

ADAPTATION OF COWDRIA RUMINANTIIUM TO MICE AND
CELL CULTURES

BY

DR. MICHAEL P. PARKLEA, B.V.M. (NAIROBI)

THIS THESIS HAS BEEN ACCEPTED FOR
THE DEGREE OF... *MSc (1991)* ...
AND A COPY MAY BE PLACED IN THE
UNIVERSITY LIBRARY.

A THESIS SUBMITTED IN PART FULFILMENT FOR THE DEGREE OF
MASTER OF SCIENCE OF THE UNIVERSITY OF NAIROBI

DEPARTMENT OF PATHOLOGY AND MICROBIOLOGY
FACULTY OF VETERINARY MEDICINE
UNIVERSITY OF NAIROBI

MARCH 1991

DECLARATION

This thesis is my original work and has not been submitted for a degree in any other University.

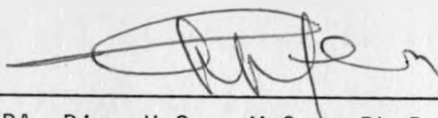


PROF. G. N. MUGERA, D.Phil., M.Sc., Ph.D.

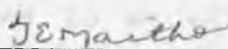
DR. MICHAEL PTOO PARKLEA

PROF. T. E. MATTHEW, B.V.M., M.Sc., Ph.D.

This thesis has been submitted for examination
with our approval as University Supervisors.



PROF. G. M. MUGERA, Dip. V.Sc., M.Sc., Ph.D.



DR. T. E. MAITHO, B.V.M., M. Sc., Ph.D.

DEDICATION

I would like to dedicate this book to my wife, Teresa, who has been my constant support and inspiration throughout my life. Her love and encouragement have been the driving force behind all my achievements. I also dedicate this book to my children, Nelly, Fabian, and Caroline, who have brought so much joy and meaning to my life. Their presence has made every challenge worth the effort. Finally, I dedicate this book to my parents, who have instilled in me the values of hard work, integrity, and perseverance. Their love and support have been the foundation of my life.

Dedicated to my wife Teresa together with our children Nelly Chepeng'at, Fabian Plimo, Caroline Chepyatich and their sibling whose welfare was a motivating factor to me.

ACKNOWLEDGEMENT

I express my sincere gratitude to my supervisors Prof. G.M. Mugeru of the Department of Veterinary Pathology and Microbiology and Dr. T.E. Maitho of the department of Public Health, Pharmacology and Toxicology for their assistance in the planning and carrying out of this research work. Their constructive criticism of the manuscript is highly appreciated. Special gratitude to Prof. G.M. Mugeru is hereby registered for his encouragement and valuable suggestions of alternative research methods when those attempted proved unworkable.

I am deeply indebted to my sponsor, Wellcome Kenya Ltd., who offered me a full scholarship to cover the whole course. Their full support during the course is highly appreciated knowing well that without their offer of the scholarship, this work would not have been done. In this connection, I record my special gratitude to Dr. G.H.M. Mulela who is the technical manager and Director of Wellcome (K) Ltd. for personally seeing to it that I received the relevant assistance from my sponsor and for procuring the experimental goats on my behalf.

The Director, International Laboratories for Research on Animal Diseases (ILRAD) kindly donated all the mice used in this experiment and the assistance is greatly appreciated. The stabilates of Cowdria ruminantium used in this research project were supplied by Dr. S. Waghela of Veterinary Laboratories, Kabeta (Tana River stabilate) and Mr. Leitch of KARI, Muguga (Transmara stabilate). Their assistance is greatly appreciated.

Many people assisted with the technical work in the laboratories and the efforts of all these people are appreciated. In this regard, Mr. Francis Njoroge of the Department of Veterinary Pathology and Microbiology played a special role of acquiring sheep and goat foetus from the abattoir and greatly assisted in the preparation of cell cultures from them. Likewise, Mr. Humphrey Kaburia of the department of Public Health, Pharmacology and Toxicology (PHPT) greatly assisted in all the experiments involving immunology. His assistance and valuable suggestions are highly appreciated. Mr. Daniel Sembe of the department of Pathology and Microbiology greatly assisted in the preparation of all the histological specimens and his efforts are also fully appreciated. Mr. Patrick Mwaurah of Vet. Labs. Kabeta greatly assisted in wordprocessing this thesis and his efforts are greatly appreciated.

My social life at campus was made more enjoyable with the companionship of fellow students especially Mr. Archangelo Byaruhanga, Dr. Badru Galiwango, Dr. Damiano Wale, Dr. William Karaba and Mr. Michael Cheruiyot. They were worthy companions and I wish them success in their academic struggles.

Finally, I am grateful to my younger brother Dr. Philip Toroitich Parklea of Kenyatta National Hospital for accommodating me in his house during the last phase of my studies.

Apart from the funds from my sponsor (above), this research was partly funded by the National Council for Science and Technology of Kenya Government (Vote No. 150-220-306). This financial support is greatly appreciated.

<u>TABLE OF CONTENTS</u>	<u>PAGE</u>
DECLARATION	ii
DEDICATION	iv
ACKNOWLEDGEMENTS	v
ABSTRACT	xv
INTRODUCTION	1
LITERATURE REVIEW	5
Aetiology	5
Epidemiology Including Vectors	8
Pathogenesis	9
Clinical Signs	10
Clinical Pathology	11
Diagnosis	17
Control	19
 <u>PART ONE: ASCERTAINING VIABILITY OF THE <u>C. RUMINANTIUM</u> STABILATE</u>	
MATERIALS AND METHODS	22
THE EXPERIMENTAL GOATS	22
THE EXPERIMENTAL MICE	22
STABILATES OF <u>C. RUMINANTIUM</u>	22
Tana River (Coast) Isolate	22
Transmara (Rift Valley Isolate .	23
INFECTION PROCEDURES IN GOATS	24
Infection of goat 425 with Tana River Isolate	24
Infection of goat 475 with Transmara Isolate	25
CLINICAL OBSERVATION OF EXPERIMENTAL GOATS	26

ORGAN SAMPLES AND IMPRESSION SMEARS TAKEN	26
Preparation of Organ Impression Smears	26
Preparation of Histological Samples	27
RESULTS FOR PART ONE	28
COURSE OF THE DISEASE IN GOAT 425 INOCULATED WITH TANA RIVER ISOLATE ..	28
COURSE OF THE DISEASE IN GOAT 475 INOCULATED WITH TRANS MARA ISOLATE ..	31
POST MORTEM FINDINGS	34
Impression Smears	34
Histological Sections	34
DISCUSSION AND CONCLUSION FOR PART ONE	35
<u>PART TWO: ADAPTATION OF <u>C. RUMINANTIUM</u> TO MICE</u>	38
MATERIALS AND METHODS	38
THE EXPERIMENTAL MICE	38
MICE INOCULATED WITH THE TANA RIVER ISOLATE	39
Intraperitoneal Inoculation of Mice .	39
Intravenous Inoculation of Mice	39
INOCULATION OF MICE WITH TRANS MARA ISOLATE	40
RESULTS OF PART TWO	41
OBSERVATION OF INFECTED MICE	41
ORGAN IMPRESSION SMEARS	42
GROSS AND HISTOPATHOLOGY	49
INDIRECT FLUORESCENT ANTIBODY TEST (IFAT)	64
Preparation of Antigen	64

RABBIT IMMUNIZATION	65
TESTING OF RABBITS IMMUNE STATUS	66
PURIFICATION OF RABBITS IMMUNOGLOBULINES	67
GEL FILTRATION CHROMATOGRAPHY OF THE PROTEIN SOLUTION	68
CONCENTRATION OF ELUATE FRACTIONS	72
DETERMINATION OF THE EFFLUENT FRACTION CONTAINING THE HIGHEST AMOUNT OF IgG.	73
ASSESSMENT OF IgG POTENCY IN FRACTION TWO	74
CONJUGATION OF IgG WITH FLUORESCCEIN ISOTHIOCYANATE (FITC)	74
REMOVAL OF UNBOUND FLUORESCCEIN DYE ..	75
ISOLATION OF OPTIMALLY-LABELLED IMMUNOGLOBULINS.....	76
PREPARATION OF UNCONJUGATED IMMUNE SERUM	77
PREPARATION OF ANTIGEN (<u>C. ruminantium</u>)	78
INDIRECT STAINING OF SLIDES	78
RESULTS OF IFAT EXPERIMENT	80
DISCUSSION AND CONCLUSION OF PART TWO	82
<u>PART THREE: ADAPTATION OF C. RUMINANTIUM TO CELL CULTURES</u>	92
MATERIALS AND METHODS	92
PREPARATION OF CELL CULTURES	92
PREPARATION OF LUNG CELLS FOR CULTURE	93
PREPARATION OF KIDNEY CELLS FOR CULTURE	95
PREPARATION OF ENDOTHELIAL CELLS FOR CULTURE	95
CARE OF THE CELL CULTURES	96

CELL PASSAGE	97
INOCULA AND INOCULUM PREPARATION	98
Inoculum One	98
Inoculum Two	99
Inoculum Three	100
INOCULATION PROCEDURES	100
Inoculation Experiment with Inoculum One	100
Inoculation Experiment with Inoculum Two	101
Inoculation Experiment with Inoculum Three	102
FIXATION AND STAINING OF INOCULATED AND CONTROL CELL CULTURES	102
Fixation and Staining of Cells Inoculated with Inoculum One ...	102
Fixation and Staining of Cells Inoculated with Inoculum Two ...	103
Fixation and Staining of Cells Inoculated with Inoculum Three	103
RESULTS OF PART THREE	103
Adaptation of <u>C. ruminantium</u> to Cell Cultures	103
RESULTS OF STAINED INOCULATED CELL CULTURES	104
DISCUSSION AND CONCLUSION OF PART THREE ..	106
REFERENCES	109

LIST OF TABLES

<u>TABLE</u>	<u>PAGE</u>
1: Mean daily rectal temperatures (°C) for goat 425 and control	29
2: Mean daily rectal temperatures (°C) for goat 475 and control	32
3: Mouse infection with the Tana River Isolate of <u>C. ruminantium</u>	40
4: Mouse infection with the Trans Mara Isolate of <u>C. ruminantium</u>	41
5: Spectrophotometric reading of eluate from Sephadex G-200	70

LIST OF FIGURES

<u>FIGURE</u>	<u>PAGE</u>
1: Mean daily rectal temperatures for goat 425 and control	30
2: Mean daily rectal temperatures for goat 475 and control	33
3: Spectrophotometric reading of eluate from Sephadex G-200	71

LIST OF PLATES

<u>PLATE</u>	<u>PAGE</u>
1: Intracytoplasmic <u>C. ruminantium</u> from Impression Smear of lymph node (Goat 475)	45
2: Smear of Aortic Intimal Scraping (Goat 425) showing <u>C. ruminantium</u> morula	45
3: Intracytoplasmic <u>C. ruminantium</u> in spleen impression smear (Goat 475)	46
4: Perinuclear <u>C. ruminantium</u> in squash smear of brain cortex (mouse)	46
5: Intracytoplasmic colony of <u>C. ruminantium</u> in liver impression smear (Goat 425)	47

6:	Intraendothelial granular colony of <u>C. ruminantium</u> in squash smear of mouse brain	47
7:	Perinuclear morula of <u>C. ruminantium</u> in squash smear of hypothalamus (Goat 425)	48
8:	Histological section of mouse kidney showing morulae of <u>C. ruminantium</u>	48
9:	Higher magnification of plate 8	51
10:	Histological section of mouse brain showing enlarged perivascular spaces	51
11:	Histological section of mouse kidney showing lymphocytic cell infiltration and other pathological changes	52
12:	Morulae of <u>C. ruminantium</u> in a histological section of cardiac muscle of mouse	52
13:	Histological section of mouse kidney showing extensive pathological changes	53
14:	Higher magnification of plate 13	53
15:	Histological section of mouse liver showing colonies of <u>C. ruminantium</u> in Kupffer cells	55
16:	Histological section of mouse brain showing enlarged perivascular spaces	55
17:	Histological section of mouse lung showing solidification and other pathological changes	56
18:	Higher magnification of plate 17	56
19:	Histological section of mouse kidney showing lymphocytic cell infiltration and other pathological changes	57
20:	Histological section of mouse kidney showing destruction of renal tubules by lymphocytic cells	57
21:	Histological section of mouse spleen showing haemorrhage and area depleted of lymphocytes	60
22:	Higher magnification of plate 22	60

23:	Histological section of mouse kidney showing lymphocytic cell infiltration and other pathological changes	61
24:	Higher magnification of plate 23	61
25:	Histological section of mouse liver showing lymphocytic cell infiltration	62
26:	Higher magnification of plate 25	62
27:	Histological section of mouse heart showing lymphocytic cell infiltration	63
28:	Higher magnification of plate 27	63
29:	Intracytoplasmic <u>C. ruminantium</u> in cultured kidney cells	105
30:	Intracytoplasmic <u>C. ruminantium</u> in cultured lung cells	105
31:	IFAT-positive confirming the presence of <u>C. ruminantium</u>	81
32:	IFAT-positive confirming the presence of <u>C. ruminantium</u> in peritoneal cell of mouse	81

ABSTRACT

Two isolates of Cowdria ruminantium were separately isolated from naturally-infected caprine and bovine species in two distant geographical locations in Kenya. The infected goat was in Tana River District (Coast Province) while infected cow was in Transmara region (Rift Valley). Two healthy adult goats were each inoculated intravenously with 10ml of either stabilate. Both goats suffered a clinical disease of heartwater and, at the height of fever, blood from them was collected in heparin (10 i.u per ml blood) and varying amounts of it inoculated into mice (60 mice per goat) as follows:-

The mice were divided at random into four groups of fifteen (15) mice each. Mice in groups one and two were inoculated intravenously with 0.1ml and 0.2ml respectively. Likewise, mice in groups three and four were inoculated intraperitoneally with 2ml and 5ml respectively.

Two mice from each group inoculated with either isolate were sacrificed every month and microscopic examination of the impression smears and histological preparations of kidney, heart, liver, spleen, lung and brain done. The C. ruminantium parasite could only be

observed for up to 196 days in the organs of mice which were inoculated intraperitoneally with either 2ml or 5ml of blood infected with the heartwater agent isolated in Tana River. No C. ruminantium parasites were observed in any of the mice infected intravenously with the same parasite and in all the sixty mice which were inoculated with the Trans Mara isolate of C. ruminantium.

Histological examination of kidney, lungs and hearts from infected mice showed pathological changes ranging from hydropic degeneration, haemorrhage, swelling, occlusion of renal ducts, lung hepatization, perivascular lymphocytic cell infiltration to necrosis of renal tubules. These changes were not associated with the presence of the parasite in the vicinity.

Indirect Fluorescent Antibody Test (IFAT) was done using infected mouse peritoneal macrophages as source of antigen (C. ruminantium). The conjugate was prepared using rabbit anti-mouse immunoglobulin G (IgG) with fluorescein isothiocyanate. The reaction procedure was conventional and observation was by a fluorescence microscope at 495nm. The reaction was positive and confirmed the presence of C. ruminantium.

Lung, kidney and endothelial cells were prepared from sheep and goat fetuses by trypsin dissociation then cultured at 37°C in Eagle minimum essential medium

(MEM) with 10% foetal bovine serum (FBS). The resulting monolayers of cells were separately inoculated with C. ruminantium either as blood stabilate or homogenised infected mice organs. The inoculum was mixed with MEM (one third by volume) prior to introducing it onto the cultured cells and incubating at 37°C for 90 to 105 minutes. The inoculum was then decanted and the culture medium reintroduced and incubated at 37°C. The culture medium over the cultured cells was changed on day two of culture and thereafter on every third day of culture. At intervals of three days, Leighton tubes, one for each cell type, had their culture media decanted and the cell contents fixed with methanol for 5 minutes prior to staining with 10% Giemsa. Microscopic examination revealed dense intracytoplasmic parasites in the cultured cells. There was no difference in the appearance of the parasites in the lung, kidney or endothelial cells.

Passage of C. ruminantium in mice was attempted by intraperitoneal inoculation of ten healthy mice with homogenised infected tissues pooled from four mice. Microscopic examination of the organ preparations of the inoculated mice (as above) did not reveal any parasites.

INTRODUCTION

Heartwater, or cowdriosis, is an acute septicaemic rickettsial disease of ruminants in Africa south of Sahara and in the Caribbean region. The disease derives its name "Heartwater" from its characteristic lesion, hydropericardium, which is found in most affected animals. It is caused by Cowdria ruminantium and is transmitted by at least five species of Amblyomma ticks. Of the tick-borne diseases in East Africa, this disease is second to East Coast fever in importance especially that it is fatal to cattle, sheep and goats. It causes indirect and direct losses to the stock owners; indirect losses being due to the expensive and repetitive tick control measures while direct ones arise from the death of affected animals.

Animals other than ruminants are refractory and difficult to infect with this disease but it is believed that wild ruminants act as reservoirs of infection. Calves, kids and lambs less than one month old have a high degree of resistance against heartwater but their resistance is not related to their maternal immunity to the disease. In this regard, the mortality rate raises with increasing age of affected animals and peaks at early maturity. It is also known that European breeds of cattle, sheep and goats are more susceptible to heartwater than are the indigenous breeds.

After artificial infection of cattle, the incubation period is an average of 14 days long but the incubation period after natural infection is usually four days longer. Affected animals may exhibit peracute, acute, subacute, mild or inapparent forms of the disease. Highly susceptible ruminants such as the European breeds usually suffer the peracute form of the disease which is characterised by sudden death after a high fever and nervous signs terminally. These signs include unsteady gait and circling hence the name "Turning sickness". At the other extreme is the inapparent form of the disease which consists of a temperature reaction only and is usually seen in Zebu cattle which are fairly resistant to the disease.

The duration of the illness prior to the animal's death determines the gross lesions encountered. The lesions encountered most often are hydrothorax, ascites, hydropericardium, splenomegaly, froth in trachea and petechial haemorrhage on the surface of the heart and lungs. There may be hyperaemia of the lungs and meninges. Histologically, necrosis and other degenerative changes may be seen in the kidneys, lungs and liver along with oedema in these organs apart from the spleen and brains of affected animals. The aetiological agent, Cowdria ruminantium, can also be

immunologic differences among themselves, whereby some cross-protect fully, others partially while others do not cross-protect at all. The objectives of this research project were:

- (1) Attempt to adapt Kenya's isolates of C. ruminantium to laboratory animals (Swiss mice). A laboratory model of heartwater would be of great potential importance for future studies on aspects such as pathogenesis, diagnosis, immunology and chemotherapy of the disease.
- (2) Attempt to attenuate the Kenyan isolates of C. ruminantium using laboratory animals. Its success would render it for use as a vaccine in future.
- (3) Attempt to grow two Kenyan isolates of C. ruminantium in cell cultures. Its success would enhance the study of the parasites biological activities, antigens and vaccine development from it.
- (4) To demonstrate the persistence of the parasite in the tissues of mice. This may provide a cheap means of transporting and "storing" C. ruminantium as an alternative to using liquid nitrogen. A study on the potential role of mice as reservoirs of heartwater disease agents can then be undertaken.

LITERATURE REVIEW

AETIOLOGY

Cowdria ruminantium is a highly pleomorphic non-motile micro-organism which may be intracellular or occur free as a morular which is often surrounded by a halo. More than one colony may be present within a single cell and the cell membrane of such an affected cell may be stretched without exhibiting any visible damage. (Prozesky and Du Plessis, 1987). The organisms within the cells are released into the general circulation following the rupture of the parasitized cells.

