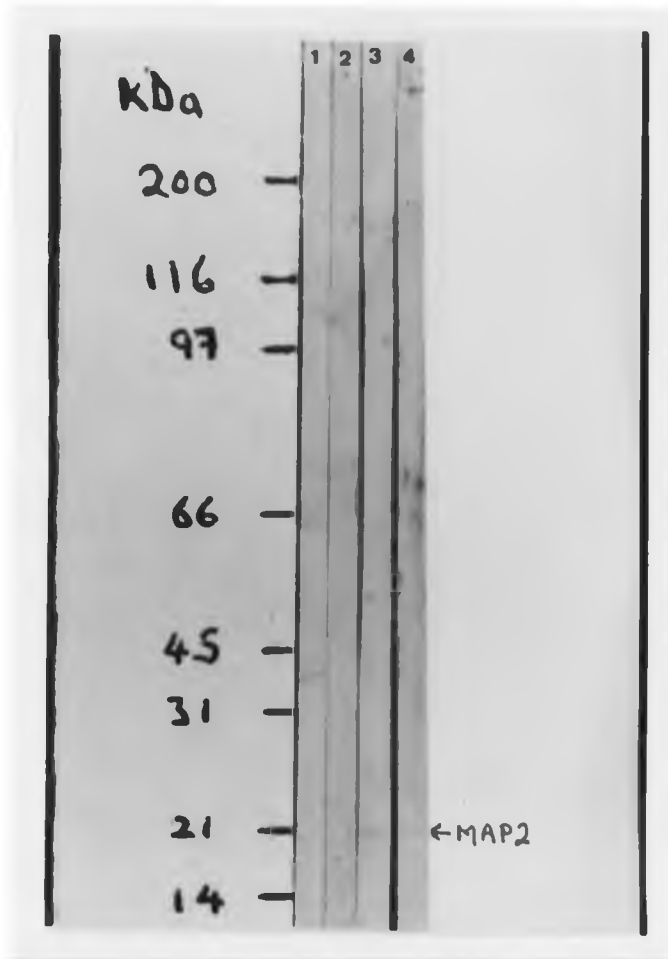


Figure 3 :

Western blot strips of recombinant MAP2 probed with pre-immune and immune rabbit sera diluted 1:5. The pre-immune rabbit sera of rabbits 634 (strip 1) and 635 (strip 2) showed no reaction in the 21kDa region, while the 2<sup>nd</sup> boost immune rabbit sera of rabbits 634 (strip 3) and 635 (strip 4) showed faint reaction bands in the 21 kDa region, where MAP2 falls (arrow).

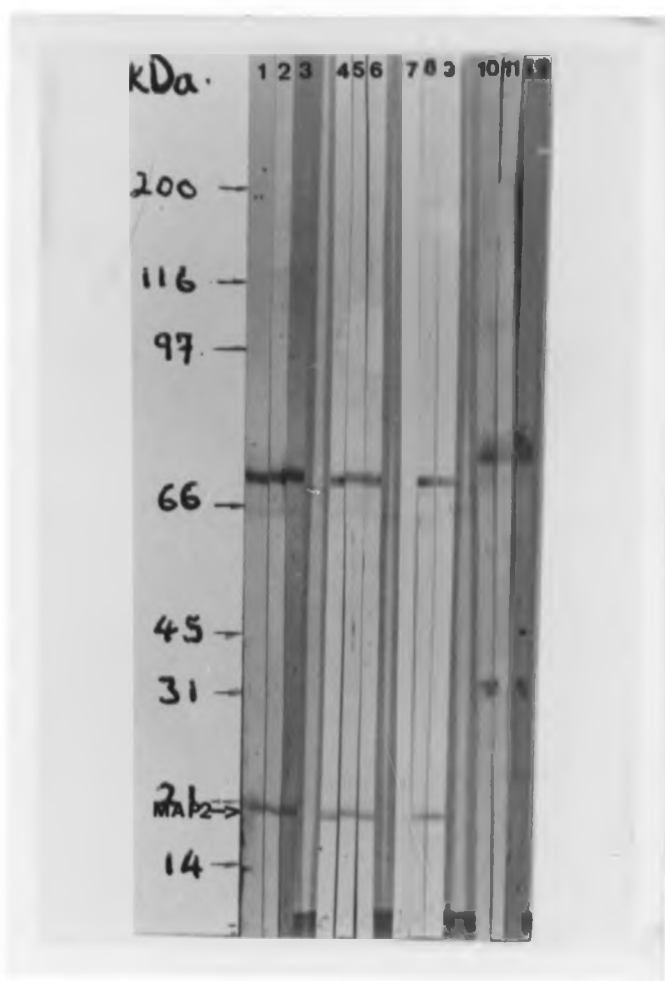


Figure 4 :

Western blot strips of EBs and BPA antigens probed with FBS-, BPA- and *E.coli*-adsorbed aMAP2 and pre-immune rabbit serum diluted 1:100. The EBs antigen strips probed with aMAP2 showed two reaction bands: one in the 21kDa region, where MAP2 falls (arrow) and another just above the 66kDa region, while the EBs antigen strip 7 probed with pre-immune rabbit serum showed no reaction band. The BPA antigen strips probed with aMAP2 showed reaction bands mid way between the 66 and the 97 kDa regions, which bands were not observed in the EBs antigen strips.

**KEY:**

Strips 1-9 = EBs. antigen

Strips 10-12 = BPA antigen.

Strip 1 was probed with FBS-adsorbed aMAP2.

Strip 2 was probed with BPA-adsorbed aMAP2.

Strip 3 was probed with non-adsorbed aMAP2.

Strip 4 was probed with sonicated *E. coli*-adsorbed aMAP2

Strip 5 was probed with whole *E. coli*-adsorbed aMAP2.

Strip 6 was probed with non-adsorbed aMAP2

Strip 7 was probed with preimmune rabbit serum.

Strip 8 was probed with aMAP2 first diluted 1:500 and then adsorbed with *E.coli*.

Strip 9 was probed with aMAP2 first diluted 1:1000 and then adsorbed with *E.coli*.

Strip 10 was probed with FBS-adsorbed aMAP2.

Strip 11 was probed with BPA-adsorbed aMAP2.

Strip 12 was probed with non-adsorbed aMAP2.

### 4.3. Clinical signs

#### 4.3.1. Group A goats

This group comprised of goat Nos 7139, 2380, 9180, and 5335, which were given a high dose (10 mls) of *C. ruminantium* blood stabilate. In this category of goats, fever (40°C or more) developed 5, 10, 11, and 12 days after intravenous inoculation, in goats No. 9180, 5335, 2380 and 7139, respectively. The onset of fever was sudden, followed by anorexia, respiratory distress, lateral recumbency, opisthotonus, extension of the legs and death. In three of the animals, death occurred within 36-48 hours after the onset of fever

#### 4.3.2. Group B goats

This group comprised of goat Nos 6170 and 8060 which were given a low dose (2 mls) of *Cowdria ruminantium* blood stabilate. In this category of goats fever developed 17 and 18 days after intravenous inoculation, in goats No. 6170 and 8060, respectively. The fever persisted for 4-5 days, but the goats continued to eat normally without overt signs of clinical illness, and rectal temperatures dropped to normal after treatment. Further challenge with another intravenous injection of *Cowdria ruminantium* blood stabilate caused no fever.

#### 4.3.3. The control goat

This was goat No.25, which received no *Cowdria ruminantium* blood stabilate. This particular goat developed no fever and showed no visible signs of sickness throughout the period of the experiment. The post-infection daily rectal temperatures of the experimental goats over a period of 30 days were as indicated in Appendix 2. The pattern of the post-infection daily rectal temperatures of goats in group A, B and the control over a period of 30 days was as shown in Figures 5 and 6, respectively.

**Fig.5: TEMPERATURE PATTERN OF A&C GOATS**

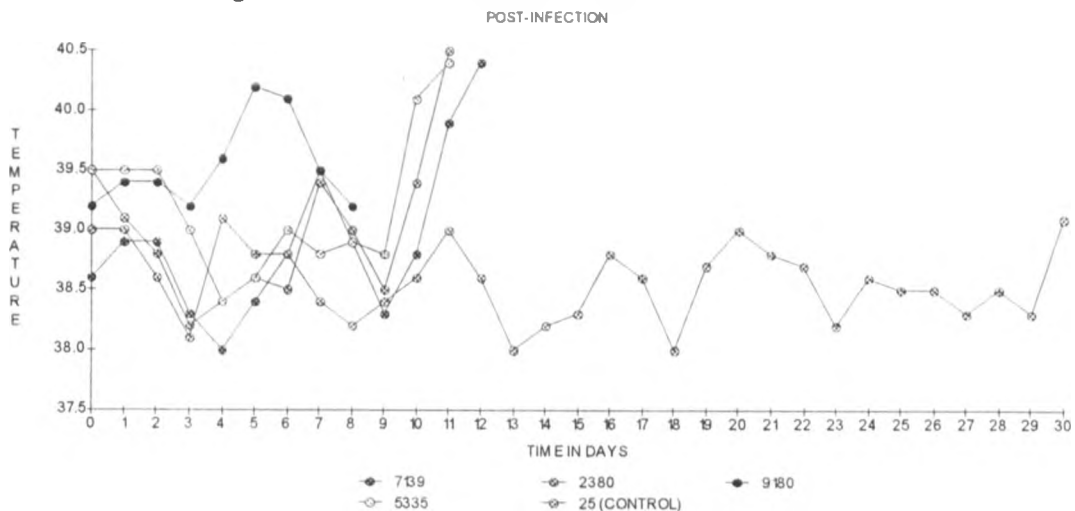


Figure 5 is a graph of post-infection daily rectal temperatures of goats in group A and C (the control) plotted against time in days, showing the pattern of daily rectal temperatures after infection. Group A goats (7139, 2380, 9180 and 5335) developed acute fever and died between day 8 and 12, while the control goat maintained the normal baseline rectal temperature.

**Fig.6:TEMPERATURE PATTERN OF B&C GOATS**

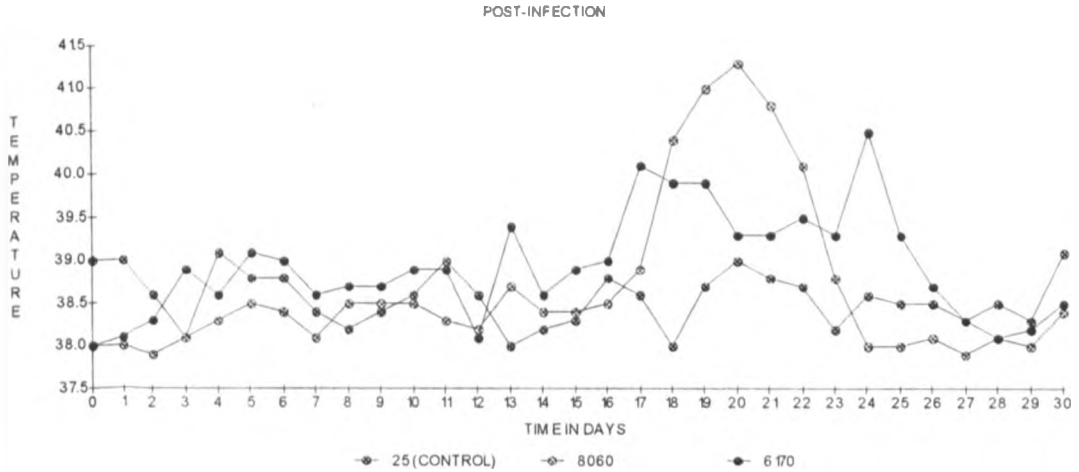


Figure 6 is a graph of post-infection daily rectal temperatures of goats in group B and C (the control goat) plotted against time in days, showing the pattern of daily rectal temperatures after infection. Group B goats (8060 and 6170) developed fever between days 17 and 18, which went down after treatment, while the control goat (25) maintained the normal baseline rectal temperature.

#### 4.4. Demonstration of *Cowdria* organisms in blood of the sick goats

Demonstration of *Cowdria* organisms in blood of sick goats was only possible by culture methods. Bovine pulmonary artery (BPA) endothelial cell cultures seeded with plasma harvested from blood collected during the febrile phase showed CPE 5-7 days after inoculation of the cultures. Scraping smears made from the CPE positive cell cultures and stained with Giemsa were positive for inclusions and elementary bodies of *C. ruminantium* (Figure 7). Uninfected BPA control cell culture scraping smears stained with Giemsa showed no inclusions and elementary bodies of *C. ruminantium* (Figure 8). Attempts to demonstrate *Cowdria* organisms in Giemsa stained blood and buffy coat smears of the sick goats were unsuccessful.

#### 4.5 Post mortem Findings

##### 4.5.1. Gross lesions

Post mortems were carried out within 6 to 8 hrs after death. Effusion of the body cavities was a common pathological change in all the four goats which died of heartwater disease. Hydrothorax and hydropericardium were a striking feature in all the four fatal cases (Figures 9, 10 and 11). The slightly turbid light yellow fluid observed in the body cavities coagulated on exposure to air. Oedema of the lungs, characterised by serous frothy fluid oozing from the cut surface, was a common finding in all the four fatal cases. The spleen was not strikingly enlarged. The kidneys were markedly swollen and pale. The heart had epicardial and endocardial haemorrhages. The brain showed congestion of the cerebral meningeal blood vessels (Figure 12), a feature which was not observed in the brain of the control goat (Figure 13). Other organs showed no striking gross pathological changes.

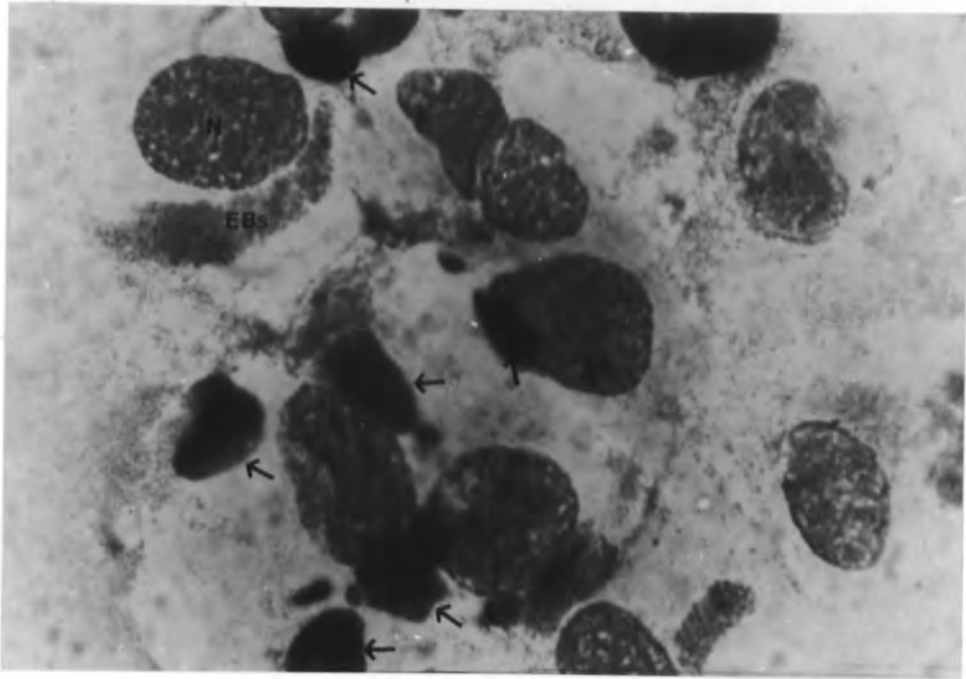


Figure 7 :

Micrograph of infected BPA cell culture scraping smear (Giemsa x 1000) showing inclusion bodies (arrows) and elementary bodies of *C. ruminantium* (EBs); and nuclei of disintegrated BPA cells (N).