Cowdria ruminantium is an organism which has been observed to have two morphological forms: electron-dense and reticulated forms in ticks (Kocan et al, 1987) and in cultured endothelial cells (Prozesky et al, 1986). Although organisms within a particular vacuole are usually of a specific form, mixed colonies have been identified in cultured endothelial cells (Prozesky, 1987a). The failure to identify mixed colonies in vertebrate hosts led Prozesky and Du Plessis (1987) to suggest the causes of mixed colonies but, the factors responsible for their formation remain largely unknown. The role which the reticulated and the electron-dense forms play in the organism's life cycle is unclear but Prozesky and Du Plessis (1987) have suggested that the reticulated forms are the predominant vegetative form.

Many researchers have attributed various modes of replication to C. ruminantium without elaboration of the circumstances which determine the mode to be adapted by the organism at various times. The modes of replication which have been reported for this organism so far are endo-sporulation (Pienaar 1970); budding (Prozesky and Du Plessis, 1987) and binary fission (Kocan et al, 1987; Munene, 1987; Prozesky and Du Plessis, 1987). Du Plessis (1970) reported that C. ruminantium initially replicate in the lymph nodes after which the organisms are released into the bloodstream and thus endothelial cells are subsequently parasitized.

Apart from recent successes in the in vitro cultivation of C. ruminantium (Bezuidenhout, 1987a; Jongejan et al, 1980), all previous attempts to cultivate the organism in cell cultures had met with little or no success. A review by Uilenberg (1983) has summed up previous work which demonstrated that such varied factors as the inoculum, cell line, medium, antibiotics and the techniques used all affect the growth of C. ruminantium. The variation of these factors in works by previous researchers may explain the difficulty previously encountered in growing this organism in tissue cultures. Jongejan et al (1980) used primary goat kidney cell cultures in their attempt to grow C. ruminantium in vitro but the organism persisted

for only 13 days. They concluded that the persistence of the organism for those few days may have been an indication that the organism simply survived but did not multiply in the tissue culture. Although these researchers were not able to detect the organism microscopically, they succeeded in infecting goats with heartwater from the cell culture. This may mean that the organisms in their culture occurred not as colonies but as scattered individual particles which would be very difficult to detect. Effective storage and preservation are important for maintaining isolates of the organism for research and for the eventual development and distribution of heartwater vaccines. In this respect, Logan (1987) sums that it has been conclusively demonstrated that C. ruminantium can be effectively preserved in a variety of organ suspensions at low temperatures (-70°C to -196°C) for indefinite periods of time. Ilemobade and Blotkamp (1975) found that an isolate of C. ruminantium from Nigeria could be frozen with or without dimethylsulfoxide (DMSO) without alteration of virulence to sheep and goats. This finding led them to conclude that those isolates, from other countries, which seem to require the use of a cryoprotectant (DMSO) appear to be more fragile than the Nigerian isolate. Stewart (1987b) cites Du Plessis (1982) who incubated macrophages and the Kumm strain of C. ruminantium in vitro and showed that the parasites entered the macrophages. According to Stewart (1987b),

this observation may explain why it is possible to infect mice, using this strain, by the intraperitoneal route whereas other mice-adapted strains must be given intravenously.

EPIDEMIOLOGY INCLUDING VECTORS

During the last Century, there was a long-standing suspicion that ticks, especially the bont tick, Amblyomma hebraeum, play a role in the transmission of heartwater. According to Bezuidenhout (1987b), this suspicion was proved correct experimentally by Lounsbury in 1900. The twelve proven Amblyomma vectors are not all of equal significance in the transmission of the disease. Bezuidenhout (1987b) and Walker (1987) suggested that the most important vector is A. variegatum followed by A. hebraeum which is apparently the major vector of heartwater in Southern Africa (Walker 1987). In an article on the ecology of ticks which are potential vectors of heartwater in Africa, Petney et al (1987) have listed the hosts, sites of attachment, life cycle, habitat requirements and seasonal abundance of Amblyomma species. They have also discussed the interactions of the ticks with other species and the role of predators and pathogens in reducing the numbers of ticks present at a given time. This information is important in the planning of control measures aimed at the vectors of heartwater.

PATHOGENESIS

Infected animals serve as a source of infection for ticks but it is not necessary for an animal to be clinically affected for it to act as a reservoir of the causative agent. In such animals, C. ruminantium infection manifests itself as a latent infection (Du Plessis et al 1984, cited by Van De Pypekamp and Prozesky, 1987). According to Oberem and Bezuidenhout (1987a), other subclinical carriers of C. ruminantium which may act as a source of organisms for ticks include wild animals such as the crowned guinea fowl (Numida meleagris) and the leopard tortoise (Geochelone pardalis).

In their efforts to determine the sites of development of C. ruminantium in the vector, Kocan et al (1987) identified the organisms in the midgut epithelial cells and salivary gland acini cells of the ticks. These findings led these researchers to conclude that the organisms initially develop in the salivary glands of the ticks. The feeding of the infected tick thus leads to transmission of the organisms through saliva to the vertebrate host. In naturally-infected animals, infective organisms are either drained from the site of infection by the afferent lymphatics or phagocytosed by leukocytes which are in turn either drained by the afferent lymphatics to the regional

lymph nodes or directly into the general circulation (Prozesky and Du Plessis, 1987). This is in general agreement with the findings of Du Plessis (1970) who reported that C. ruminantium initially replicate in the lymph nodes after which the organisms are released into the bloodstream and thus endothelial cells become parasitized. Depending on the host, these organisms appear to have a predilection for endothelial cells in selected organs. In ruminants, the highest concentration of organisms is found in the brain followed by the kidneys whereas in mice infected by the Welgevonden strain, the highest concentration of organisms is in the lungs (Prozesky and Du Plessis, 1985). Further research to determine the factors which enable these organisms to concentrate in their predilection sites in different organs of the various animal species is desirable.

CLINICAL SIGNS

Heartwater is caused by Cowdria ruminantium and its clinical manifestation is frequently characterized by fever and nervous signs. The nervous signs are frequently seen in animals affected by the peracute and acute forms of heartwater and these signs are attributed to oedema of the brain (Van De Pypekamp and Prozesky 1987). These researchers also reported that in few cases of non-fatal heartwater, complications such as permanent blindness, torticollis and permanent

recumbency are manifested occasionally. The macroscopical lesions on most animals with heartwater include hydropericardium, effusion into body cavities, oedema of the lungs and lymph nodes, and splenomegaly (Prozesky 1987c). In spite of these findings, macroscopical lesions alone are not sufficient to make a diagnosis of heartwater. Confirmation requires the demonstration of C. ruminantium in either brain smears or histopathological sections of organs.

CLINICAL PATHOLOGY

Camus and Barre (1987) reported that rickettsemia in goats reacting to heartwater is limited to the fever period only but Uilenberg (1983) had earlier reported that an animal's blood is infective for ticks from the start of the febrile reaction until a few weeks after recovery. This report is in agreement with the findings of Ilemobade (1978) who found blood to be infective 40 days after recovery in one case and 50 days after challenge in another case. In this regard, recovered animals are held suspect as carriers of heartwater. In a recent experiment, some experimental goats being used by Barre and Camus (1987) recovered spontaneously from heartwater. This development convinced these researchers to acknowledge that recovered goats are possible carriers of the parasites but they doubt the significance of goats as reservoirs of infection.

Apart from the presence of heartwater organisms in most organs, the histopathological lesions are not so obvious. The mild cytopathic changes in parasitized cells indicate that the damage caused by the organisms is most probably not responsible for the increased vascular permeability because the damage to capillary endothelial cells caused by the organisms is limited (Prozesky, 1987c). The absence of a direct link between the presence of the colonies of the heartwater agent and cytopathic changes of the cells harbouring them had earlier been confirmed by Prozesky and Du Plessis (1985). According to these researchers, their findings complement Cowdry's (1925a) observation that the organisms cause little damage to parasitized cells.

Van Amstel et al (1987) cite Clark (1962) who reported that the light-yellow transudates in fatal cases of heartwater frequently coagulate on exposure to air. This coagulation is an indication that large protein-like fibrinogen can pass through capillary walls in the course of this disease. More recently, Prozesky (1987c) confirmed this observation but could not explain how these large molecules pass through the blood vessels when it is widely acknowledged that C. ruminantium organisms cause minimal damage to endothelial cells. This is a pointer to the fact that the pathogenesis of heartwater is one very important aspect of the disease which is not well understood

currently. Despite this obscurity, Du Plessis et al (1987) stress that there is a general agreement that increased permeability of smaller blood vessels plays an important role in the pathogenesis of the disease. Opinions differ, however, on how the increased permeability is caused. Vascular lesions in the brain were at first ascribed to the direct necrotizing effect of the heartwater agent (Pienaar et al 1966 cited by Du Plessis et al, 1987). This theory lasted only a few years and according to Du Plessis et al (1987), Pienaar (1970) subsequently favoured the toxin hypothesis which most researchers subscribed to at that time. To date, such a toxin has not been demonstrated and no experimental evidence supports this theory. Likewise, Du Plessis et al (1987) cite Du Plessis (1975) who attempted to explain the lesion by advancing a hypothesis that vaso-active substances liberated by mast cells are responsible for the increased permeability. In response to this hypothesis, unsuccessful attempts were made to demonstrate a rise in blood levels of serotonin or histamine in sheep and mice with fatal heartwater. According to Du Plessis et al (1987), the hypothesis of vaso-active substances is invalid and they have gone ahead to suggest that there is an involvement of an immune-complex type of hypersensitivity reaction in the pathogenesis of heartwater. They state that this may be triggered by

the release of pharmacologically-active substances through a mechanism which is yet still to be determined through further research.

It has been known for many years that cattle of different breeds and ages have varied susceptibility to heartwater. Although the great differences in susceptibility between local and exotic breeds in Africa have been confirmed, Uilenberg (1983) concluded that the innate resistance has nothing to do with Zebu influence. This means that Zebus which have not been exposed to heartwater remain fully susceptible despite being of Zebu breed.

Du Plessis and Malan (1987b) reported that the suckling-calf resistance to heartwater is at its strongest within the first month after birth but that the resistance isn't absolute. This resistance diminishes with advancing age and appears to be at its lowest about 9 to 12 months. Though the mechanism of the suckling-calf resistance is poorly understood, these researchers clarified that the resistance bears no relationship to the susceptibility or immunity of the dam and must not be confused with passive immunity transmitted through colostrum. On the other hand, Du Plessis and Malan (1987b) confirmed earlier suggestions that conglutinin is the serum factor which plays a role in the non-specific resistance of older cattle and that

conglutinin-associated resistance to heartwater takes effect at a much later stage than the suckling-calf resistance. Since the former strengthens as the latter wanes, this researcher thus concluded that the resistance of cattle from calf to adulthood has both specific and non-specific components and that the effects of the two overlap. In an attempt to explain the non-specific resistance, Matheron et al (1987) suggested that genetic effect may be one of the most important factors involved in individual resistance. Their hypothesis is that a recessive sex-linked gene could be involved in the control of resistance.

Hiltenberg (1983) was of the opinion that animal resistance to heartwater is a resistance against severe reaction and, consequently, not an absence of susceptibility. From this suggestion, it follows that the resistant as well as susceptible animals are prone to infection but they will respond differently to the infection depending on their previous heartwater histories. This is in agreement with Alexander (1931) - cited by Stewart (1987a) - who reported that no immunity could be demonstrated in any farm animal that had not passed through an attack of the disease.

Reports on resistance to heartwater have also been made on sheep and goats but there is controversy on the resistance of indigenous small ruminants. Some authors noted a higher resistance on the part of the local

breeds while other researchers deny this or observed heavy losses among them (Ilemobade 1977, Karar, 1960). In an attempt to settle this argument, Matheron et al (1987) have presented data which tends to support the opinions of both the opposing groups of researchers depending on how their findings are interpreted. As stated earlier by Uilenberg (1983), variation in resistance to heartwater may not be a question of breed but of population. Matheron et al (1987) tended to accept that view when they stated that differences in susceptibility do not appear to be linked to any particular breed or species but probably depend mainly or exclusively on inherited resistance acquired by local livestock. Though these researchers are in favour of acquired resistance only, Gradwell et al (1976) blamed innate as well as acquired immunity as the causes of the resistance of several species of wild ruminants which they couldn't infect artificially with heartwater.

The mechanism by which the immune response develops remains largely unknown especially that Du Plessis (1984) is cited by Stewart (1987a) as having found no correlation between antibody titres and the immune status of calves to heartwater. It is unclear whether sterile immunity or premunition plays a role in the maintenance of protective immunity but Ilemobade (1978) demonstrated the persistence of C. ruminantium

in the blood of animals for upto 50 days. More recently, Stewart (1987b) cited Du Plessis (1982) as having been able to recover the organisms from the tissues of mice 365 days after infection.

Stewart (1987b) cites Du Plessis (1982) who reported that the protective immune response which develops in mice is apparently cell-mediated in nature and that humoral antibodies do not appear to play any role in infection with at least the Kumm strain of heartwater. The various immune mechanisms which occur in heartwater as well as other Rickettsia has recently been reviewed by Tringali et al (1983)

Logan et al (1987) studied the cross-immunity between four isolates of C. ruminantium in goats and mice. Two of the isolates (Kwanyanga and Kumm) were from South Africa; one from Mali and the fourth from Guadealope (Gardel). In this experiment, these isolates demonstrated antigenic variation.

DIAGNOSIS

Prior to the report by Purchase (1945) of the brain smear technique for heartwater diagnosis currently in use, demonstration of C. ruminantium in smears used to be made from the intima of large blood vessels and in tissue sections (Jackson, 1931 - cited by Prozesky, 1987d). The method of Purchase (1945)

involves opening an animal's skull for samples but Schreuder (1980) has reported an easier method of obtaining brain samples for smears through the foramen occipitale thus obviating the need to open the skull in large animals. In his report, Purchase (1945) reported that capillaries were not easily detected in the cerebellum as in the cerebrum but this was contradicted by Schreuder (1980) who reported that the vessels were readily detected both in the cerebellum and cerebrum.

Apart from the above method of post mortem diagnosis of heartwater, researchers have sought other diagnostic methods which are mainly immunological. Two recent reports by Viljoen et al (1987) and Du Plessis and Malan (1987a) have summarized the immunological methods for the detection of antibodies to C. ruminantium that have so far been recorded in the literature. They include Indirect Fluorescent Antibody test (IFAT) and Enzyme - linked immunosorbent assay (ELISA) technique whereby the latter technique is reported by Viljoen et al (1987) to be 500 times more sensitive than the other known methods used in C. ruminantium assays.

Heartwater in mice can also be confirmed microscopically and serologically as mentioned for cattle. Likewise, Prozesky (1987b) reported that conclusive confirmation of the presence of C. ruminantium in mice can also be effected by

subinoculating infective mouse tissues into susceptible ruminants and subsequently demonstrating the organism in the latter's tissues.

CONTROL

Neitz and Alexander (1941) were among the earliest to develop a vaccine against heartwater. Their method was used until recently when Bezuidenhout (1981) reported an alternative cheaper method of vaccination. He introduced the use of homogenized C. ruminantium - infected Amblyoma hebraeum nymphs as inoculum but this did not solve the usual problems caused by live and virulent vaccines because the vaccine had these two qualities. While the immunity elicited with the tick vaccine was similar to that stimulated by the blood vaccine, Van Der Merwe (1987) lamented the great disadvantage of the former as being the allergic shock that sometimes occurred after intravenous administration of the vaccine thus requiring intensive treatment of affected stock with antihistamines. This expense becomes even greater if the infection-and-treatment method of vaccination is practiced. This method entails the close monitoring of vaccinated animals and the application of treatment as soon as the febrile reaction commences as summarized by Uilenberg (1983).

Although infected brain material has been found to be infective if administered subcutaneously (Ilemohade and Blotkamp, 1978), only blood and nymph suspension have ever been produced and used commercially as vaccines. Methods of production and the limitations of both of these vaccines have been reviewed by Uilenberg (1983). Apart from this review, Oberem and Bezuidenhout (1987b) have also summarized the present-day techniques of vaccine production in details. The fact that this is a live virulent vaccine precludes its use in heartwater-free areas such as United States of America where potential vectors exist (Barre et al, 1987; Uilenberg et al, 1984).

Of all the animal diseases where vaccination is practiced, heartwater is the only one where intravenous inoculation is still a prerequisite for successful vaccination unlike the case of the other diseases whose vaccines are administered subcutaneously (Bezuidenhout et al, 1987). Despite this observation, these researchers carried out experiments which showed that there is a high chance of success of subcutaneous and intramuscular inoculation against heartwater.

Du Plessis and Malan (1987c) reported that immunogenicity formed against heartwater depends on the pathogenicity of the infecting organism. This finding implies that avirulent organisms would be less immunogenic and lack protective ability as a vaccine.

This is contrary to the findings of Bezuidenhout et al (1987) who reported that some of their experimental sheep developed immunity after only a slight temperature reaction or no reaction at all. Their report is in agreement with what was reported earlier by Mare (1972) that immunity to heartwater may develop without a strong vaccination reaction. It also agrees with Alexander (1931) - cited by Stewart (1987a) - who reported that the severity of the heartwater reaction does not have any influence upon the degree of immunity so long as a reaction is produced.

Apart from these views on the relationship between the severity of the disease and the resulting immunity, other factors seem to play a role in the immunity of mice to heartwater. Bezuidenhout et al (1987) concluded that in mice, the strain or isolate of C. ruminantium is a very important factor in the success rate of intraperitoneal and subcutaneous inoculations.

The control of heartwater by means of tick control using acaricides has been practiced by farmers for a long time. Bezuidenhout and Bigalke (1987) reported that the main objective of strategic control of ticks is to minimize "Tick worry" without interfering too drastically with natural transmission of heartwater. This would still allow natural immunization of young animals and the maintenance of their immunity thus

minimizing deaths from the disease. Tick control coupled with vaccination of livestock against heartwater affords the livestock better defence against the disease.

PART ONE

1. ASCERTAINING THE VIABILITY OF THE COWDRIA RUMINANTIUM STABILATE

1.1 MATERIALS AND METHODS

1.1.1 THE EXPERIMENTAL GOATS

The four goats (Capra hircus) used in this experiment were purchased from Yatta in Machakos district and transported by vehicle to the department of Veterinary Pathology and Microbiology. They were sprayed with an acaricide (Delnav Wellcome (K) Ltd.) on the second day of their arrival. The goats were housed in concrete-floored stalls and provided with hay, wheat bran and water throughout the duration of the experiment.

1.1.2 THE STABILITATES OF COWDRIA RUMINANTIUM

1.1.2.1 THE TANA RIVER (COAST) ISOLATE

It was obtained from the Veterinary Laboratories, Kabete, as a blood stabilate which had been preserved in liquid nitrogen (-196°C) for ten days. Originally,

the stabilate was obtained from a febrile (40.6°C) naturally-infected goat at Galana Ranch of Tana River district (Kenya). Soon after drawing the goat's blood from the jugular vein into a sterile flask containing heparin (10i.u heparin: 1ml blood), it was mixed with dimethylsulfoxide (DMSO) whereby the latter was 10% of the final volume. The DMSO is a cryoprotectant. The blood was then dispensed into 20ml plastic vials having screw lids. The vials were then snap-frozen by being immersed in liquid nitrogen and maintaining it therein until needed.

After removing the stabilate from liquid nitrogen, it was transported to the Department of Veterinary Pathology and Microbiology where it was thawed on a table top at room temperature.