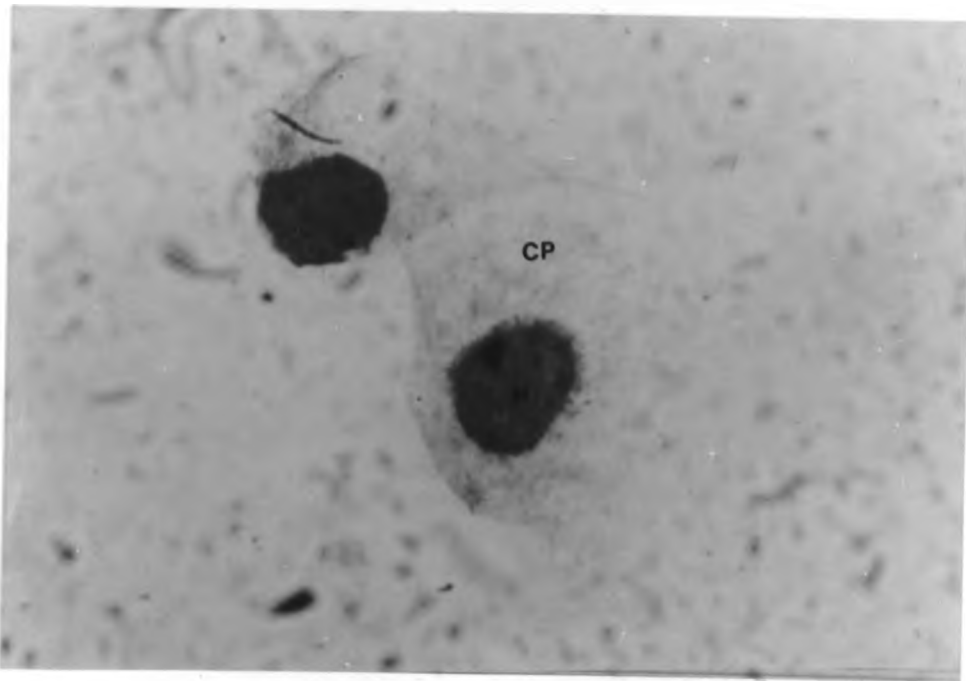


Figure 8 :

Micrograph of un-infected BPA cell culture (control) scraping smear (Giemsa x 1000). The cells are intact, with their nuclei (N) and cytoplasm (CP) clearly demarcated, and are without inclusion bodies and EBs of *C. ruminantium*.

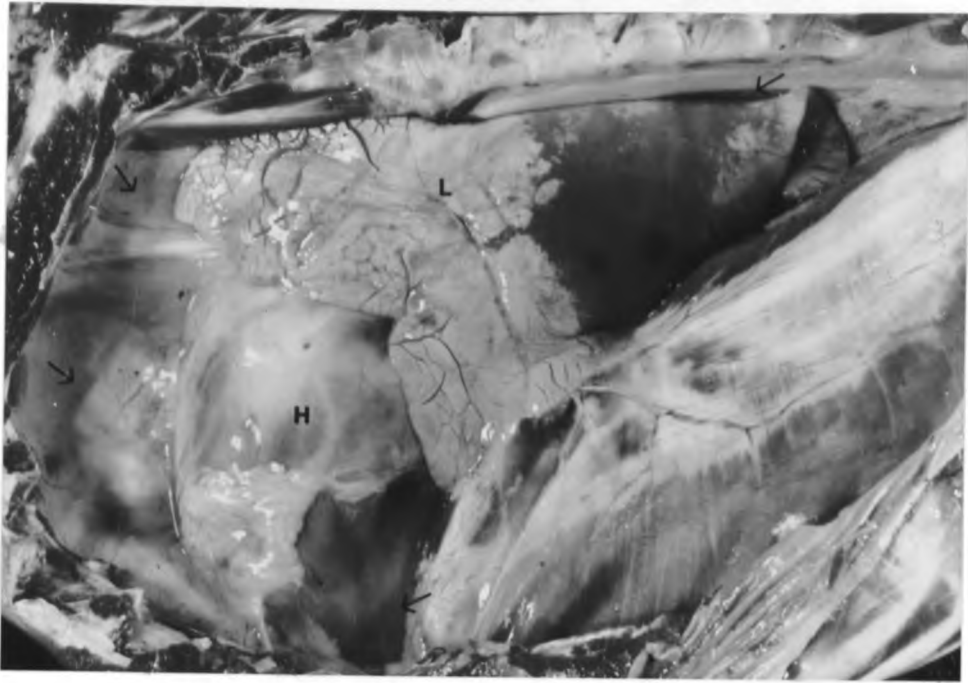


Figure 9 :

Top view of opened thoracic cavity of goat No.5335, showing fluid in the thoracic cavity ( hydrothorax) and pericardial sac (hydropericardium) (arrows), the lung (L) and the heart.



Figure 10

Top view of opened thoracic cavity of goat No. 7139, showing fluid in the thoracic cavity (hydrothorax) (big arrows), in the pericardial sac (hydropericardium) (small arrow), the heart (H), petechial and diffuse haemorrhages (1 and 2) on the lungs (L).





Figure 11 :

Photograph of the pluck of goat No.7139, showing, fluid in the pericardial sac (hydropericardium) (arrow), heart (H) and lungs (L).



Figure 12 :

Photograph of the brain of goat No. 5335, showing congestion of the cerebral meningeal blood vessels ( arrows ) .

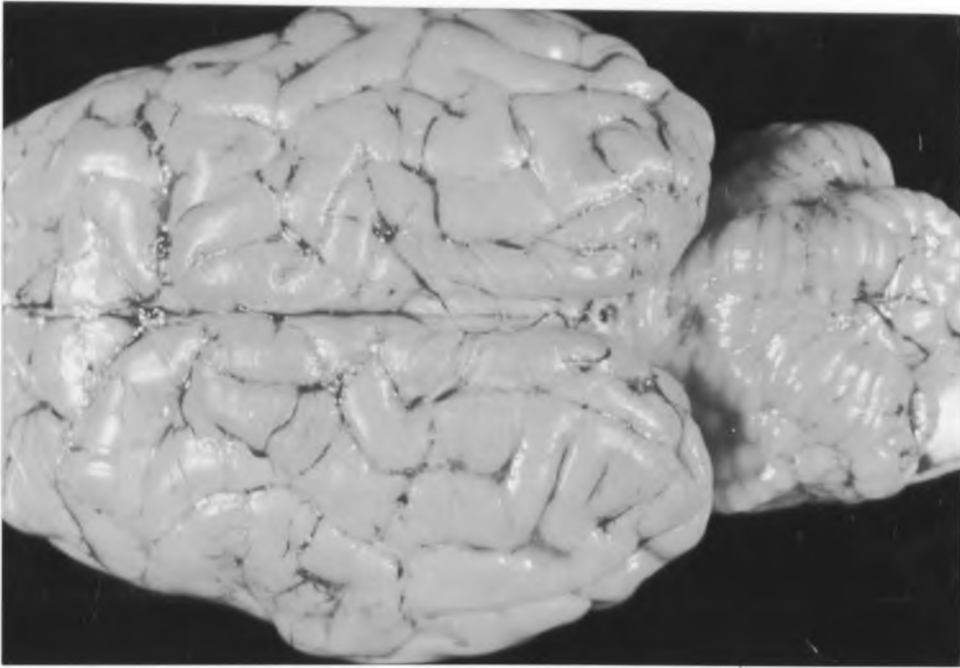


Figure 13 :

Photograph of the brain of the control goat. There was no congestion of the cerebral meningeal blood vessels, compared to the brain in figure 12.

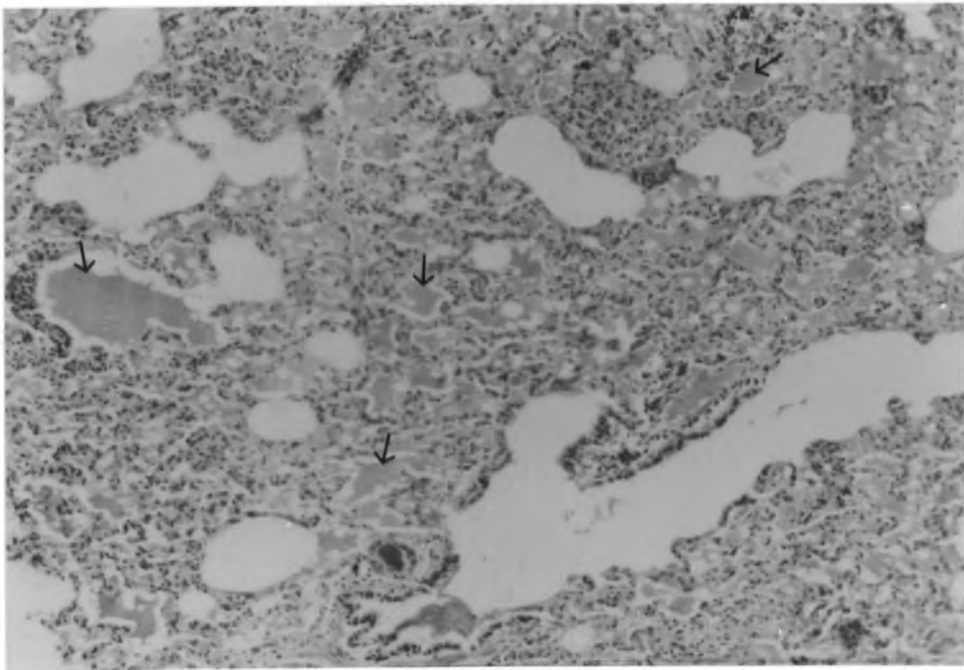


Figure 14 :

Micrograph of lung tissue section of a goat which died of heartwater. The pink staining homogenous material (arrows) in the bronchi, bronchioles and alveolar spaces is suggestive of lung oedema,(H & E x 100).