1.1.2.2 THE TRANSMARA (RIFT VALLEY) ISOLATE

This was obtained from Kenya Agricultural Research Institute (KARI), Muguga, as a cryopreserved blood stabilate. It had initially been obtained by venipuncture of the jugular vein from a naturally-infected bovine from Transmara region of Rift Valley province. The container into which the blood was drawn contained ethylenediamine tetra-acetic acid (EDTA) at 1mg to 1ml blood. The DMSO was added at 10% of the final volume then dispensed into slender glass vials of 2.8ml each. These vials were then stored overnight at

-80°C then transferred into liquid nitrogen (-196°C) where the vials remained until the stabilate was required.

For this work, six of the vials were removed from the liquid nitrogen and placed in a large plastic beaker packed with ice. This was then transported immediately by vehicle to the department of Veterinary pathology and Microbiology where it was allowed to thaw slowly in the melting ice. The contents of all the six vials were then pooled in a cold universal bottle standing in ice ready for injection into the experimental animals.

1.1.3 INFECTION PROCEDURES IN GOATS

Two adult female goats (Capra hircus) of the small East African, breed were used in this experiment. This was done after the goat, together with 2 other control goats, had been in a stall for acclimatization for seven days during which time their rectal temperatures was taken and recorded twice daily. The pre-infection temperature range was 38.9°C to 39.2°C. During that period, the goats were kept and fed as in section 1.1.1.

1.1.3.1 INFECTION OF GOAT 425 WITH THE TANA RIVER ISOLATE

As soon as the stabilate was completely thawed, it was mixed by gentle inversion movements of the vial. An 18 gauge needle and a sterile disposable syringe was

used to draw 10ml of the stabilate which was then injected gently into the jugular vein of the experimental goat. The post-infection rectal temperature was taken and recorded twice daily. Its averages are shown in Table 1 and Figure 1. When the temperature rose to 41.7°C on day 14 post-infection, the goat was well restrained and 120ml of blood drawn from it by jugular venipuncture using a 14 gauge needle without a syringe. On venipuncture, the blood flowed freely through the needle into a clean sterile flask containing heparin (Paines & Byrne Ltd, Greenford, England) at a ratio of 10i.u heparin to 1ml blood. The blood was constantly swirled to facilitate proper mixing of the blood and heparin and thus prevent clotting.

1.1.3.2 INFECTION OF GOAT 475 WITH TRANSMARA ISOLATE

The thawed stabilate of the Transmara isolate of C. ruminantium was drawn into a 10ml syringe with 18 gauge needle and injected intravenously into the jugular vein of the experimental goat. The goat's rectal temperature before and after infection was monitored as done under section 1.1.3.1 and is shown in Table 2 and figure 2. When the temperature rose to 40.9°C on day 16, the goat was bled in a similar manner to goat 425 (section 1.1.3.1).

1.1.4 CLINICAL OBSERVATION OF THE EXPERIMENTAL GOATS

After drawing blood from goats 425 and 475 for inoculation into mice, the course of the disease in both goats was monitored daily. Their daily mean temperature are shown in Tables 1 and 2, and figures 1 and 2 respectively. The heartwater disease in goat 425 which was inoculated with the Tana River isolate of C. ruminantium had an incubation period of 12 days while in goat 475 (Transmara isolate) the disease had an incubation period of 14 days.

1.1.4.1 ORGAN SAMPLES AND IMPRESSION SMEARS TAKEN

From each goat and each mouse which was subjected to post mortem examination, the following samples were taken and prepared appropriately for microscopic examination:

- (a) Impression smears of the kidney, spleen, liver and lung in separate microscope glass slides.
- (b) Squash smear of the brain according to the method of Purchase (1945).
- (c) Kidney, liver, heart, spleen, lung and brain; all these were fixed in 10 per cent (10%) formalin for histological examination.

1.1.4.2 PREPARATION OF ORGAN IMPRESSION SMEARS

The impression smears of each organ were made on a separate clean and dry glass slide. After cutting the desired organ with a clean scalpel blade, excess fluid

on the cut surface was removed by gently pressing a clean tissue paper on it to absorb the fluid. The impression smears were then made by gently pressing the glass slides on the cut surface of the organ. The slide was then dried immediately by waving it in the air.

The smears were fixed by flooding the slides with methanol for three minutes after which they were stained with 10 per cent (10%) Giemsa stain for 20 minutes. The stained smears were then washed with tap water and left to dry at room temperature before being observed microscopically.

1.1.4.3 PREPARATION OF HISTOLOGICAL SAMPLES

All the organ samples, from the goats and mice, which were taken for histological examination were trimmed to an approximate thickness of one centimetre then submerged in fresh 10% formalin. After fixing for at least 72 hours, sections were made from the fixed tissues, mounted and stained according to Kiernan (1983).

From each organ sample, two histological sections were made, mounted on separate slides then stained either with Giemsa or Hematoxylin and eosin (H&E).

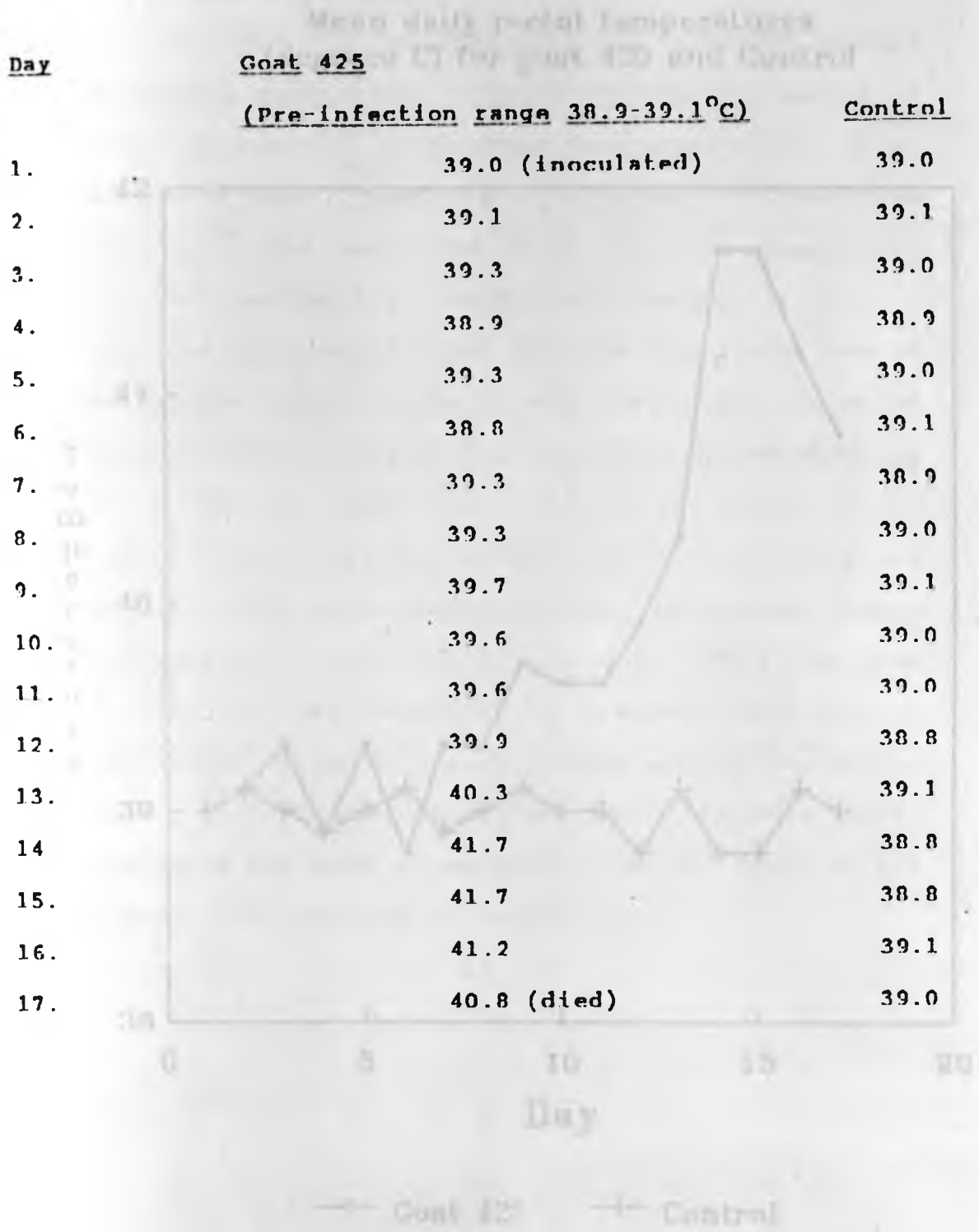
1.2 RESULTS FOR PART ONE

ASCERTAINING THE VIABILITY OF THE C. RUMINANTIUM STABILATE

1.2.1 COURSE OF THE DISEASE IN GOAT 425 INOCULATED WITH TANA RIVER ISOLATE

The disease syndrome in this goat lasted 5 days. At the onset of fever ($>40^{\circ}\text{C}$) the goat lost appetite and fed only occasionally and at other times, it would stop masticating the hay in its mouth. Such hay could remain in the mouth for upto 10 minutes then the goat would masticate slowly. The goat could drink a little water then just stand near the water trough but with a tendency to lean against an adjoining wall. It was dull and held its head low most of the time. When encouraged to move with a gentle push, it would hold its head high and take a few steps before stopping and lowering the head again. During the last clinical examination, the rectal temperature had dropped from 41.7°C to 40.8°C . The goat was found dead four hours after the last clinical examination and it was immediately subjected to standard necropsy examination.

Table 1: Mean daily rectal temperatures ($^{\circ}\text{C}$) for goat 425 and control



Mean daily rectal temperatures
(degrees C) for goat 425 and Control

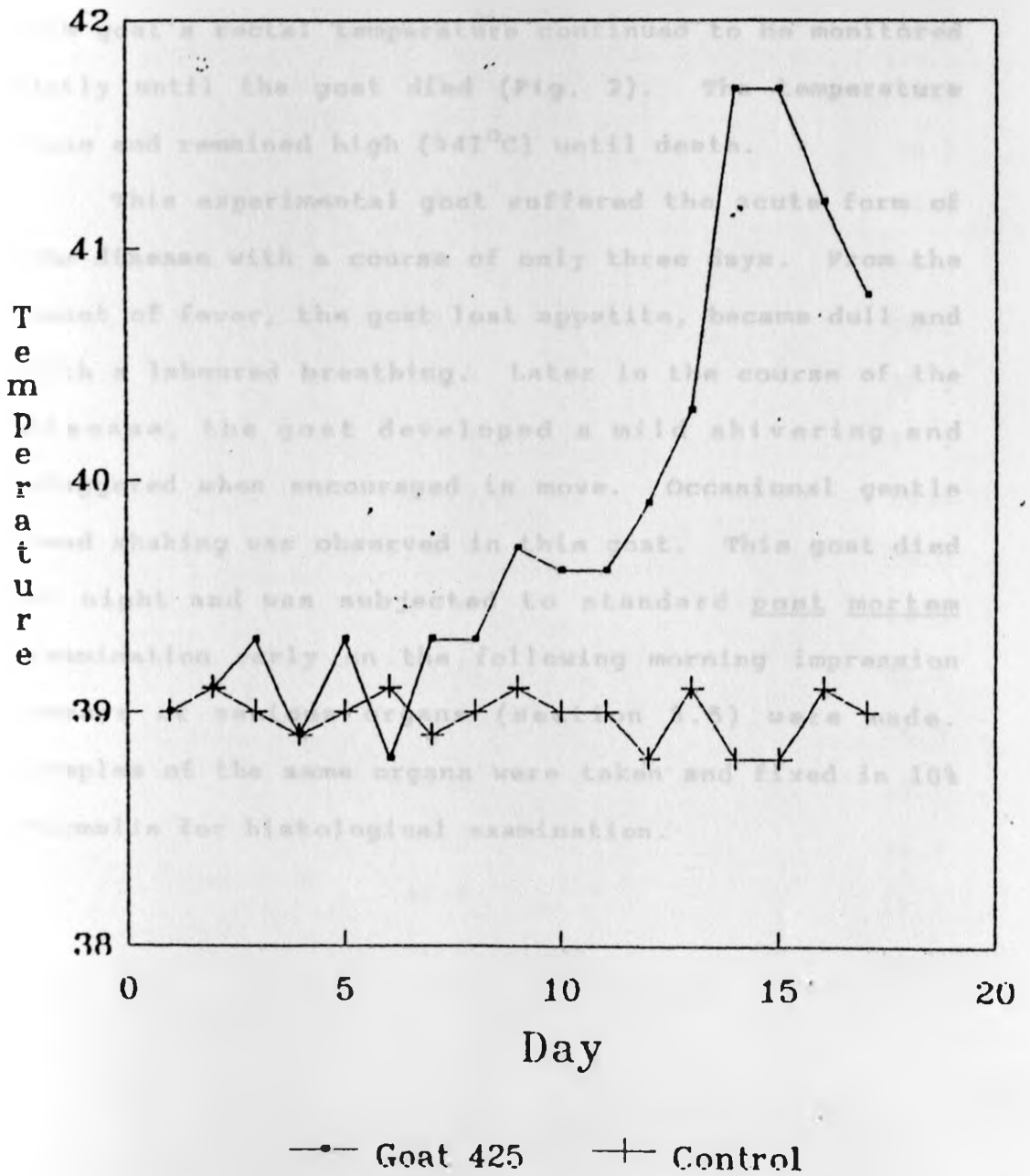


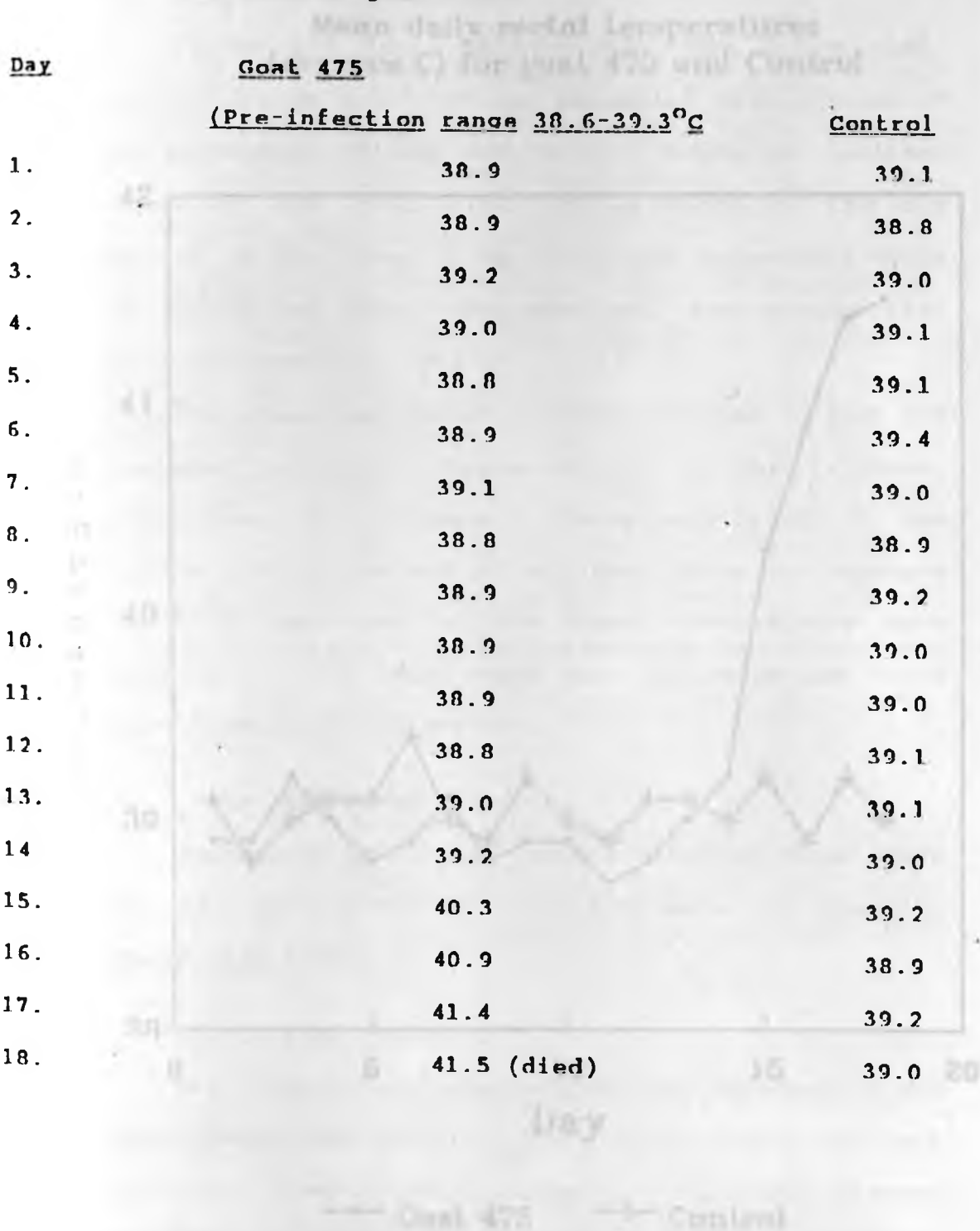
Figure 1

1.2.2 COURSE OF THE DISEASE IN GOAT 475 INOCULATED WITH TRANS MARA ISOLATE

This goat was infected with the Transmara isolate of Cowdria ruminantium. After drawing the amount of blood required for inoculation into experimental mice, the goat's rectal temperature continued to be monitored daily until the goat died (Fig. 2). The temperature rose and remained high ($>41^{\circ}\text{C}$) until death.

This experimental goat suffered the acute form of the disease with a course of only three days. From the onset of fever, the goat lost appetite, became dull and with a laboured breathing. Later in the course of the disease, the goat developed a mild shivering and staggered when encouraged to move. Occasional gentle head shaking was observed in this goat. This goat died at night and was subjected to standard post mortem examination early on the following morning impression smears of various organs (section 3.5) were made. Samples of the same organs were taken and fixed in 10% formalin for histological examination.

Table 2: Mean daily rectal temperatures ($^{\circ}\text{C}$) of goat 475 and control goat



Mean daily rectal temperatures
(degrees C) for goat 475 and Control

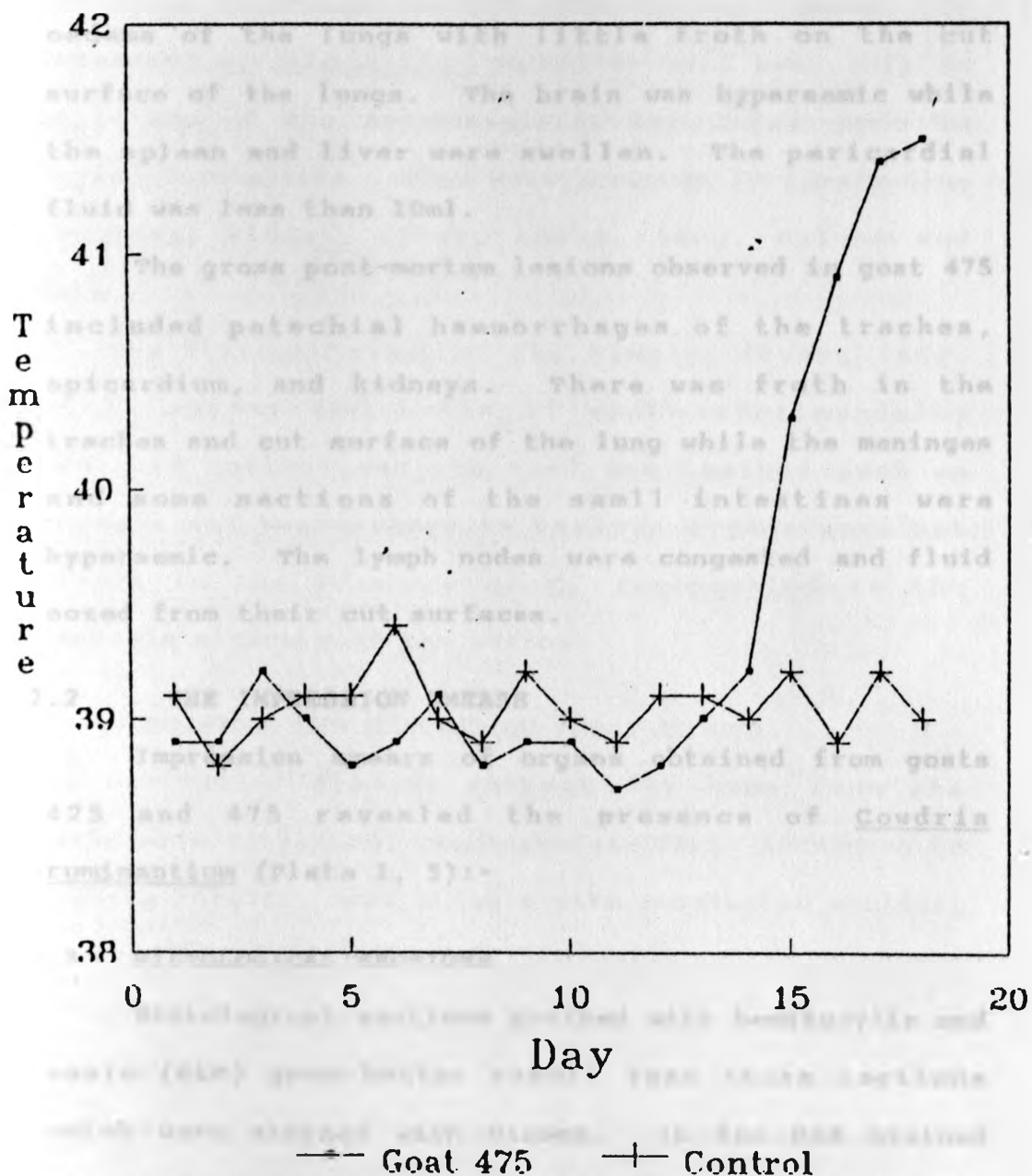


Figure 2

1.2.2.1 POST MORTEM FINDINGS

After the death of goat 425, the gross post-mortem lesions seen included petechial haemorrhages of the epicardium, kidney and liver. There was ascites, oedema of the lungs with little froth on the cut surface of the lungs. The brain was hyperaemic while the spleen and liver were swollen. The pericardial fluid was less than 10ml.

The gross post-mortem lesions observed in goat 475 included petechial haemorrhages of the trachea, epicardium, and kidneys. There was froth in the trachea and cut surface of the lung while the meninges and some sections of the small intestines were hyperaemic. The lymph nodes were congested and fluid oozed from their cut surfaces.

1.2.2.2 THE IMPRESSION SMEARS

Impression smears of organs obtained from goats 425 and 475 revealed the presence of Cowdria ruminantium (Plate 1, 5):-

1.2.2.3 HISTOLOGICAL SECTIONS

Histological sections stained with hematoxylin and eosin (H&E) gave better result than those sections which were stained with Giemsa. In the H&E stained specimen, C. ruminantium stained purple while the surrounding stained pink thus giving a definite contrast (Plate 12, 15, 20).

The detection of C. ruminantium parasites in the organs of experimental animals occurred in those animals whose organ impression smears had revealed the presence of the parasite. Of the six organ samples taken from each experimental and control animal for histology, C. ruminantium parasites were seen only in the organs of the experimentally inoculated goats in varying frequencies. They were observed in (descending frequency) kidney, liver, heart, lung, spleen and brain.

The histopathology of the kidney, liver, lung, heart, spleen and brain of each experimentally inoculated animal was observed and lesions such as necrosis and haemorrhage in various organs were not related to the presence of C. ruminantium in the immediate vicinity of the lesion.

1.2.2.4 DISCUSSION AND CONCLUSION FOR PART ONE

Heartwater disease derives its name from its characteristic lesion: hydropericardium. According to Prozesky (1987c), most animals with heartwater manifest gross lesions which include hydropericardium but this lesion was not observed in the two experimental goats used in this experiment. This observation is in agreement with the findings of Mebus and Logan (1988) who reported that many animals which die of this disease do not always exhibit hydropericardium and that this lesion is more frequent in cattle than in sheep

and goats. Likewise, this experiment confirmed their report on the occurrence of ascites, oedema of the lungs, splenomegally and haemorrhages of the epicardium, liver and kidneys. Prozesky (1987c) and Prozesky and Du Plessis (1985) reported the absence of a direct link between the presence of C. ruminantium parasites and the cytopathic effects of the cells harbouring those parasites. This was confirmed in this experiment but without explanation of the pathogenesis leading to the development of the lesions, especially the haemorrhage and the increased vascular permeability. One of the most recent explanation of the increased vessel permeability is from Du Plessis et al (1987) who suggested the occurrence of immune-complex type of hypersensitivity reaction. The result of this experiment cannot rule out this possibility especially that degenerative changes observed in histological preparations of the lung and kidneys had no relation with the presence of the rickettsial parasites in the immediate vicinity.

The degree of severity of observed nervous signs in the two goats was not dramatic. This result may be explained by the conclusion of Uilenberg (1983) who reported that pathological lesions develop according to the duration of the disease. He stated that the longer the disease persists, the more frequently the exudates and several of the other lesions are found and

that in peracute cases, oedema of the lungs, may be the only striking lesion observed. Likewise, the pathological picture is believed to vary with the strain of cowdria responsible for death. The Kenya isolates used in this experiment may be a type which neither causes hydropericardium nor serious nervous symptoms to goats. Wassink et al (1986) reported the existence of a South African isolate of C. ruminantium which is atypical in that it does not produce any nervous signs in goats. There might exist a Kenyan isolate which is atypical in its ability to cause some pathological lesions and its further investigation is warranted.

The two Kenya isolates of C. ruminantium used were equally fatal to goats despite their having been initially isolated from goat and bovine respectively. Further research on the ability of these two isolates to cause nervous signs and such lesions as hydropericardium ought to be done. This is especially meant to test the viability of the stabilates before being used on experiment on mice (part two).

PART TWO

2.0 ADAPTATION OF C. RUMINANTIUM TO MICE

2.1 MATERIALS AND METHODS

2.1.1 THE EXPERIMENTAL MICE

A total of 150 adult male mice (Swiss strain) were obtained from International Laboratory for Research on Animal Diseases (ILRAD) and transported by vehicle in deep-litter cages to the Department of Veterinary Pathology and Microbiology. They were fed ad libitum on commercially-prepared feed (mice pencil, Unga Feeds Ltd., Nairobi) and water in bottles was freely available to them throughout the duration of the experiment.

All the mice used in this experiment were adult males of the white Swiss strain variety. Seventy five mice were chosen at random and placed in five groups of 15 mice each whereby mice in the fifth group were to serve as controls. Each group was housed in a deep-litter cage and fed ad libitum on commercial feed (mice pencil) and water in bottles was freely available to them before and throughout the duration of the experiment.

All the experimental mice were kept in cages in the same room in which the goat was being bled in order to minimize the time lapse between the bleeding of the goat and the injection of the mice.

2.1.2 MICE INOCULATION WITH THE TANA RIVER ISOLATE

2.1.2.1 INTRAPERITONEAL INOCULATION OF MICE

Each experimental mouse was restrained by firmly grasping the loose skin at the back of the neck between the thumb and index finger. The tail and rear legs were also held to prevent violent struggling.

A 21 gauge needle and 5ml syringe were used and the needle was inserted lateral to the midline in the ventral abdomen whereby the stabilate was administered intraperitoneally. Mice in two of the four groups were inoculated intraperitoneally (i.p) with 2ml and 5ml respectively of stabilate (table 3) while those in the remaining two groups were used for intravenous (i.v) inoculations.

2.1.2.2 INTRAVENOUS (I.V) INOCULATION OF MICE

The stabilate was administered into two groups consisting of 15 mice each whereby each group was inoculated with 0.1ml and 0.2ml respectively (table 3).

Each experimental mouse was restrained as described under section 2.1.2.1 except that the tail was left unrestrained. The tail was grasped between the thumb and the index finger then it was swabbed with xylene followed by an antiseptic (70% Alcohol).

A 2ml syringe and a 26 gauge needle were used to administer the stabilate intravenously near the distal portion of the lateral tail vein. The volume of stabilate administered intravenously to each mouse in the various groups is shown in table 3.

During the i.v administration of the stabilate, a little amount of it was inadvertently deposited extravascularly in some mice but no effort was made to quantify the amount of stabilate involved.

TABLE 3: Mouse Infection with the Tana River Isolate of C. ruminantium

ROUTE OF INFECTION	VOLUME OF STABILATE (ml)	NO. OF MICE
Intravenous (i.v)	0.1	15
	0.2	15
Intraperitoneal (i.p)	2	15
	5	15

2.1.1.3 INOCULATION OF MICE WITH TRANSMARA ISOLATE

Blood used in this experiment was drawn from goat 475 (>41°C) as done under section 1.1.2.1.

A repetition of the intraperitoneal and intravenous procedures listed under sections 2.1.2.1 and 2.1.2.2, respectively, was done using the Transmara isolate of C. ruminantium. A total of sixty mice were injected either intraperitoneally (i.p) or intravenously (i.v) with varying amounts of infected

blood as shown in table 4. Once more, the amount of blood which may have been deposited extravascularly is unknown.

TABLE 4: Mouse Infection with the Transmara Isolate of C. ruminantium

ROUTE OF INFECTION	VOLUME OF STABILATE (ml)	NO. OF MICE
Intravenous (i.v)	0.1	15
	0.2	15
Intraperitoneal (i.p)	2	15
	5	15

2.2 RESULTS OF PART TWO

2.1.4 OBSERVATION OF INFECTED MICE

Out of the 30 mice which were inoculated intraperitoneally with 5ml of infected blood (Tana River or Transmara isolate), 4 died within five days of infection (3 Tana River and 1 Rift Valley). No post mortem examination was carried out on them because it was assumed that the deaths were a result of the large volume of blood in the peritoneal cavity which may have caused shock or respiratory failure.

All the surviving mice in both groups were slow in their movement and exhibited piloerection. One mouse which received 5ml of infected blood intraperitoneally (Trans Mara) died on day 17 post infection. Post mortem examination on it revealed a large abscess in

the abdomen in a sac adhering to the liver and the peritoneum close to the linear alba. This abscess arose from the intraperitoneal experimental injections. Impression smears of its organs together with the histological preparations of those organs did not reveal any Cowdria ruminantium parasites. One mouse which received 5ml IP of the Tana River isolate died on day 28 post infection. Its post mortem examination revealed a mottled liver, froth in the trachea, discoloured lungs and a congestion of the meninges. The impression smear of its organs together with their histological preparations revealed C. ruminantium parasites.

All the mice (30) which were inoculated intravenously with either the Tana River isolate (15) or the Transmara isolate (15), remained active and their hair coats were smooth without signs of piloerection. They appeared normal throughout the duration of the experiment and none died other than those which were sacrificed at the rate of two mice from each group per month for six months.

2.2.1 ORGAN IMPRESSION SMEARS

The impression smears of mouse organs which revealed the presence of C. ruminantium (Plates 12, 15) were as follows:

- (1) Nine out of fifteen mice infected I.P with 2ml stabilate (Tana River isolate)

- (ii) Eleven out of fifteen mice infected I.P with 5ml stabilate (Tana River isolate)

On the other hand, no C. ruminantium parasites were observed microscopically in the impression smears of organs obtained from the experimental animals which were inoculated as follows:

- (i) All the thirty mice which were inoculated intravenously (i.v) with either 0.1ml (15 mice) or 0.2ml (15 mice) of blood infected with the Tana River isolate from goat 425.
- (ii) All the thirty mice which were inoculated intravenously (i.v) with infected blood from goat 475 (Transmara isolate) either with 0.1ml (15 mice) or 0.2ml (15 mice).
- (iii) All the thirty mice which were inoculated intraperitoneally (i.p) with either 2ml (15 mice) or 5ml (15 mice) of infected blood from goat 475 (Transmara isolate).
- (iv) All the fifteen non-inoculated control mice.

The C. ruminantium parasites were observed microscopically from the organs of mice as follows:

- (i) 20 out of 30 mice inoculated i.p with Tana River isolate.
- (ii) 0 out of 30 mice inoculated i.v with Tana River isolate.
- (iii) 0 out of 30 mice inoculated i.p with Transmara isolate.

(iv) 0 out of 30 mice inoculated i.v with Transmara isolate.

(v) 0 out of 15 control mice.

The parasites which were observed are shown on plates 8, 9, 12, 13 and 15. The impression and squash smears together with the histological sections revealed morulae or single dense forms of the parasites.

The result shows that it is only the Tana River isolate of C. ruminantium which could be recovered in mice after being inoculated intraperitoneally with infected goat's blood. The parasites which were observed were photographed (Plates 1, 2, 3, 5) while brain squash smears revealing the parasite after being stained (Giemsa) were also photographed (Plates 4, 6, 7). Only in goats were impression smears of lymph nodes taken.

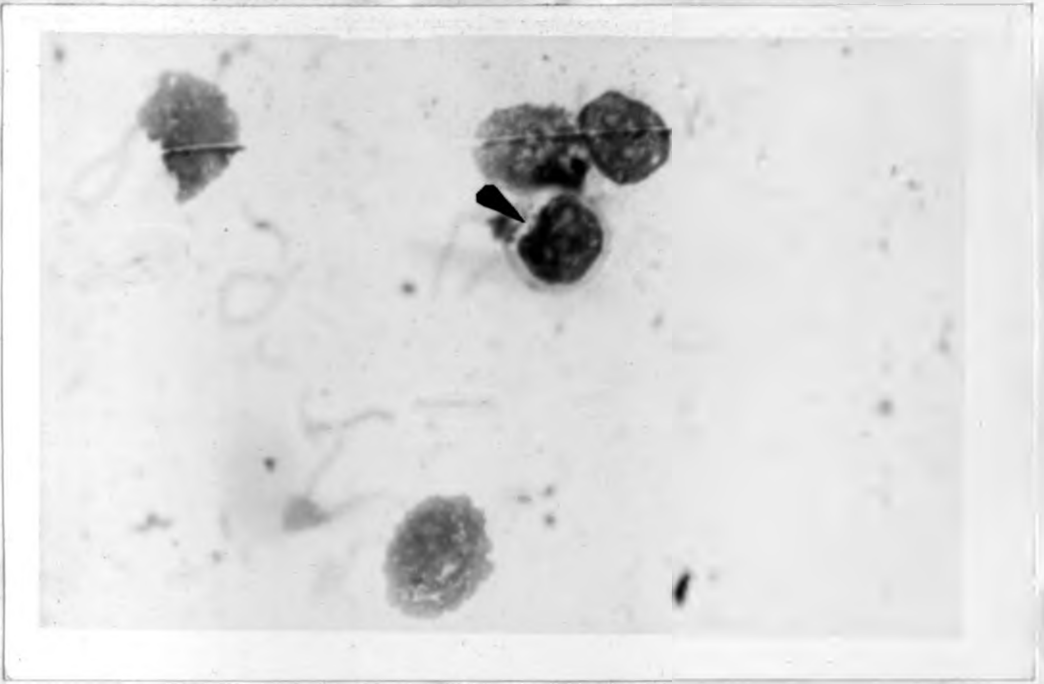


PLATE 1: Impression smear of mesenteric lymph node (goat 475) showing intracytoplasmic C. Ruminantium within a mononuclear cell (Giemsa x 630)

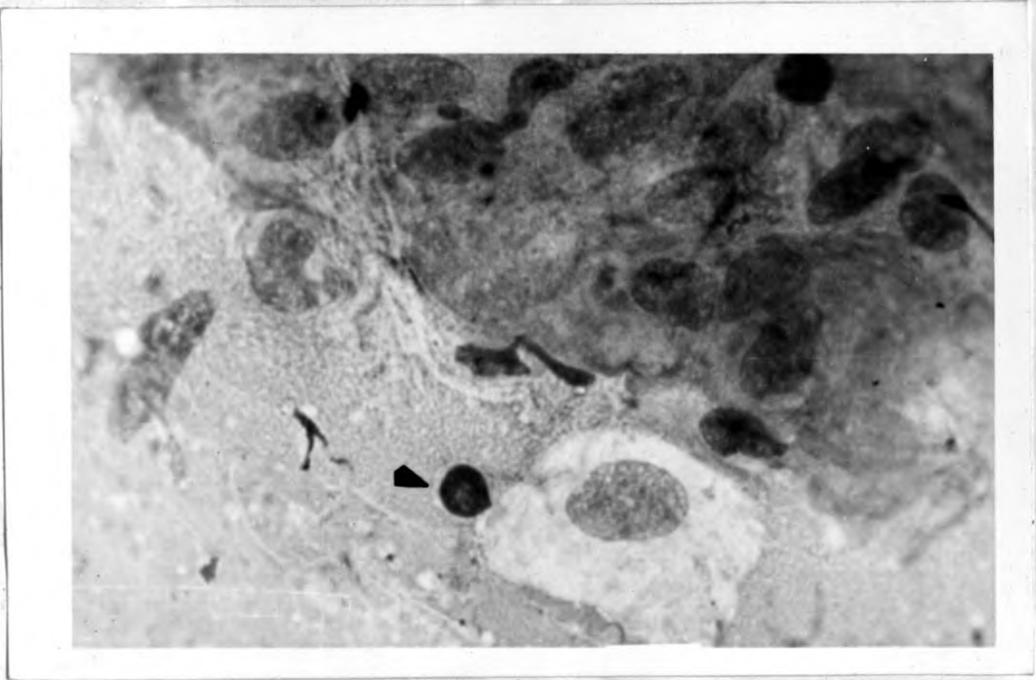


PLATE 2: Smear of aortic intimal scraping (goat 425) showing granular C. ruminantium morula with a surface projection suggestive of its recent extrusion from the adjacent cell (Giemsa x 1000).

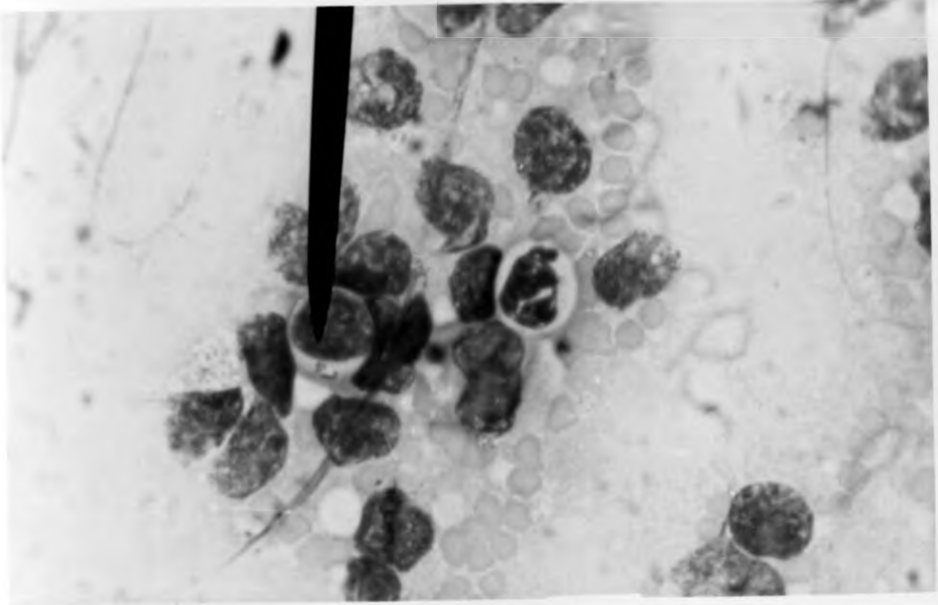


PLATE 3: Spleen impression smear (Goat 475) showing intracytoplasmic C. ruminantium colonies in a mononuclear cell (pointer). Three colonies are round while one is elongated and adhering to host cell membrane (Giemsa x 1000).