#### 4.5.2. Histopathology

##### Lungs

The lung tissue sections from the fatal cases of heartwater had a pink (eosinophilic) staining homogeneous material in the bronchi , bronchioles and alveolar spaces which was suggestive of lung edema (Figure 14). Such pink staining homogeneous material was not observed in the the lung tissue sections of the control goat (Figure 15).

##### Kidneys

The kidney tissue sections from the fatal cases of heartwater had focal areas of tubular and glomerular degeneration and loss of tissue architecture which were suggestive of nephrosis (Figure 16). Such microscopic lesions were not observed in the control goat kidney tissue sections (Figure 17).

##### Brain

Increased perivascular space, which was suggestive of cerebral oedema, was a common finding in all the brain tissue sections of the fatal cases (Figure 18). Such an increase in the perivascular space was not observed in the control goat brain tissue sections (Figure 19). The other organs showed no significant microscopic pathological changes.

#### 4.6 Brain squash smears

Demonstration of *Cowdria* organisms in Giemsa stained brain squash smears was successful in all the four fatal cases. Colonies (morulae) of *Cowdria ruminantium* organisms were observed in endothelial cells of brain blood capillaries ( Figures 20 and 21 ).

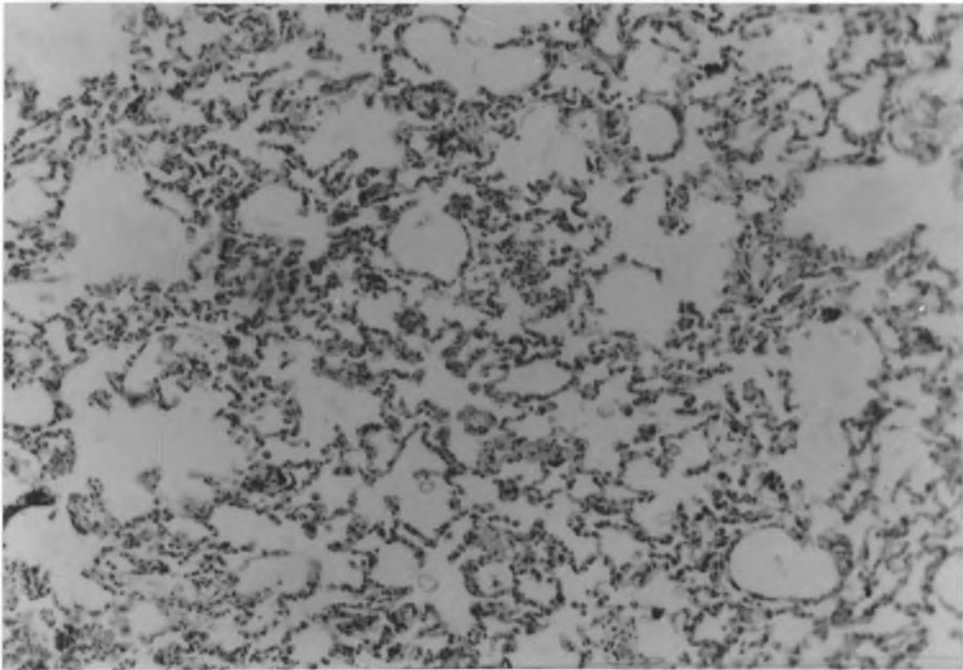


Figure 15 :

Micrograph of lung tissue section of the control goat (H & E x 100). The pink staining homogeneous material was missing, compared to the lung tissue section in Figure 14.

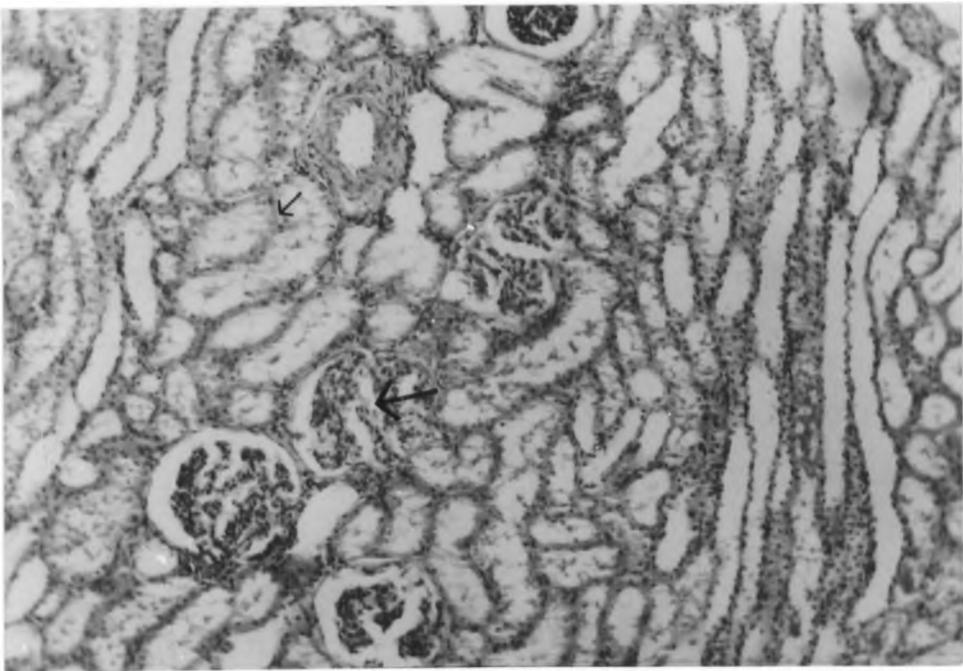


Figure 16 :

Micrograph of kidney tissue section of a goat which died of heartwater, showing focal areas of tubular (small arrow) and glomerular (big arrow) degeneration (H & E x 100).

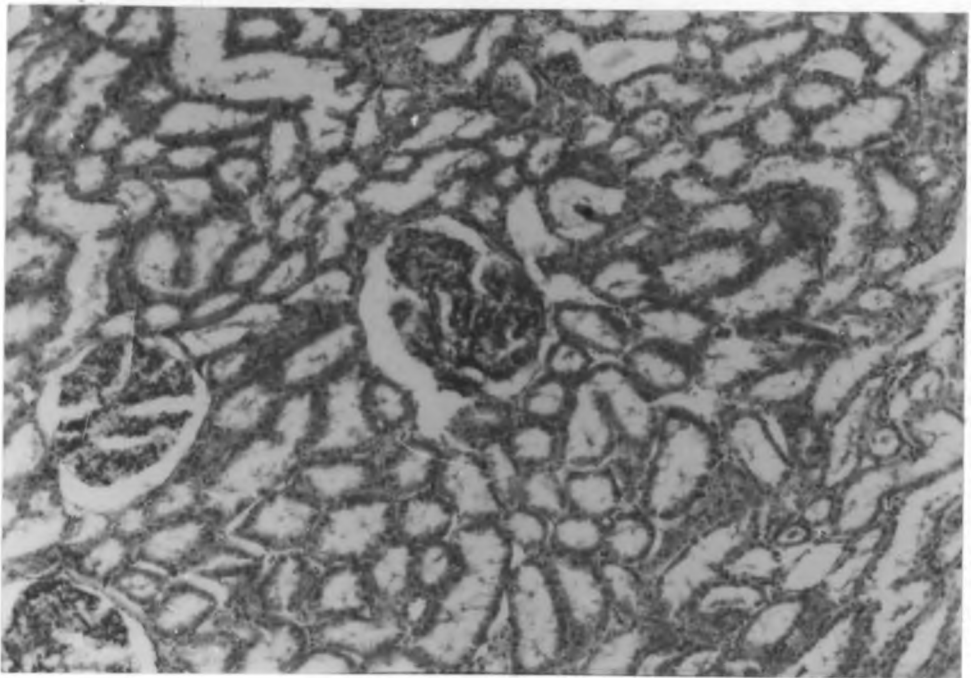


Figure 17 :

Micrograph of kidney tissue section of the control goat (H & E x 100). There were no significant microscopic pathological changes, compared to the kidney tissue section in Figure 16.