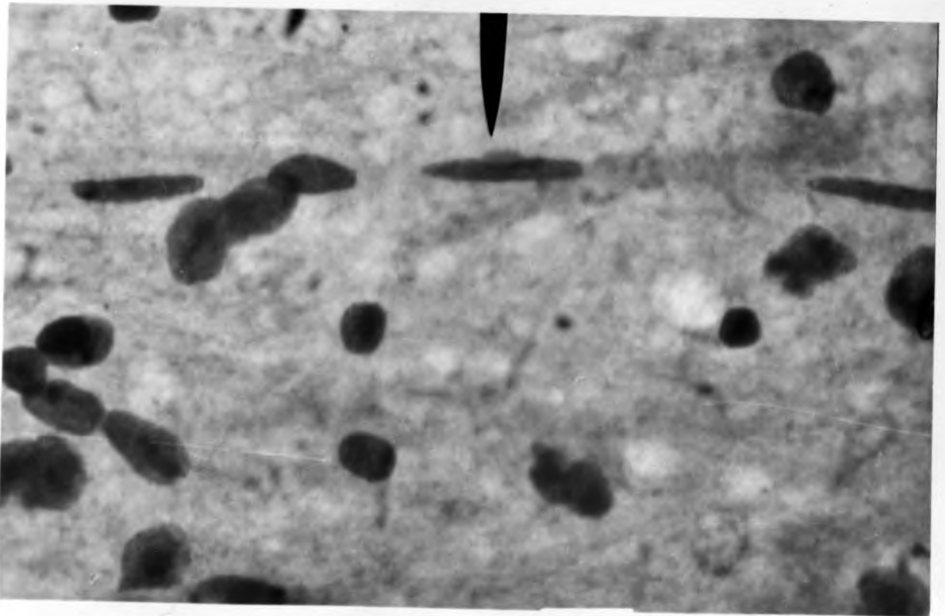


PLATE 4: Squash smear of brain cortex (mouse) showing a perinuclear colony of C. ruminantium (pointer) which has deformed the nucleus of a capillary endothelial cell (Giemsa x 1000).

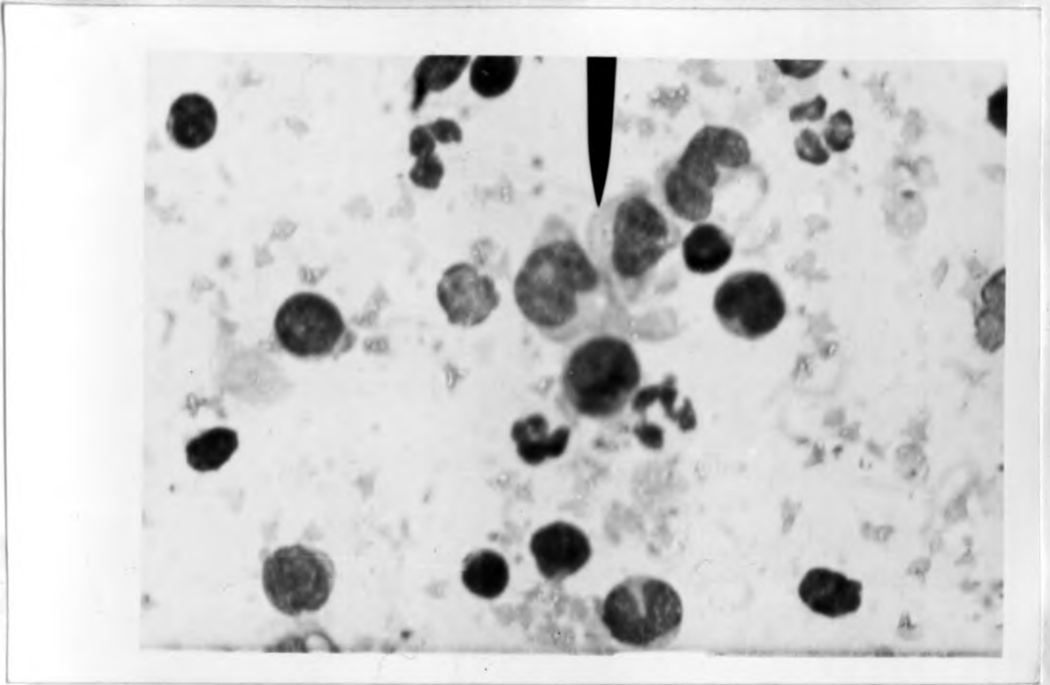


PLATE 5: Impression smear of liver (Goat 425) showing intracytoplasmic colonies of C. ruminantium (pointer) in a mononuclear cell (Giemsa x 630).

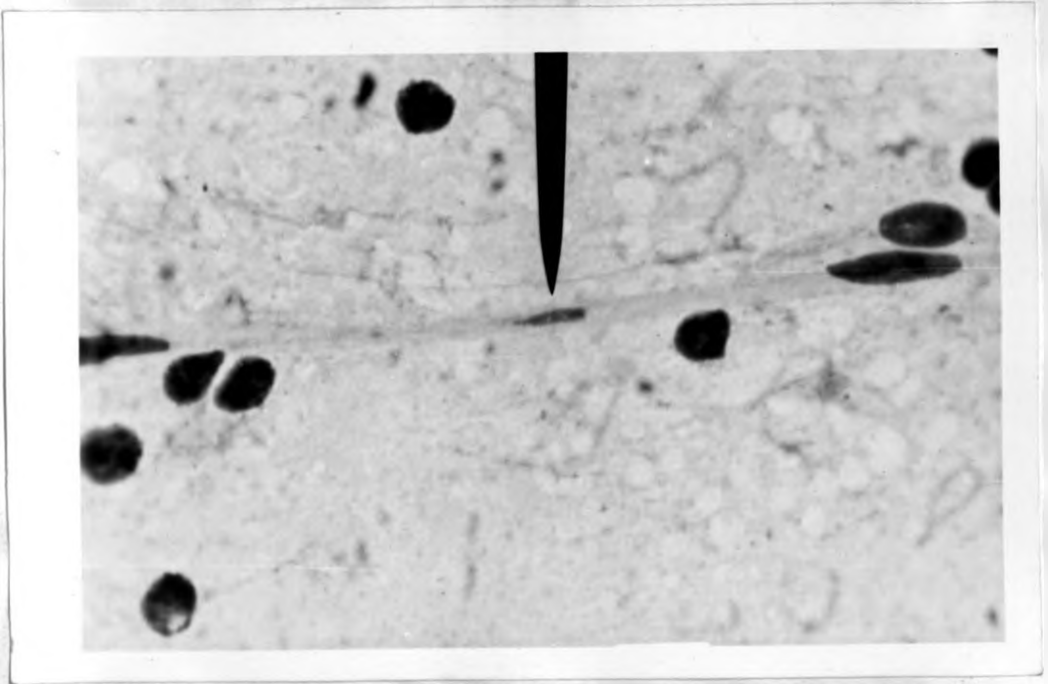


PLATE 6: Brain squash smear (mouse) showing a granular colony of C. ruminantium (pointer) within a blood capillary (Giemsa x 630).

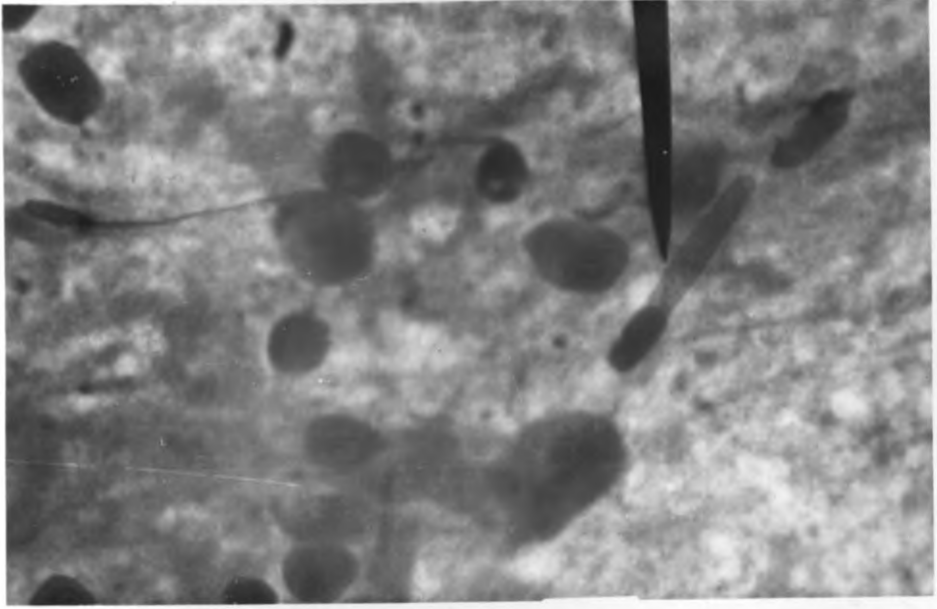


PLATE 7:

Squash smear of hypothalamus (Goat 425) showing granular perinuclear morula of C. ruminantium (pointer) (Giemsa x 630).

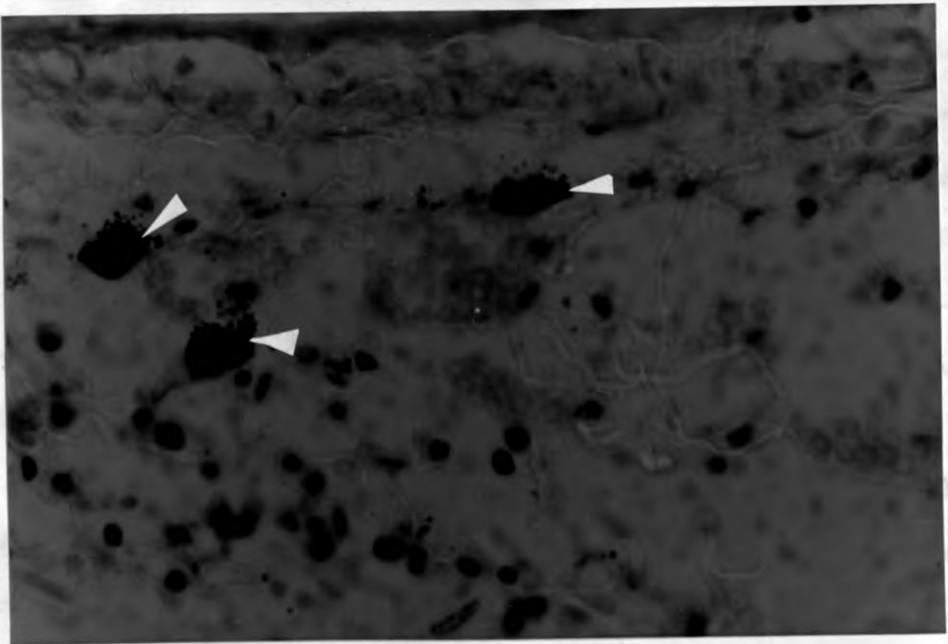


PLATE 8:

Histological section of mouse kidney (day 196 post infection) showing two morulae of C. ruminantium in blood capillaries of the renal cortex (H&E x 630).

2.2.2 GROSS AND HISTOPATHOLOGY

KIDNEY

Grossly, some experimental mice had obviously enlarged kidneys with no other visible lesions while other mice had normal-sized kidneys but with elevated nodular grey-white lesions on the renal cortex. The pin-head sized lesions were visible when the non-adhering renal capsule was removed. Some mice had pale or mottled kidneys as the only gross lesion on these organs.

Microscopically, there were interstitial haemorrhages accompanied by lymphocyte infiltration (Plate 11, 19). Most of the cell infiltration observed was mainly perivascular affecting the renal capillaries but also involved the interstitial stroma and around the glomeruli. Many degenerative changes were observed in the kidneys of the experimental animals. They ranged from the presence of hyaline casts in the lumina of renal tubules (Plate 14), adhesion of glomerulus to Bowman's capsule (Plate 11, 19) to necrosis of renal tubules characterised by the loss of cell nuclei (Plate 11, 13, 14). The architectural arrangement of the affected kidneys was distorted especially that some necrotic renal tubules were occluded either by casts or by swelling. In all affected mice, congestion of the blood vessels and haemorrhage were constantly observed lesions.

The degenerative changes observed in the kidneys of experimental mice were not restricted to those mice from which C. ruminantium parasites were recovered. The lesions were also observed in some mice which were inoculated with infected blood but no parasites were observed in their tissues on histological examination of their organs. On the other hand, no degenerative changes were observed in the kidneys of control mice.

Microscopic examination of the grey-white nodular spots on the kidneys revealed that the spots are formed by proliferating lymphoid cells (plate 23, 24). Intracellular and extracellular forms of C. ruminantium parasites were observed in some of the nodules formed by the proliferating lymphoid cells (plate 20). The extracellular parasites were in clusters or morulae which stained purple while the intracellular parasites occurred singly or paired in each cell and stained purple also (plate 20).



PLATE 9: Higher magnification of plate 8 showing a large colony of C. ruminantium with scattered pairs suggestive of the parasite's division by binary fission (H&E x 1000).

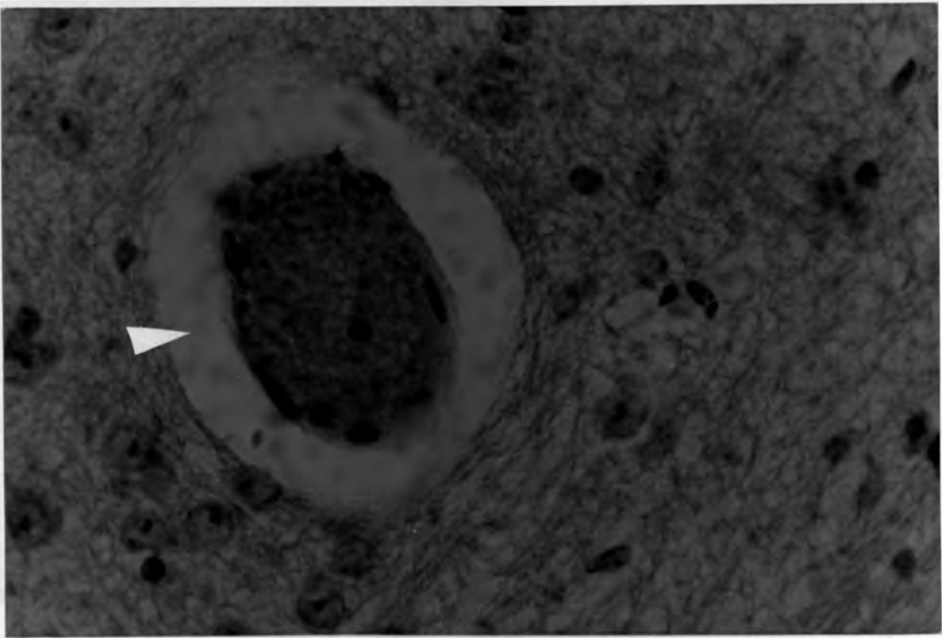


PLATE 10: Histological section of a mouse brain showing enlarged perivascular space and perivascular lymphoid cell infiltration (H&E x 1000).

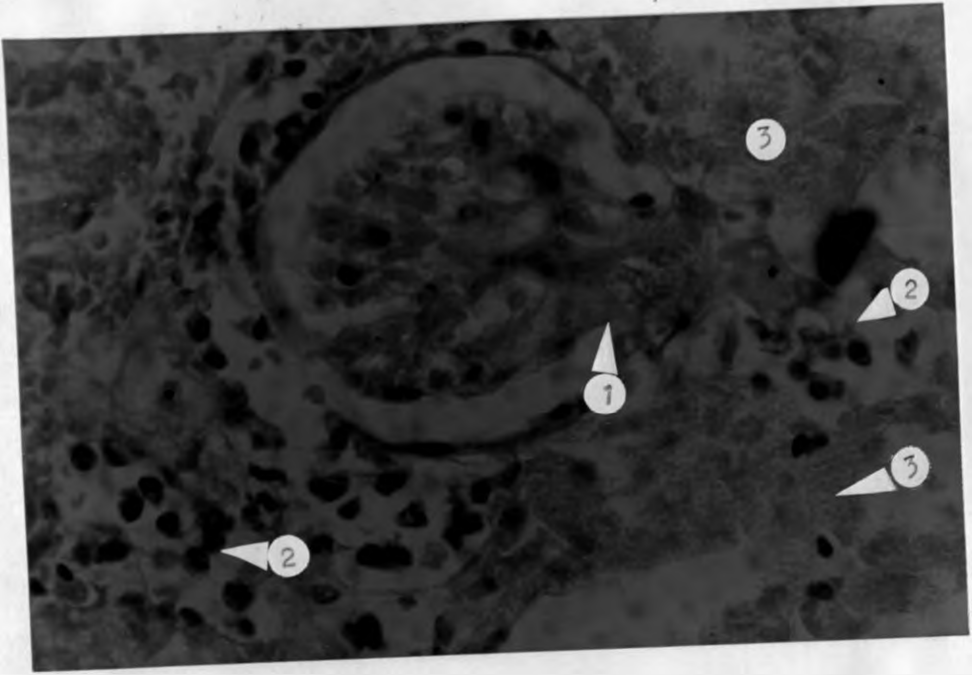


PLATE 11: Mouse kidney showing adhesion of glomerulus to Bowman's capsule (1); perivascular lymphocytic cell infiltration in interstitial stroma (2) and necrosis of renal tubules (lost nuclei) (3) (H&E x 1000).

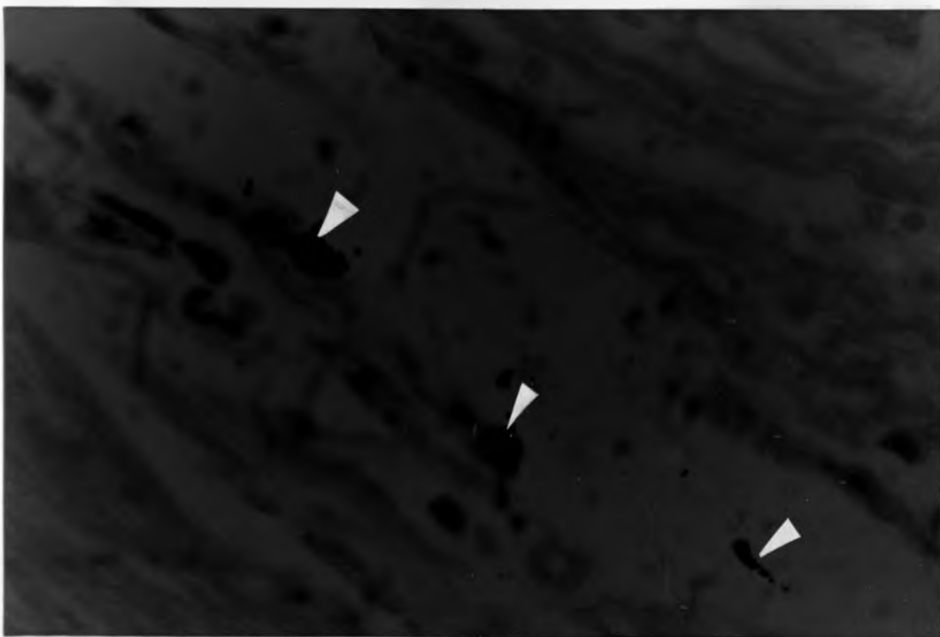


PLATE 12: Three morulae of *C. ruminantium* on the periphery of an arteriole in the cardiac muscle of a mouse (Giemsa x 630).