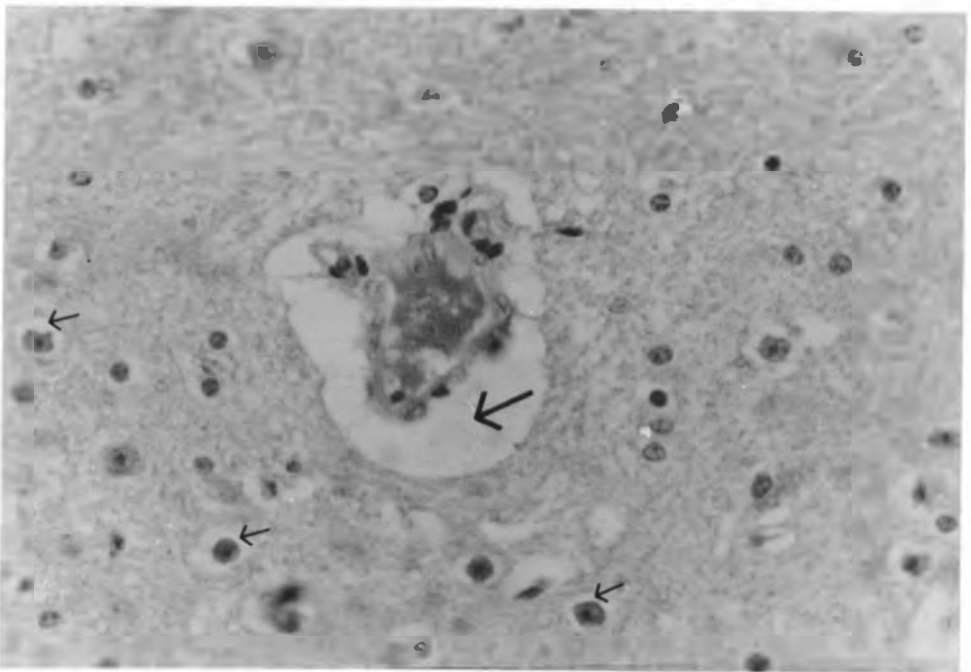


Figure 18 :

Micrograph of brain tissue section of a goat which died of heartwater ,showing increased perivascular space (big arrow), and clear spaces around the glial and brain cells (small arrows), which are suggestive of cerebral oedema, (H & E x 400).

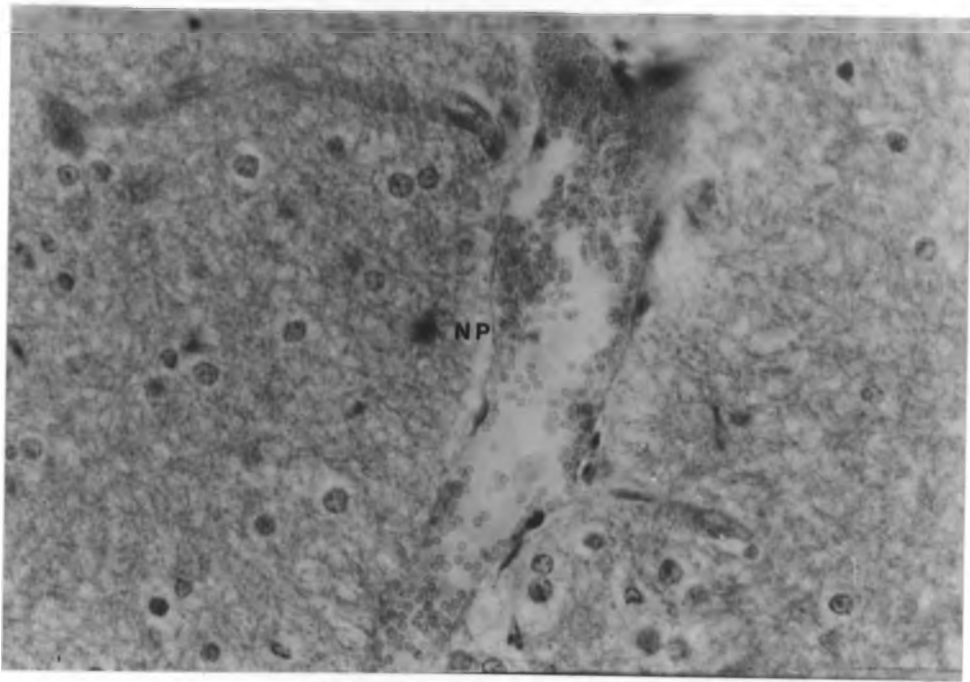


Figure 19 :

Micrograph of brain tissue section of the control goat showing no significant increase in the perivascular space (NP), and no significant clear spaces around glial and brain cells, compared to the section in Figure 18,(H& E x 400).

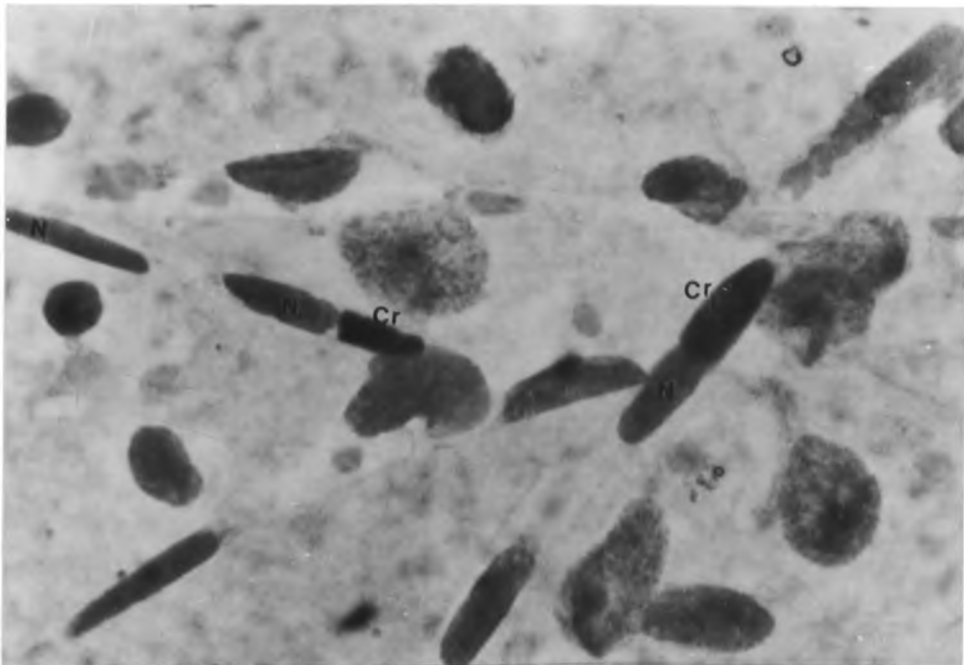


Figure 20 :

Micrograph of brain squash smear of a goat which died of heartwater, showing colonies of *C. ruminantium* (Cr) in blood capillary endothelial cells, adjacent to the nuclei (N) of the endothelial cells, (Giemsa x 1000).

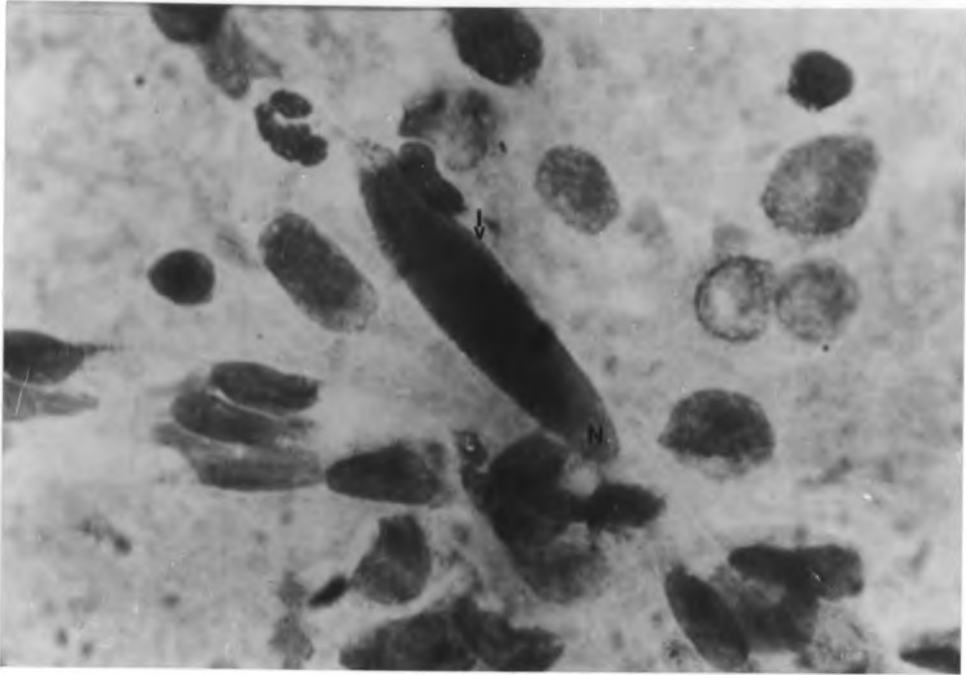


Figure 21 :

Micrograph of brain squash smear of a goat which died of heartwater , showing a big colony of *Cowdria ruminantium* organisms (arrow) partially obscuring the ucleus of the blood capillary endothelial cell (N), (Giemsa x1000).

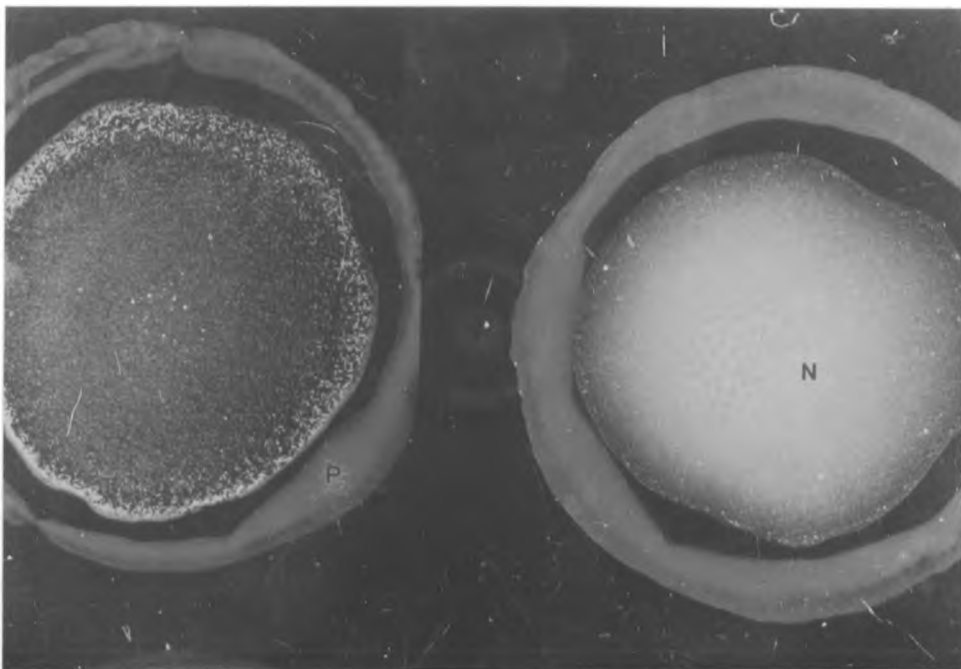


Figure 22 :

Latex slide agglutination antibody test results of evaluation of latex beads coated with crude *Cowdria ruminantium* antigen against known positive and negative sera. Positive serum showed agglutination (P), while negative serum showed no agglutination

#### 4.7 Latex slide agglutination assays

When the latex beads coated with crude *C. ruminantium* antigen (latex slide agglutination antibody test) were evaluated against known negative and positive sera, the beads did not agglutinate with the negative serum but agglutinated with the positive serum (Figure 22). When the latex beads coated with aMAP2 Ig (latex slide agglutination antigen test) were evaluated against sonicated EBs, NP-40 and SDS-PAGE sample buffer-solubilised EBs, the beads did not agglutinate with the sonicated EBs but weakly agglutinated with the solubilised EBs. (Table 2) The use of latex beads coated with aMAP2 Ig (latex slide agglutination antigen test) to detect *Cowdria* antigen in plasma of sick goats was unsuccessful. All the plasma samples collected during the febrile reaction did not agglutinate with the beads coated with aMAP2 Ig and were all recorded as negative (Table 3). The results of the use of latex beads coated with crude *Cowdria* antigen to detect *C. ruminantium* antibodies in post infection sera collected over a period of 8 weeks varied in the three categories of the experimental goats. Group A goats (7139, 2380, 9180 and 5335) sera were negative (showed no agglutination), while sera from goats in group B (6170 and 8060) started showing agglutination during the 6th week after inoculation (24 days after the onset of the febrile reaction). The control goat (25) and pre-infection (week 0) sera were negative (Table 4). The latex slide agglutination antibody test set up using pre-infection serum (week 0) week 6 and 7 post-infection sera of a goat from group B (6170) along with PBS as a control, for non specific agglutination of the latex beads, did not agglutinate with PBS and pre-infection serum but agglutinated with the post infection sera (Figure 23). The results of the 81 randomly collected field goat serum samples tested using latex beads coated with recombinant *Cowdria* MAP2 were as indicated in Appendix 3. Of the 81 random serum samples tested, 19 (23.4 %) were positive (P), 11 (13.6%) weak positive (WP) and 51 (63 %) negative (N) (Fig.25).



#### 4.8. Phase separation of *Cowdria* integral membrane proteins in Triton X-114 .

The Western immunoblot of the separated aqueous and detergent phases of Triton X- 114 extracts of *Cowdria* antigen showed a reaction band in the 21kDa. region of the aqueous phase lane. Such a band was not observed in the 21 kDa region of the detergent phase lane (Figure 24).

Table 2 :

Results of the evaluation of latex beads coated with aMAP2 against sonicated EBs, NP-40 and SDS-PAGE sample buffer solubilised EBs.

ANTIGEN	SONICATED EBs	NP-40 SOLUBILISED EB	SDS-PAGE SAMPLE BUFFER SOLUBILISED EBs
LATEX BEADS COATED WITH aMAP2	N	WA	WA

Sonicated EBs showed no agglutination (N), while solubilised EBs showed weak agglutination (WA).

Table 3 :

Latex slide agglutination antigen test results

PLASMA	7139	2380	9180	5335	8060	6170	CONTROL (25)
LTEX BEADS COATED WITH aMAP2 Ig	N	N	N	N	N	N	N

All the 7 goats were negative (N). The figures in the table are goat identification numbers.

Table 4 :

Latex slide agglutination antibody test results.

	[ W E E K S ]								
Tag No	0	1	2	3	4	5	6	7	8
7139	N	N	N	Dead					
2380	N	N	N	Dead					
9180	N	N	N	Dead					
5335	N	N	N	Dead					
8060	N	N	N	N	N	N	A	A	A
6170	N	N	N	N	N	N	A	A	A
25	N	N	N	N	N	N	N	N	N

Sera collected from goat Nos 7139, 2380, 9180 5335 (group A goats) and 25 (the control goat), showed no agglutination (N), while sera collected from goat Nos 8060 and 6170 ( group B goats) started showing agglutination (A) from week 6.

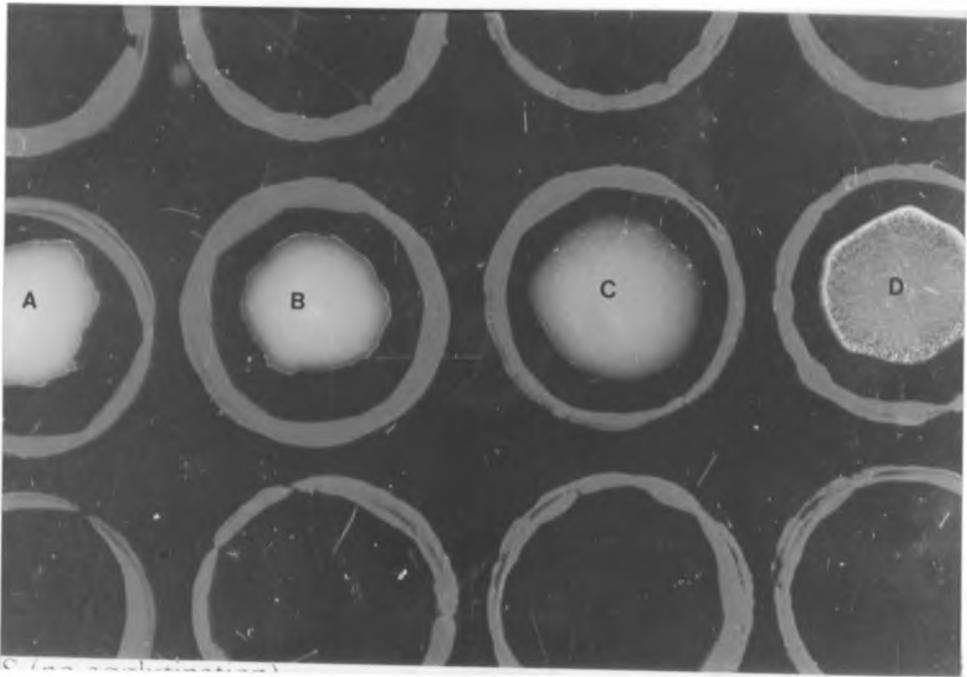


Figure 23 :

Latex slide agglutination antibody test set up using pre-infection and post-infection goat sera along with PBS, as a control, for non specific agglutination of the latex beads. The PBS control (A) and pre-infection serum (B) showed no agglutination, week 6 post infection (C) showed weak agglutination, while week 7 post-infection serum (D) showed strong agglutination.