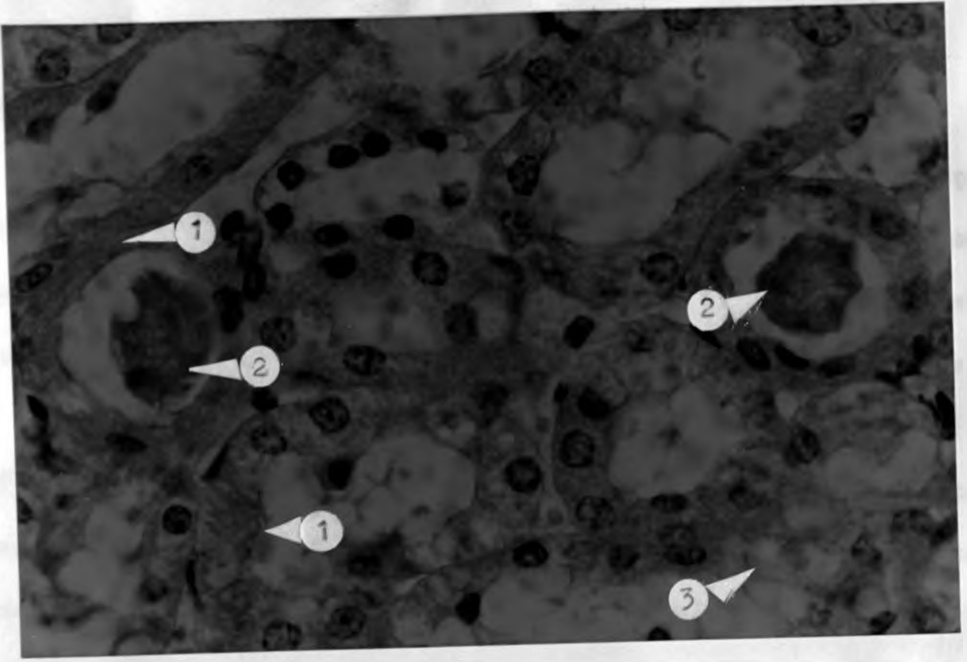


PLATE 13: Mouse kidney showing necrosis of renal tubules (1) with casts occluding their lumina (2) and a destruction of the renal architectures (3). C. ruminantium parasites are in endothelial cells of blood capillary (H&E x 630).

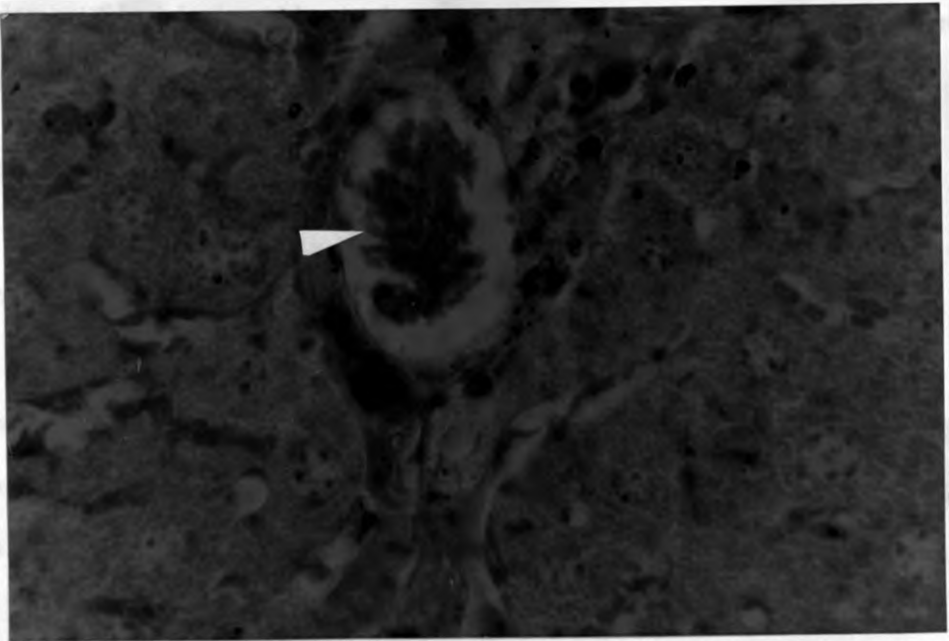


PLATE 14: Higher magnification of plate 13 showing casts in renal tubules (pointer) and generalised necrosis causing loss of renal architecture (H&E x 1000).

LUNGS

Grossly, a few experimental mice had pale lungs with little froth in the trachea while other mice had firm lobes of the lungs. Such affected lobes were at times darker than the rest of the lung lobes.

Microscopically, there was lymphoid cell infiltration in the lungs of many experimental mice whereby the lungs exhibiting this lesion were from mice whose kidneys had the lesion also. The lymphoid cell infiltration was especially concentrated around the pulmonary Blood vessels (Plates 17 & 18) thus causing congestion of such affected vessels. The infiltrating cells were also in the inter-alveolar septae and therefore compromised the alveoli. There was often oedema fluid in the alveoli whose walls were damaged and haemorrhagic.

HEART

Grossly, the blood vessels on the heart were congested in all experimental mice.

Microscopically, the experimental mice had lymphoid cell infiltration in their myocardium and, there was atrophy and necrosis of myocardial fibres in the areas with extensive lymphoid infiltration. The cell infiltration was closely associated with the blood vessels of the heart (Plates 27 & 28). In some cases, intermuscular haemorrhage was extensive especially in those cases with congested blood vessels.

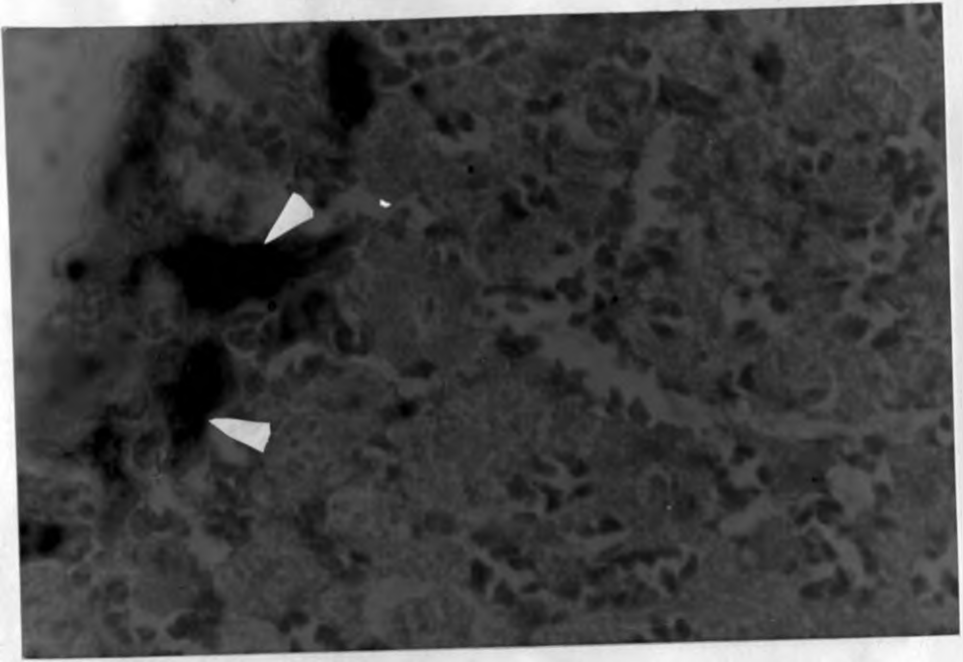


PLATE 15: Mouse liver showing colonies of *C. ruminantium* in Kupffer cells on day 169 post infection (H&E 630).

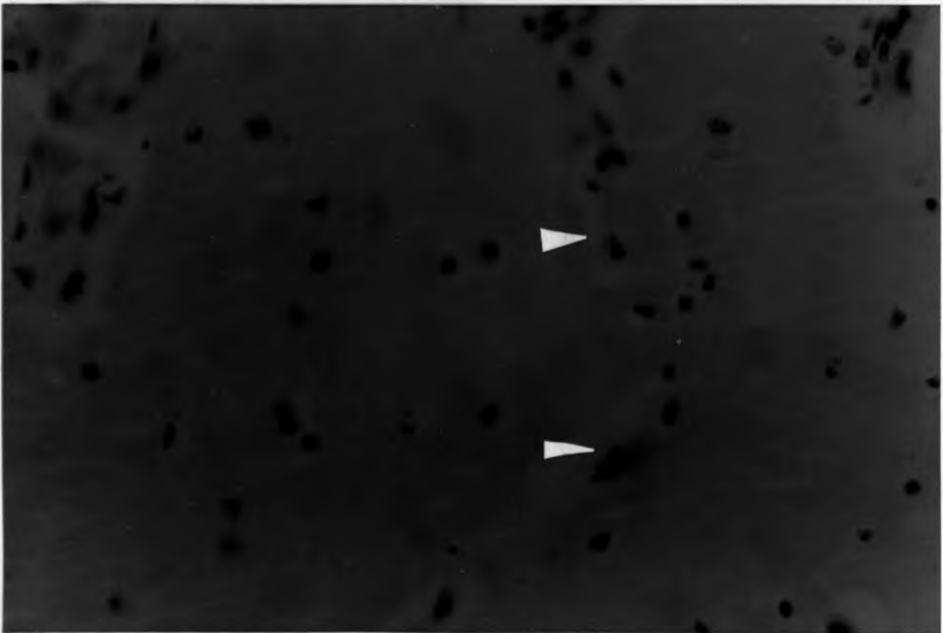


PLATE 16: Mouse brain showing enlarged perivascular spaces suggestive of oedema (H&E x 630).

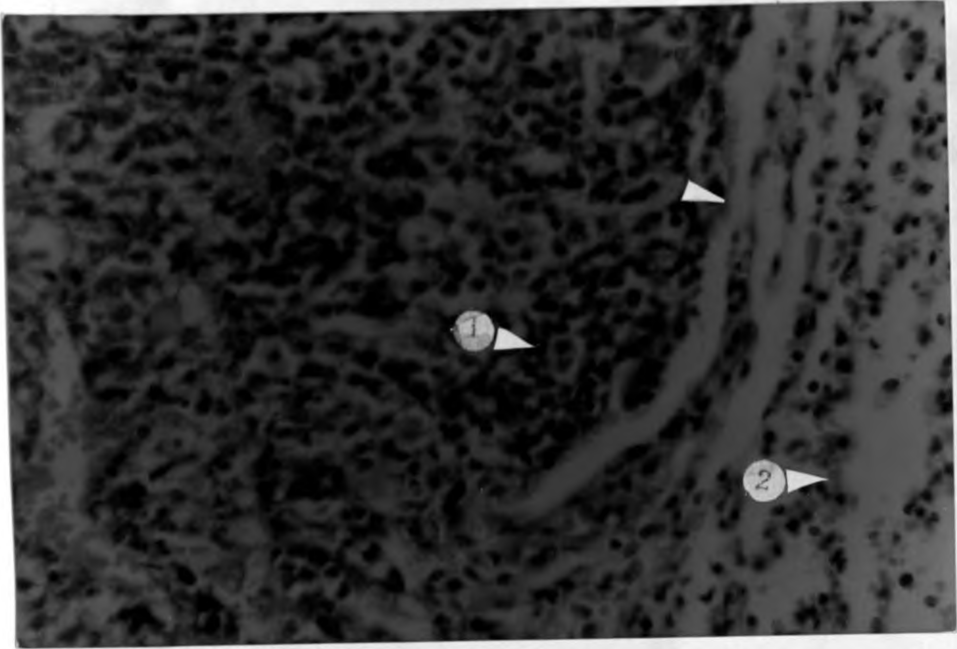


PLATE 17: Mouse lung showing solidification as a result of mononuclear cell infiltration (1) haemorrhage (2) and alveolar collapse (arrow) (Giemsa x 400).

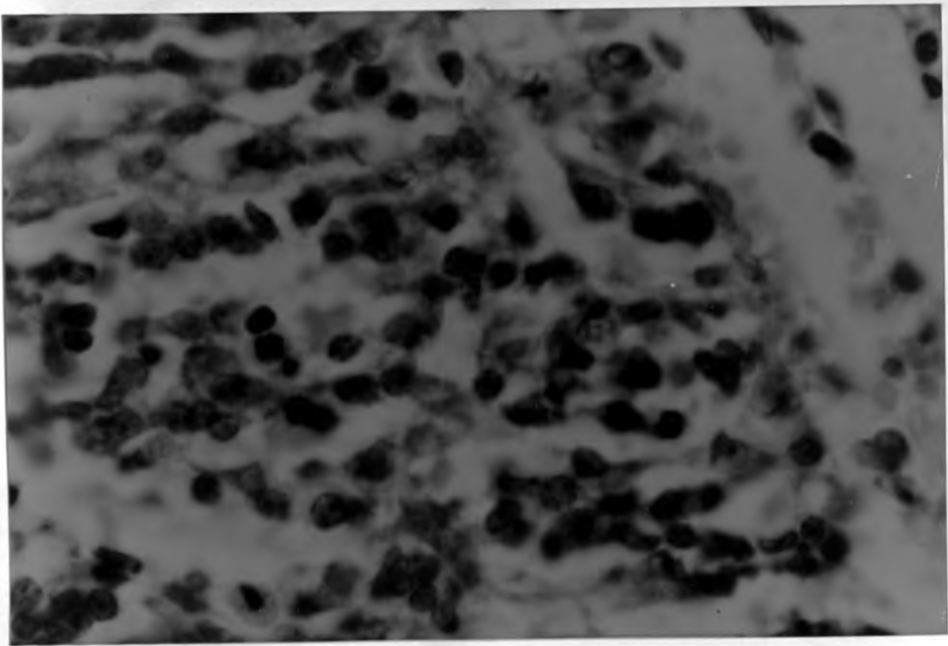


PLATE 18: Higher magnification of plate 17 showing the infiltrating mononuclear cells and extensive destruction of the lung (Giemsa x 1000).

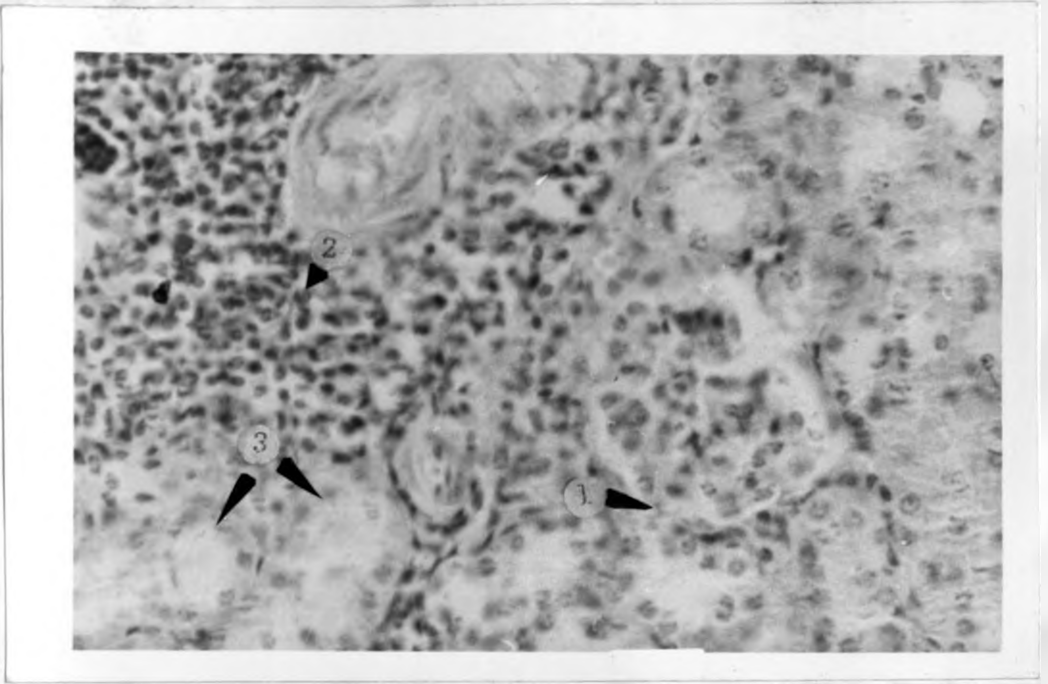


PLATE 19: Mouse kidney showing glomerula adhesion to Bowman's capsule (1), perivascular lymphocyte cell infiltration (2); and necrosis of renal tubules (3) (H&E x 400).

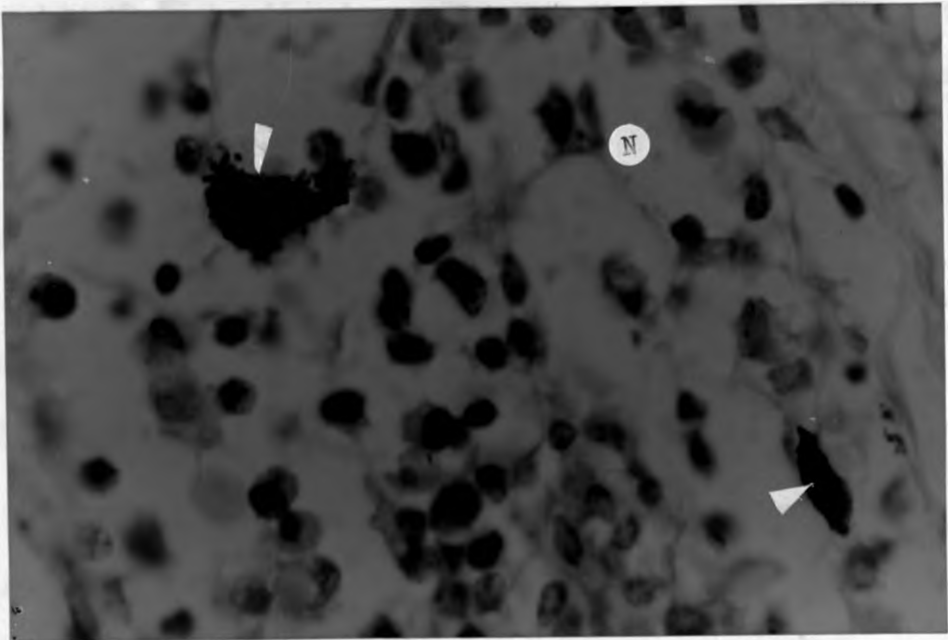


PLATE 20: Mouse kidney showing destruction of renal tubules by infiltrating lymphoid cells. Necrosis of tubules (N) is evident. Note morulae of *C. ruminantium* (arrow) (Giemsa x 1000).