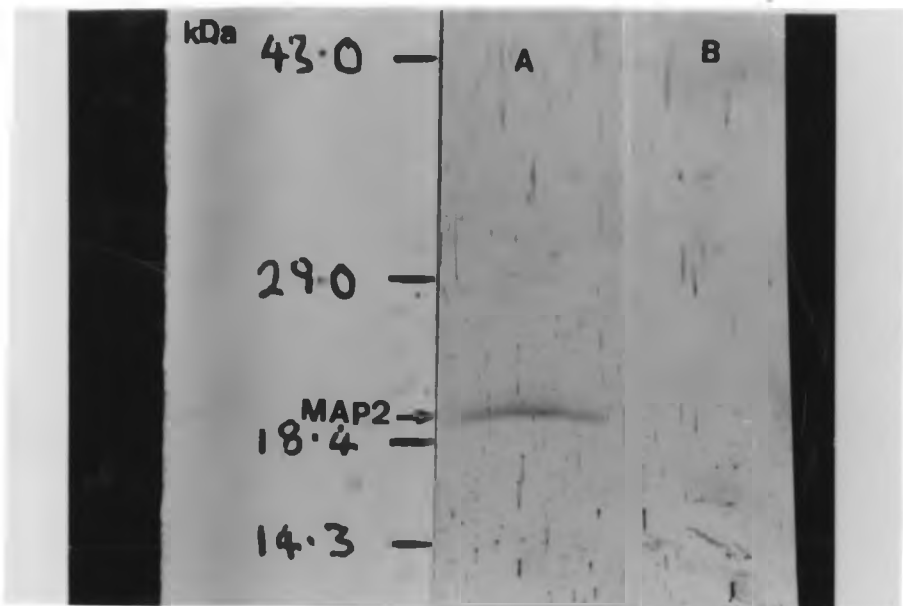


Figure 24

Western blots of Triton X- 114 aqueous and detergent phase extracts of *Cowdria* antigen probed with aMAP2. The aqueous lane (A) showed a reaction band in the 21kDa region, where MAP2 falls (arrow), which was not observed in the detergent lane (B).

Fig.25:LATEX RESULTS OF FIELD SERA

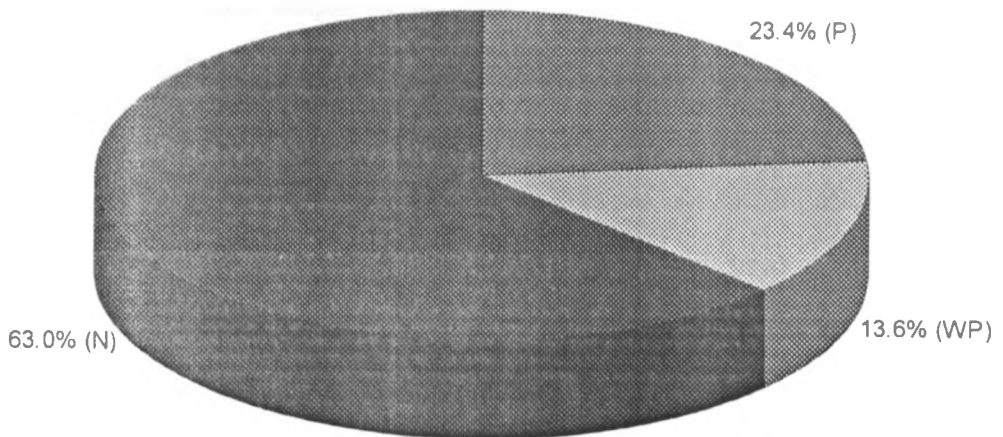


Figure 25 is a pie chart showing a summary of latex slide agglutination antibody test results of the 81 random field goat sera. 23.4% were positive (P), 13.6% were weak positive (WP) and 63.0% were negative (N).

## 5 DISCUSSION

The current diagnostic methods for cowdriosis using the Indirect fluorescent antibody test (IFAT), Enzyme linked immunosorbent assay (ELISA), DNA probes, infection of susceptible animals, and observations at post-mortem, require sophisticated laboratory equipment and well trained personnel. The IFAT and ELISA (Du Plessis, Bezuidenhout, Brett, Camus, Jongejan, Mahan and Martinez, 1993) tests are highly sensitive and simple to perform, but require specialised equipment and laboratories. The DNA probes (Mahan *et al.*, 1991) are more specific, but involve separation of DNA from the cells, a process which is long, tedious and requires expensive reagents. Therefore these diagnostic tests are not suitable for routine use in the field. The need for a simple diagnostic test for cowdriosis preferably at the pen side necessitates the development of a latex slide agglutination test that is simple, rapid and does not require sophisticated laboratory equipment nor specially trained personnel and is therefore suitable for routine use in the field. Available tests developed for serodiagnosis of heartwater, however, cross-react with *Ehrlichia* and *Chlamydia* species and therefore cannot solely be relied upon to make a confirmatory diagnosis of heartwater (Du Plessis *et al.*, 1987b, Jongejan, 1991b). In the present study, an attempt was made to develop a latex slide agglutination test using a recombinant major antigenic protein-2 (MAP2) (Mahan, McGuire, Semu, Bower, Jongejan, Rurangirwa and Barbet, 1994) of *Cowdria ruminantium*. Goats experimentally infected with *Cowdria* and confirmed to be infected using clinical, serological, and necropsy findings were used as a source of plasma and serum for validating the latex slide agglutination test.

Experimental goats infected with a Kiswani isolate of *Cowdria* exhibited a temperature of 40°C while the control goat did not develop fever and maintained a temperature close to the normal range of 37-39.7°C. Goats infected with a high dose developed a fever 5-12 days after intravenous inoculation with the *Cowdria* stabilate while goats inoculated with the low dose developed fever 17-18 days later. This indicated that the incubation period was influenced by the dose, an observation noted

earlier by Neitz (1968) and Uilenberg (1983). The clinical picture observed indicated that the disease in group A goats was a peracute syndrome. Group B goats were treated to prevent death and therefore allow harvesting of plasma and serum. Goats in group A died of heartwater as was confirmed by clinical and post-mortem observations, isolation of *Cowdria* in BPA cultures and demonstration of *Cowdria* in brain smears.

In order to characterise the antigen targeted for use in developing the Latex slide agglutination test, Western blots of crude *Cowdria* antigen and recombinant MAP2 were performed and probed with rabbit anti-MAP2 serum. In the crude *Cowdria* antigen Western blots, a 66 kDa antigen was identified in addition to the 21 kDa antigen (MAP2). Activity to the 66 kDa antigen in the rabbit anti-MAP2 serum persisted even after the serum was adsorbed using fetal bovine serum, bovine pulmonary artery endothelial cell lysate and *E. coli*. Therefore, the 66 kDa antigen may be a precursor of the 21 kDa antigen or an unrelated antigen but with shared epitopes. In dot-blots, rabbit anti-MAP2 serum reacted strongly with elementary body lysates and recombinant MAP2. The serum did not react with BPA lysates.

Latex beads coated with crude *Cowdria* antigen agglutinated with hyperimmune heartwater control serum but not with the preimmune control serum showing that the assay could detect *Cowdria* antibodies in the immune serum. In the Latex slide agglutination antibody test where day 0-12 sera of group A goats was tested, *Cowdria* antibodies were not detected. This was probably because the course of the disease in this particular group of animals was acute and the animals did not have adequate time to produce high titres of antibodies to *Cowdria*. Alternatively, the *Cowdria* specific antibodies produced at that time could have formed immune complexes with the circulating *Cowdria* antigen, and could thus not be detected by the latex antibody agglutination test. However, specific antibodies were detected in sera from goats inoculated with low dose of *Cowdria* stabilate and were subsequently treated. The goats had ample time to respond optimally to the challenge and therefore developed high titres of antibodies. It was not possible to detect *Cowdria* in blood of

infected goats during the febrile reaction using the Latex slide agglutination antigen test. This is probably because of either low rickettsemia, low levels of MAP2 from lysed elementary bodies in circulation, coating of circulating EBs with host proteins or the Latex slide agglutination antigen test was not sensitive enough. In addition, failure to detect elementary body antigens in circulation can be attributed to the fact that MAP2 is an internal and not an integral membrane protein as demonstrated by the phase partitioning of MAP2 in the aqueous phase. Therefore, MAP2 in intact elementary bodies was inaccessible to the monospecific rabbit anti-MAP2 serum. To further test this observation, intact and solubilized elementary bodies were tested with latex beads coated with rabbit anti-MAP2 serum. A weak agglutination reaction was observed with the solubilised elementary bodies and no agglutination was observed with the intact elementary bodies. This observation indicated that solubilization of elementary bodies exposed epitopes recognised by the rabbit anti-MAP2 serum. To further validate the Latex slide agglutination antibody test, 81 field serum samples randomly collected from a heartwater endemic area were tested. Of the 81 random serum samples tested, 19 (23.4%) were positive, 11 (13.6%) weak positive (border line) and 51 (63%) negative. The low level of positives detected (23.4%) may probably indicate that the latex slide agglutination antibody test is not very sensitive (but this hypothesis needs to be tested with at least 50 serum samples collected from confirmed cases of heartwater), the infection rate in the vector ticks is low or farmers practice stringent tick control. The sensitivity and specificity of the latex slide agglutination test used to detect other parasites can reach 98% and 99% respectively (Rurangirwa *et al.*, 1987; Kayang, Bosompem, Assoku and Awumbila, 1997), however, these parameters were not tested in the present study. Cross reactions with *Ehrlichia* and *Chlamydia* species may exist but are not a major constraint since some of the *Ehrlichia* and *Chlamydia* species do not infect domestic ruminants. Where they do infect domestic ruminants more specific tests such as DNA probes and PCR may be required (Mahan *et al.*, 1991; Peter, Deem, Barbet, Norval, Simbi, Kelly and Mahan, 1995).

The ability of the Latex slide agglutination antigen test to detect *Cowdria* antigen in the blood of the sick animals can be improved by coating the latex beads with monoclonal antibodies to MAP2 or by identifying a specific *Cowdria* surface antigen and using monoclonal antibodies to the specific surface antigen to coat the latex beads. The latex slide agglutination antibody test suffers the setback of being non specific due to antibody cross reactions between *Cowdria* and *Ehrlichia* species (Logan *et al.*, 1986; Camus, 1987 and Jongejan *et al.*, 1989). However, this limitation can be minimized by coating the latex beads with recombinant MAP1-B protein which does not cross react with *Ehrlichia* species which infect domestic ruminants (Van Vliet *et al.*, 1995).

### 5.1. Conclusion

The present study has shown that the latex agglutination test can detect *Cowdria ruminantium* antibodies in sera of animals which do not suffer acute heartwater, but was unable to detect *Cowdria* antigen in plasma of sick animals. The Latex agglutination assay was simple, easier to perform compared to IFAT, ELISA, DNA probes and PCR and it was possible to read the results visually within 5 minutes. Therefore, if improved, the latex agglutination test would be suitable for field use and ideal for testing of small numbers of animals as well as for bulk and batch testing.



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## **7. Appendices**

Appendix 1 :

Record of the preinfection daily rectal temperatures ( $^{\circ}\text{C}$ ) of the experimental goats over a period of 30 days.

Day	Animal No.						
	7139	2380	9180	5335	25	8060	6170
1	37.6	38.7	37.8	38.7	38.9	38.6	38.7
2	37.3	38.3	38.3	39.1	38.8	39	38.8
3	37.1	38.7	37.8	38.5	38	38.2	37.9
4	37.8	38.5	37.8	38.3	38.3	38	38.3
5	38.8	38.9	37.3	38	38.5	38	38.2
6	39	39.7	37.8	38.4	38.4	39.1	38.2
7	37.7	39.6	37.9	38.8	38.6	39.5	38
8	38	39.1	38.9	38.1	38.1	38.1	38
9	37.5	38.2	38.6	38.4	37.8	38.2	38.3
10	37.8	38	37.5	38.6	38.7	38.5	38.3
11	37.8	37.8	37	38.4	37.7	37.8	38.1
12	37.6	37.2	37	38.4	38.3	37.9	38.1
13	37.8	38.5	37.5	38.9	38.4	38	38.1
14	37.8	38.4	37.5	38.8	38.3	38.2	38.3
15	37.6	38.1	37.8	38.8	38	38.3	38.6
16	38	38.4	38.1	38.7	38.3	38.3	38.1
17	37.6	37.9	38.4	38.7	38.6	38.4	38.5
18	37.3	37.6	37.9	38.4	38	38	38
19	37.4	37.3	37.1	38.4	38.6	38	38.1
20	37.4	37.6	37.2	38.1	38.1	37.6	37.7
21	37.9	37.6	37.3	38.5	38.1	37.6	37.6
22	37.4	37.8	37.4	38.3	38.2	38.4	37.9
23	37.5	37.7	37.5	38.2	38.5	38.5	38.3
24	37.8	37.4	37.2	38.3	38	38	37.5
25	37.8	37.9	38.1	38.8	38.5	38.3	38
26	38	39	38.5	38.7	38.7	38.7	38.2
27	37.6	37.7	38.3	38.3	38.9	38.3	38.7
28	37.6	38.1	38.5	38.2	38.5	38	38.6
29	37.5	38	39.1	39.1	38.1	38.4	38.1
30	39	39.3	39.4	39.1	38.7	38.5	38.5

Appendix 2 :

Record of the postinfection daily rectal temperatures ( $^{\circ}\text{C}$ ) of the experimental goats over a period of 30 days.

Day	Group A				[[Control]]	Group B	
	7139	2380	9180	5335	25	8060	6170
0	38.6	39.5	39.2	39.5	39	38	38
1	38.9	39.1	39.4	39.5	39	38	38.1
2	38.9	38.8	39.4	39.5	38.6	37.9	38.3
3	38.3	38.2	39.2	39	38.1	38.1	38.9
4	38	38.4	39.6	38.4	39.1	38.3	38.6
5	38.4	38.6	40.2	38.6	38.8	38.5	39.1
6	38.8	38.5	40.1	39	38.8	38.4	39
7	39.5	39.4	39.5	38.8	38.4	38.1	38.6
8	38.9	39	39.2	38.9	38.2	38.5	38.7
9	38.3	38.5	Dead	38.8	38.4	38.5	38.7
10	38.8	39.4		40.1	38.6	38.5	38.9
11	39.9	40.5		40.4	39	38.3	38.9
12	40.4	Dead		Dead	38.6	38.2	38.1
13	Dead				38	38.7	39.4
14					38.2	38.4	38.6
15					38.3	38.4	38.9
16					38.8	38.5	39
17					38.6	38.9	40.1
18					38	40.4	39.9
19					38.7	41	39.9
20					39	41.3	39.3
21					38.8	40.8	39.3
22					38.7	40.1	39.5
23					38.2	38.8	39.3
24					38.6	38	40.5
25					38.5	38	39.3
26					38.5	38.1	38.7
27					38.3	37.9	38.3
28					38.5	38.1	38.1
29					38.3	38	38.2
30					39.1	38.4	38.5

The figures (numbers) in the top row represent animal identification numbers .

Appendix 3 :

Latex slide agglutination antibody test results of field goat sera

TAG No.	REACTION	TAG No.	REACTION
POS.CONTROL	P	2471	N
NEG.CONTROL	N	2435	N
2485	P	4792	N
2476	P	4716	N
3098	P	4746	N
2444	P	4770	N
2474	P	4717	N
2402	WP	4722	N
2500	P	4747	N
2488	P	4741	N
2472	P	4727	N
2437	WP	4754	P
2478	P	4757	N
2475	WP	4752	P
2441	P	4793	WP
2461	WP	4796	N
2497	WP	2248	N
2491	P	3606	N
2481	WP	2248	N
2404	P	2248	N
2411	P	2248	N
3002	WP	2261	N
2436	N	3606	N
2499	N	2261	WP
2477	N	2261	WP
2479	N	2261	WP
2414	N	2254	N
2416	P	3601	N
2446	N	2254	N
3001	N	2254	P
2439	N	2254	N
2447	N	2260	N
2493	P	3606	N
2422	N	2260	N
2463	N	2260	N
2420	P	2260	N
2442	N	4462	N
2401	N	3606	N
2406	N	4462	N
2434	N	4462	N
2412	N	4462	N
3095	N		

P = Positive; WP = Weak positive (Border line) and N = Negative.