LIVER

Grossly, mice had enlarged livers but only a few of the mice had the nodular grey-white lesions on their livers. Those mice which had the lesions in their liver also had the same lesion on other organs such as the kidneys. Some livers appeared mottled whereby patches of paleness were visible grossly.

Microscopically, there were lymphocytic cell infiltration in the livers of those mice which had a similar lesion (infiltration) in other organs. The lymphocytic cells infiltrated mainly the centrilobular zones of the liver and these were closely associated with congestion and haemorrhage. Degenerative lesions varying from hydropic degeneration to necrosis characterised by the loss of cell nuclei were observed. The colonies of C. ruminantium in the Kupffer cells of the liver stained purple blue (H&E Plate 25 & 26).

BRAIN

Grossly, the majority of brains of the experimental mice appeared normal except for a few in which the surface blood vessels appeared congested.

Histologically, only two mice had brains (Plates 10 & 16) which showed enlarged perivascular spaces indicative of oedema. Few of the blood vessels were congested. Of all the organs examined, the brains of the experimental animals showed least lesions in extent and severity.

SPLEEN

Only four experimental mice had grossly enlarged spleens while a few others had soft pulp but were of normal size.

Histologically, some cases showed haemorrhage and lymphocyte depletion in the white pulp leading to their replacement with fibrin material (Plates 21 & 22). The nuclei of some of the lymphocytes were pyknotic especially along the periphery of the areas already depleted of lymphocytes.

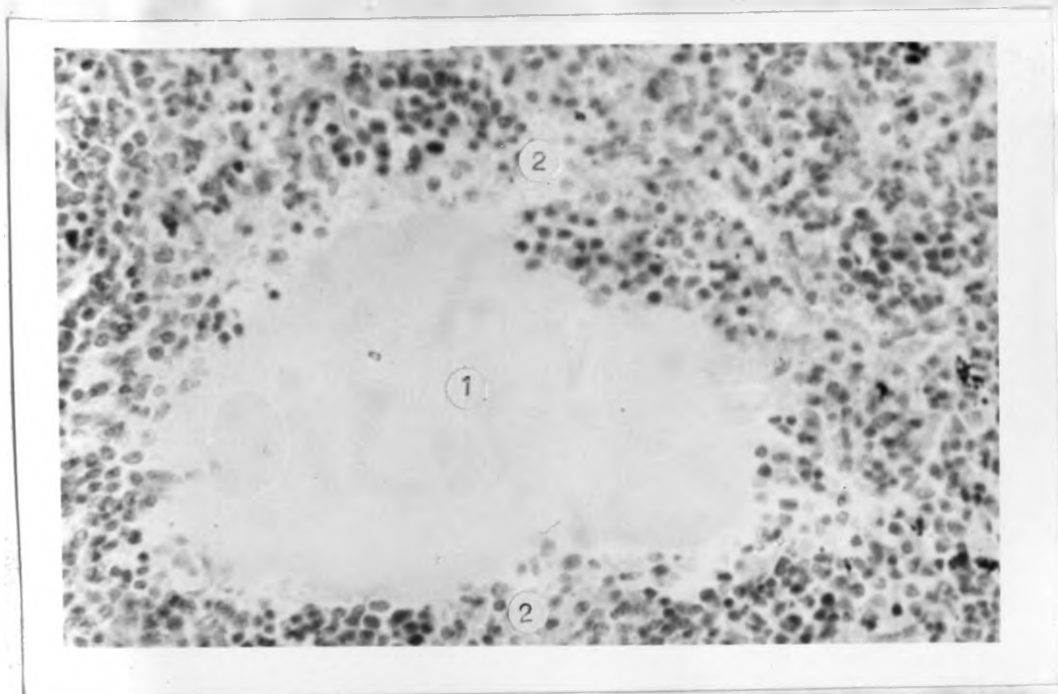


PLATE 21: Mouse spleen showing an area of lymphocyte depletion replaced by fibrin (1) and surrounded by a haemorrhagic zone (2) (H&E x 400).

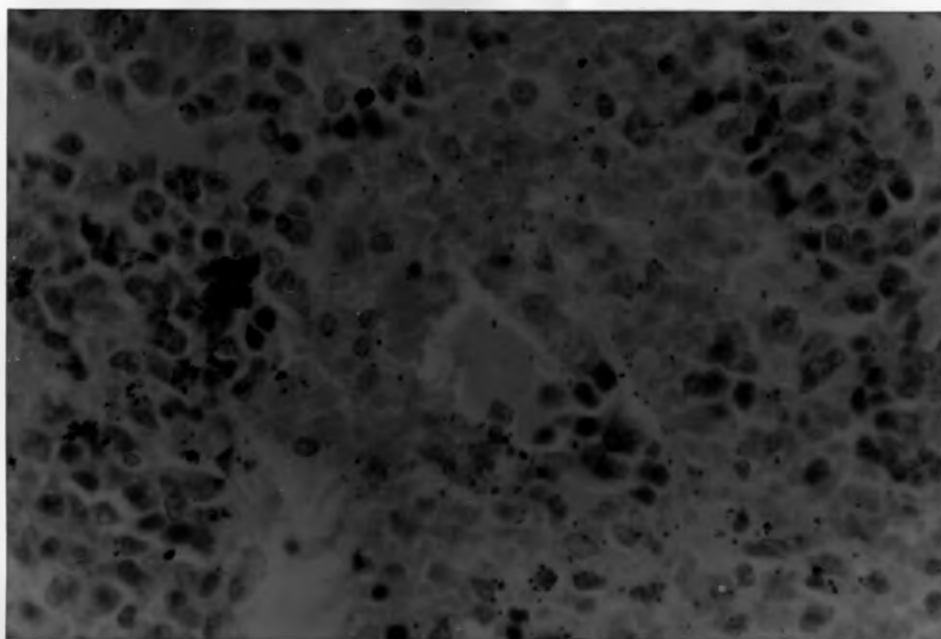


PLATE 22: Coloured photomicrograph of mouse spleen shown in plate 21 (H&E 400).

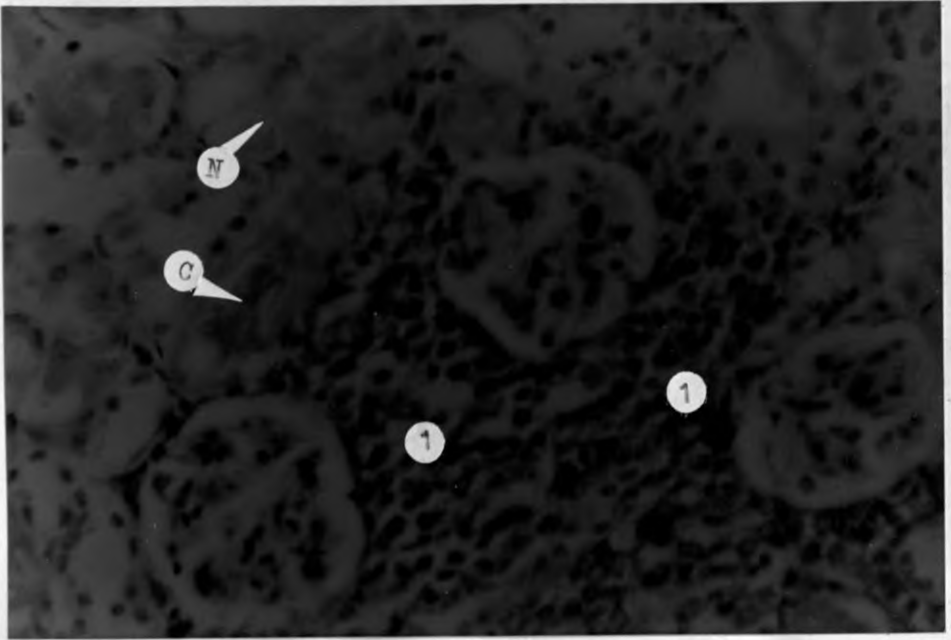


PLATE 23: Mouse kidney showing mononuclear cell infiltration of stroma (1) tubular necrosis (N) and cast deposit (C) (Giemsa x 400).

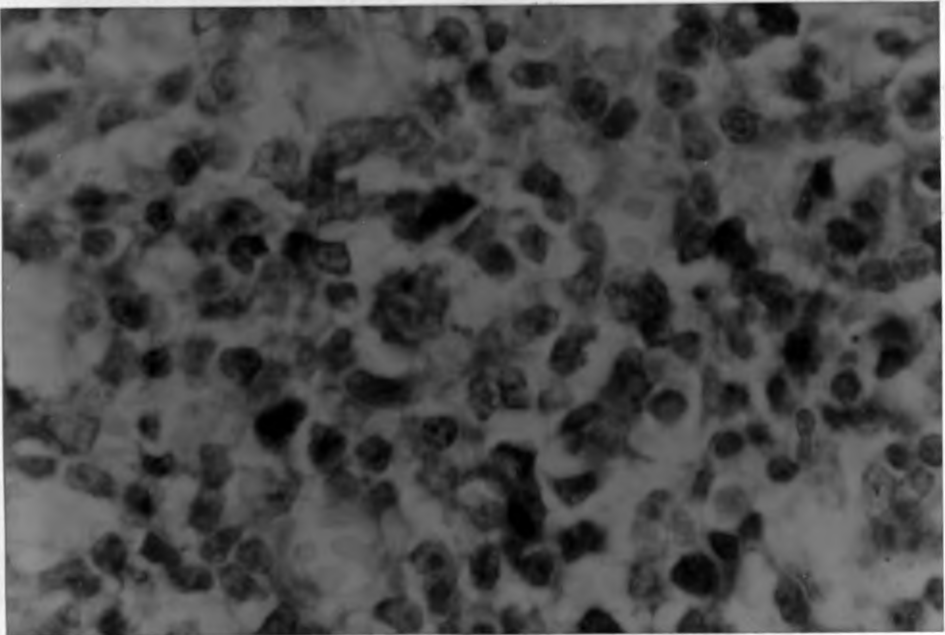


PLATE 24: Higher magnification of plate 23 showing the infiltrating cells (Giemsa x 1000).

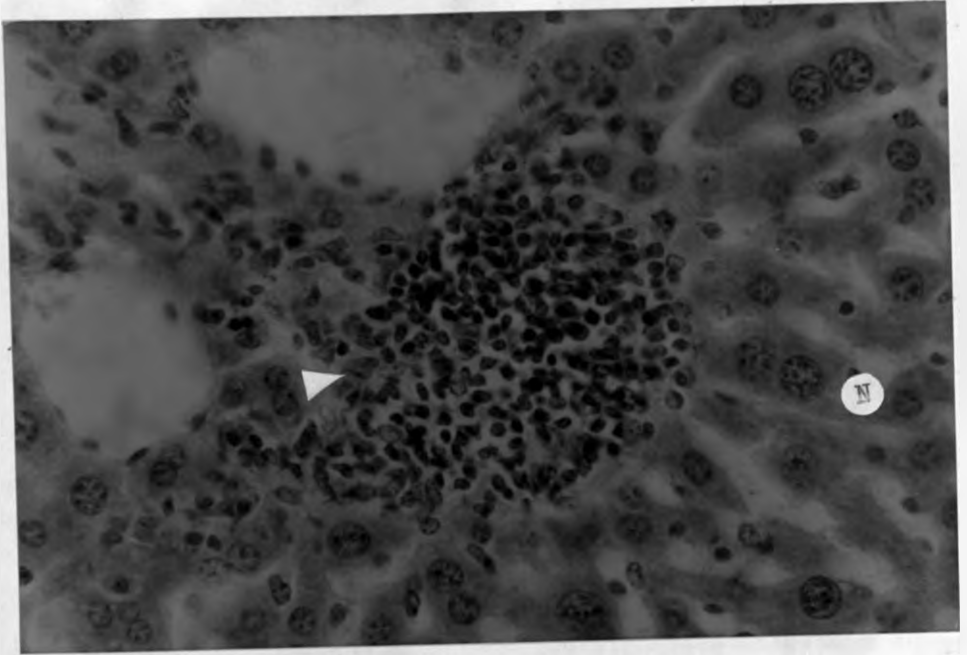


PLATE 25: Mouse liver showing areas of mononuclear cell infiltration (dark) and normal areas (N) (Giemsa x 100).

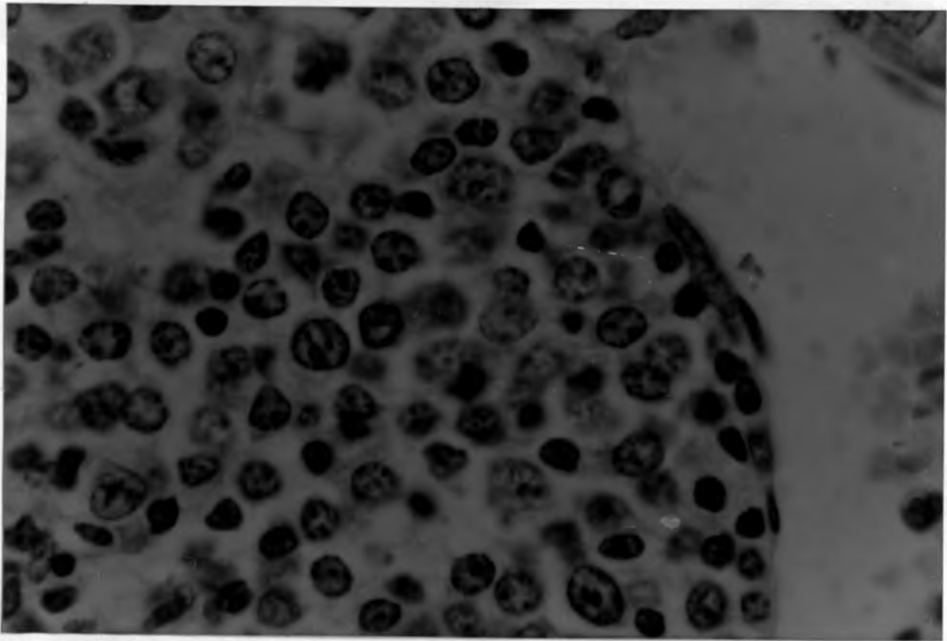


PLATE 26: Higher magnification of plate 25 showing the infiltrating mononuclear cells (Giemsa x1000).

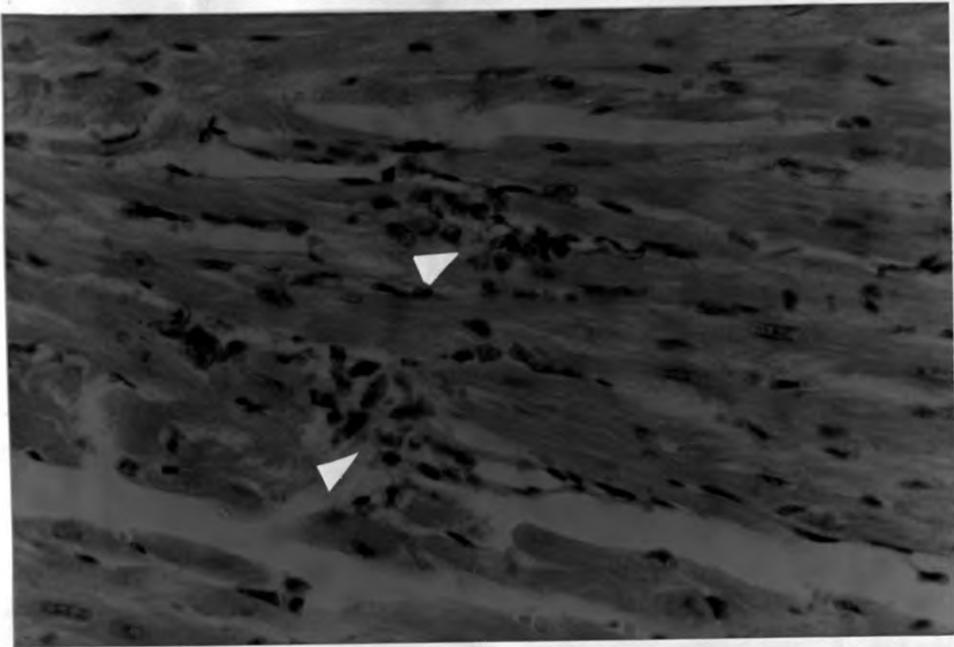


PLATE 27: Mouse heart showing lymphoid cells replacing the muscle fibres (H&E x 400).

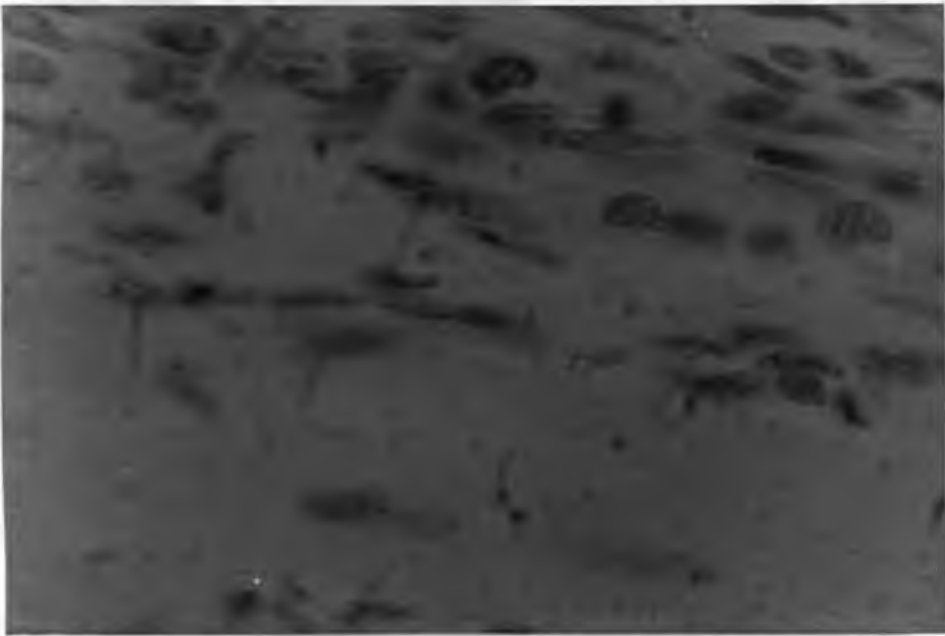


PLATE 28: Higher magnification of plate 27 (H&E x 1000).

2.1.5 INDIRECT FLUORESCENT ANTIBODY TEST (IFAT)

This was carried out as a confirmatory test of the presence of C. ruminantium in the tissues of the mice which were experimentally infected with this disease agent.

2.1.6 PREPARATION OF ANTIGEN (Rabbit Anti-mouse Immunoglobulin)

Whole serum from six healthy mice (Swiss strain) was used as antigen to immunise the rabbit.

Each of the six mice was first anaesthetized by being put in a large beaker containing cotton wool soaked with chloroform. The mouse was then removed when unconscious, but still alive, and its forelegs pinned onto a wooden board to hold the mouse on dorsal recumbency. A shallow skin incision was then made from the neck to the umbilicus region and the skin flayed laterally to expose the abdominal and thoracic muscles. An incision was then made through the thorax along the costo-chondral junction to expose the beating heart and the lungs. Blood was aspirated directly from the right ventricle using a 21-gauge needle and a 5-ml syringe thus killing the mouse in the process. The blood was transferred into clean sterile Bijou bottles and allowed to clot overnight on a table top. Blood from three mice was pooled in each bottle.

When the clotting process was completed, the clot of blood in the bottles was hooked out to leave the

clear serum. The serum in the two bottles was then pooled in a conical centrifuge tube and centrifuged at 740 G. for six minutes. The clear serum was pipetted into a clean sterile Bijou bottle while the cell sediment left in the centrifuge tube was discarded. An aliquot of 3ml serum was mixed with an equal volume of complete Freund's adjuvant (Difco labs; Detroit, Michigan U.S.A.). This was effected with the use of a 21-gauge needle and a 10ml-syringe whereby the contents were thoroughly mixed by repeatedly aspirating and forcefully expressing out the mixture until a homogenous white emulsion was obtained.

2.1.7 RABBIT IMMUNISATION

The rabbit was immunised by injecting it intramuscularly with 3ml of the antigen-adjuvant emulsion.

A booster dose of the antigen (mouse serum) was administered intramuscularly to the rabbit 15 days after the first injection. The booster serum antigen was mixed with incomplete Freund's adjuvant (sigma labs; st. Louis, MO, USA) in a syringe to obtain a uniform emulsion, as above, prior to injecting 3ml of it into the rabbit. The booster dose of antigen was injected into the muscles of a different leg from the one into which the first antigen was administered. The rabbit was examined daily for any sign of disease or excessive reaction against the antigen.

2.1.8 TESTING OF THE RABBIT'S IMMUNE STATUS

The extent of the rabbit's immune reaction to the mouse serum was tested by immunodiffusion on day 6 following the administration of the booster dose of the serum (antigen). For this test, fresh mouse serum (antigen) was prepared as done under section 2.1.6 while the rabbit serum was prepared as follows:

The dorsal surface of the rabbit's ear was wetted with soapy water and the hair on it shaved to expose the marginal ear vein along the rear border of ear.

The shaved area was then swabbed with cotton wool soaked in 70 per cent methanol then left to dry. The surface was next smeared with a thin coating of petroleum jelly to prevent premature clotting of blood. A diagonal incision was then made across the marginal ear vein, without severing it, while holding the base of ear with the thumb and finger to occlude venous return. The blood was collected in a large centrifuge tube coated internally with a thin film of petroleum jelly. When 10ml of blood had been collected, the pressure at the base of the ear was removed and a piece of cotton wool was pressed onto the incision site to stop bleeding. The rabbit was cleaned and returned to its cage.

A one per cent (1%) agar gel molten over a water-bath was poured onto glass slides to a depth of 0.3cm and left to set. After congelation, five wells were

punched into the agar to form a sign of the cross. The four peripheral wells were equidistant from the central well. The central well was filled with mouse serum while the remaining four peripheral wells were filled with the rabbit serum (antiserum). The glass slides were then left overnight on a flat surface in a humid chamber at room temperature for immunodiffusion to take place.

The sets of wells exhibited a continuous line of precipitation running between the central well and the four peripheral wells. This is indicative of a strong immune reaction of the immunised rabbit.

2.1.9 PURIFICATION OF THE RABBIT'S IMMUNOGLOBULINS

Serum was obtained from a rabbit by bleeding and processing the blood as done under section 2.1.8. The isolation and purification of immunoglobulin G (IgG) was achieved by ammonium sulphate precipitation of the serum proteins followed by gel exclusion chromatography.

In this process, 10ml concentrated ammonium sulphate solution was added in drops to an equal volume of serum in a beaker on a magnetic stirrer, to precipitate the serum proteins. The contents were then centrifuged at 1660G for six minutes. The supernatant was discarded while the precipitate sediment was dissolved in phosphate buffered saline (PBS), PH 7.2, to make a total volume of 6ml. The protein in this

solution was re-precipitated as above using 3ml (33.33%) of concentrated ammonium sulphate and once again centrifuged at 1660G for 10 minutes. The supernatant was discarded while the sediment was redissolved in 5ml PBS.

Desalting of the protein solution was effected by putting it in a washed cellophane bag (Visking size 9-36/32, medicell International Ltd. London) and dialysing against one litre of PBS over a magnetic stirrer. Fresh PBS was used each day for three days and sodium azide was put in the PBS used for dialysis each time.

2.1.10 GEL FILTRATION CHROMATOGRAPHY OF THE PROTEIN SOLUTION

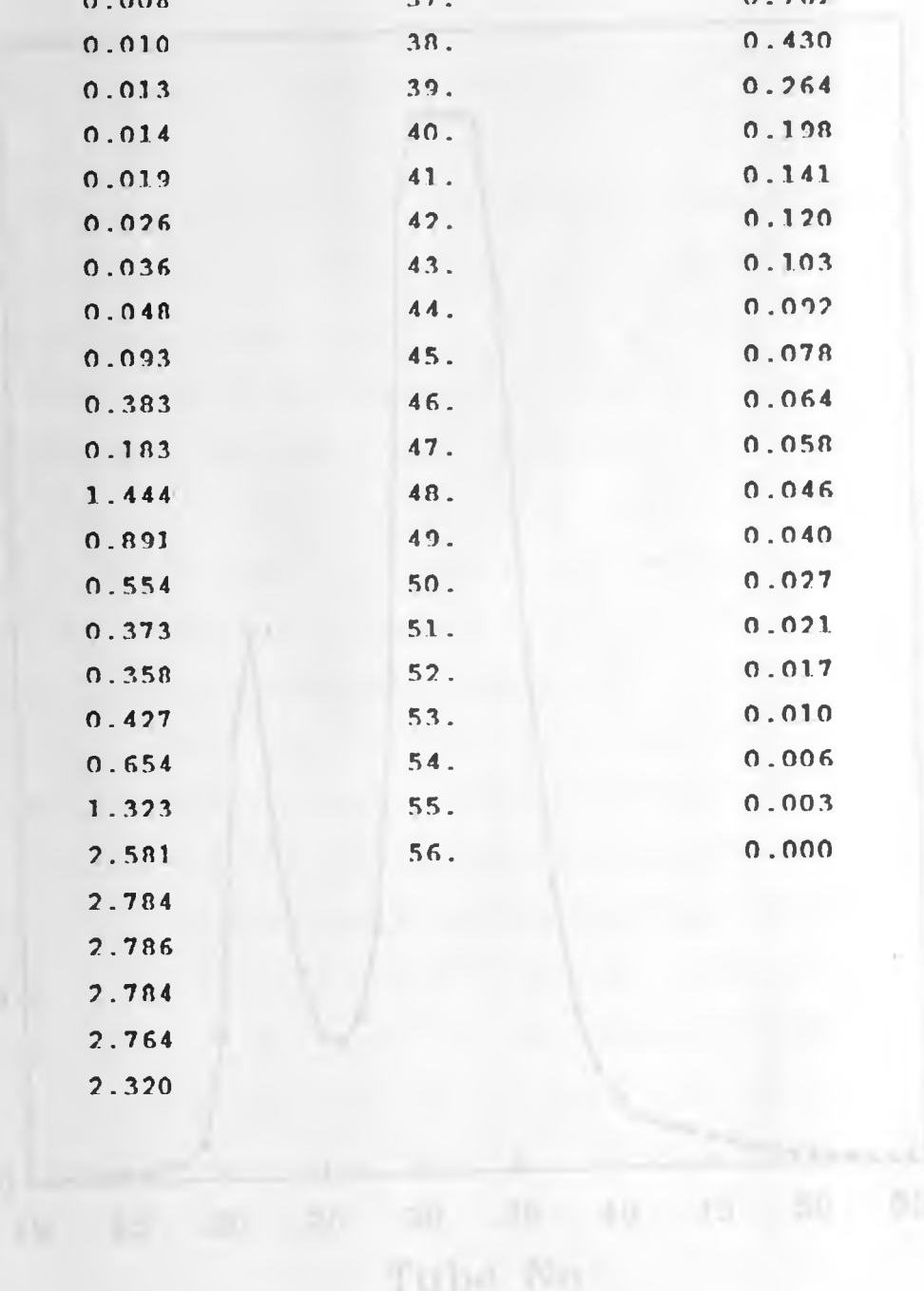
This was effected using Sephadex G-200 (Sigma Chemical Co. St. Louis, MO, USA), tubing and glassware for column operation as described by Fahey and Terry (1978). In this experiment, reverse-flow chromatographic arrangement was used whereby the sample and buffer enter the gel column at the bottom and emerge at the top of the column as a result of a constant pressure from a peristaltic pump (LKB, Bromma, Sweden). The serum sample (5ml) was introduced into the column by placing the free end of the in-flow tubing into the sample and allowing the serum to be drawn in. When it was all drawn in, the free end of the inflow

tubing was immediately placed in a reservoir of PBS which was constantly drawn after the serum to maintain an air-free continuous flow throughout the system. The eluate was collected in a fraction collector (LKB, Bromma, Sweden) which was coupled to a spectrophotometer for graphically recording light absorbance of the emerging effluent. The fraction collected in each test tube was 8ml. After calibrating the spectrophotometer using PBS by setting the optical density (OD) reading at zero, a sample of eluate from each test tube was put separately in provided cuvette and its OD read in an ultra-violet spectrophotometer (Beckmann, USA) at a wave length of 280nm and recorded (table 5) and the readings used to plot a graph (Figure 3).

Three pools of the eluates in the test tubes comprising tubes 13 to 26, 28 to 42 and 44 to 52, respectively, were made and designated as fractions one, two and three respectively.

Table 5: Spectrophotometric reading of eluate from Sephadex G-200

TUBE NO.	OPTICAL DENSITY	TUBE NO:	OPTICAL DENSITY
10.	0.000	36.	1.385
11.	0.008	37.	0.762
12.	0.010	38.	0.430
13.	0.013	39.	0.264
14.	0.014	40.	0.198
15.	0.019	41.	0.141
16.	0.026	42.	0.120
17.	0.036	43.	0.103
18.	0.048	44.	0.092
19.	0.093	45.	0.078
20.	0.383	46.	0.064
21.	0.183	47.	0.058
22.	1.444	48.	0.046
23.	0.891	49.	0.040
24.	0.554	50.	0.027
25.	0.373	51.	0.021
26.	0.358	52.	0.017
27.	0.427	53.	0.010
28.	0.654	54.	0.006
29.	1.323	55.	0.003
30.	2.581	56.	0.000
31.	2.784		
32.	2.786		
33.	2.784		
34.	2.764		
35.	2.320		



Optical Density of column eluates

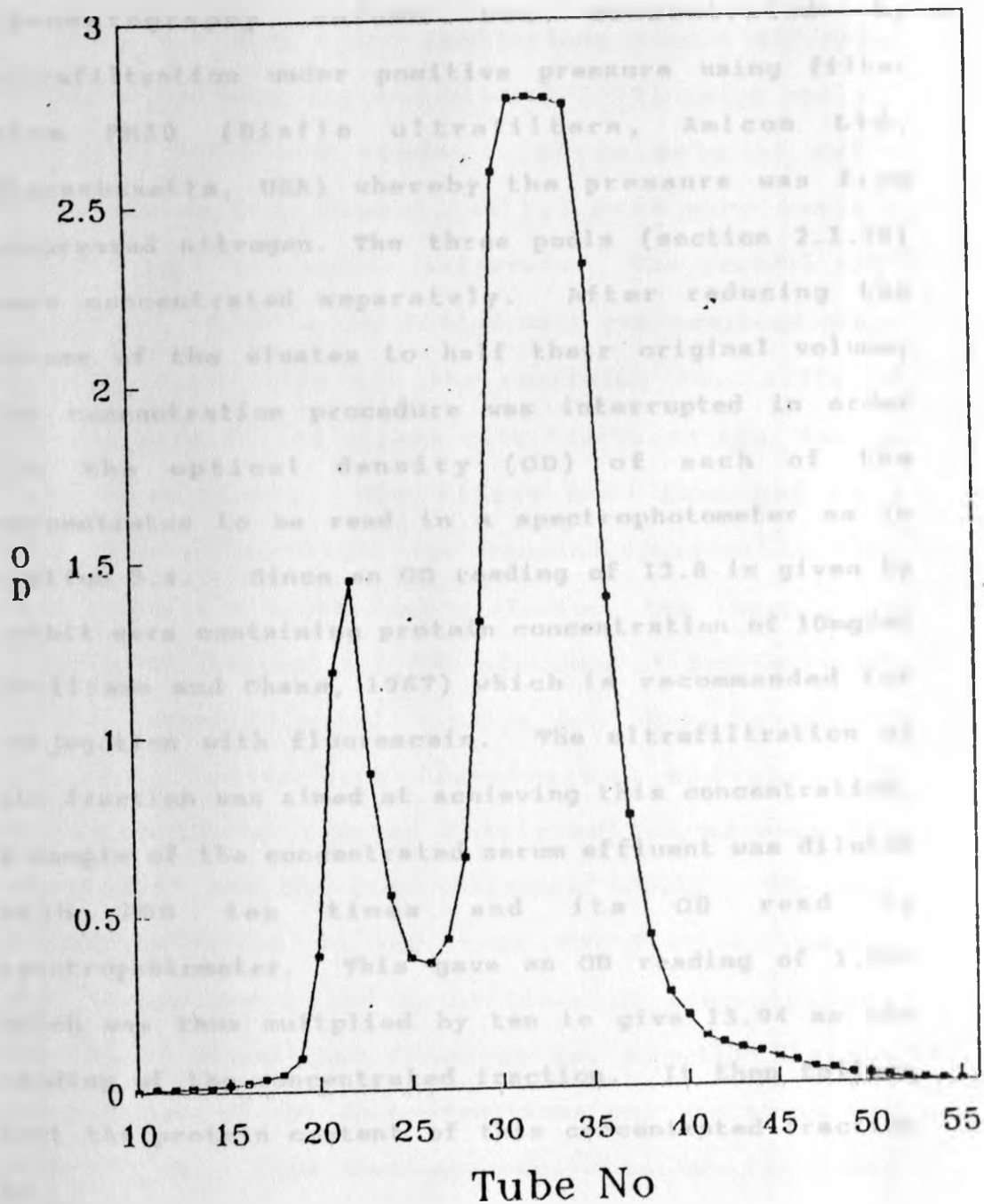


Figure 3

2.1.11 CONCENTRATION OF ELUTE FRACTIONS

The immunoglobulin fractions obtained from the chromatography column was concentrated by ultrafiltration under positive pressure using filter size PM10 (Diaflo ultrafilters, Amicon Ltd, Massachusetts, USA) whereby the pressure was from compressed nitrogen. The three pools (section 2.1.10) were concentrated separately. After reducing the volume of the eluates to half their original volume, the concentration procedure was interrupted in order for the optical density (OD) of each of the concentrates to be read in a spectrophotometer as in section 5.4. Since an OD reading of 13.8 is given by rabbit sera containing protein concentration of 10mg/ml (Williams and Chase, 1967) which is recommended for conjugation with fluorescein. The ultrafiltration of the fraction was aimed at achieving this concentration. A sample of the concentrated serum effluent was diluted with PBS ten times and its OD read by spectrophotometer. This gave an OD reading of 1.394 which was thus multiplied by ten to give 13.94 as the reading of the concentrated fraction. It then follows that the protein content of this concentrated fraction is

$$\begin{aligned} & \frac{(13.94 \times 10)\text{mg/ml}}{13.8} \\ & = 10.01\text{mg/ml.} \end{aligned}$$

2.1.12 DETERMINATION OF THE EFFLUENT FRACTION CONTAINING THE HIGHEST AMOUNT OF Ig G.

This was done using Ouchterlony simple diffusion technique (Ouchterlony and Nilsson 1978) using one per cent agar on glass slide. Three sets of wells consisting of five depots (wells) each were punched into the agar at regular intervals. The central well in each set of wells was filled with pre-prepared goat-antirabbit IgG while all the remaining four wells of each set were filled either with fractions one, two or three respectively. The slides were then put in a humid chamber overnight for immunodiffusion to take place. After a night-long diffusion, the three glass slides were examined for the presence of precipitation and its intensity noted. Slide two whose peripheral wells were filled with fraction two had the most intense continuous line of precipitation between the central well and the four peripheral wells. The sets of wells on slides one and three with fraction one and three respectively, had faint lines of precipitation. This result shows that fraction two had the highest concentration of IgG while fractions one and three had traces of it. From then onwards, fraction two alone was used for other subsequent experiments by virtue of its having the highest concentration of IgG.

2.1.13 ASSESSMENT OF IgG POTENCY IN FRACTION TWO.

The activity of the isolated IgG (fraction two) against its specific antigen (mouse serum) was tested by the Ouchterlony plate technique (Ouchterlony and Millson 1978) involving immunodiffusion (section 2.1.12). The central well was filled with IgG (fraction two) while the four peripheral wells were filled with fresh mouse serum (antigen) then left in a humid chamber overnight. When the results was read, the gel showed strong precipitation reaction between the central well and the peripheral wells therefore confirming that the isolated IgG was still potent against its specific antigen (whole mouse serum).

2.1.14 CONJUGATION OF IgG WITH FLUORESCHEIN ISOTHIOCYANATE (FITC)

12ml of protein solution (10mg/ml approx.) was dialysed against 0.25mol. carbonate buffer, pH 9.5 to introduce this buffer into the protein solution while minimising the amount of PBS in the solution. The dialysis was effected using a cellophane bag (Visking, size 9-36/32, Medicell International Ltd. London) for 24 hours. After dialysis, the protein solution had a pH of 9.5. The required conjugation ratio of fluorescein to protein is 40 microgrammes to 1mg (Johnson et al, 1978) and the total protein in 12ml solution was 120mg which therefore required (120 x 40 microgrammes = 4800ug.

= 4.8 mg of FITC.

This amount of fluorescein dye was measured and added dry to the protein solution according to Johnson et al, (1978) and placed on a magnetic stirrer. The stirring to dissolve the dye was done gently overnight at 4°C in a dark room.

2.1.15 REMOVAL OF UNBOUND FLUORESCHEIN DYE

This was achieved by gel chromatography using sephadex G25 (Sigma Chem. Co. Ltd. St. Louis, Mo, USA). The gel powder (20g) was swelled overnight at 4°C in excess phosphate buffered saline, pH 7.2, 8.0M then packed in a 40cm perspex column (Pharmacia, Sweden) and allowed to stabilize for 24 hours with a constant flow of PBS through it.

The conjugate was applied on top of the gel and its elution carried out with PBS supplied by a peristaltic pump while the fractions were collected using a fraction collector as in section 2.1.10. The colour of the eluate collected in test tubes was used to determine those test tubes whose contents were to be pooled, concentrated and used for further experiments. The test tubes of interest here had yellow contents while the rest had colourless liquid.

The 25ml of yellow eluate collected was concentrated by ultrafiltration as in section 2.1.11 to 13ml.

2.1.16 ISOLATION OF OPTIMALLY-LABELLED IMMUNOGLOBULINS

This was done according to a modification of the method of Riggs *et al* (1960). It involves the elution of the conjugate using DE-52 cellulose (Whatman, England) and phosphate buffer with sodium chloride (NaCl) of varying molarities. The cellulose was equilibrated overnight with PBS, 0.0175mol/L, pH 6.3 while the conjugate was also equilibrated in the cold with the same buffer overnight by dialysis through cellophane bag (Medicell International Ltd., London).

After the equilibration, the conjugate (13ml) was applied onto the cellulose and eluted gradually using phosphate buffer with varying molarities of sodium chloride as follows:-

- (1) 100ml phosphate buffer 0.0175mol/L, pH 6.3,
- (2) 100ml phosphate buffer 0.0175mol/L, pH 6.3 with 0.125mol/L of sodium chloride.
- (3) 100ml phosphate buffer 0.0175mol/L, pH 6.3 with 0.25mol/L of sodium chloride.

The effluent from the column was collected by a fraction collector (LKB, Sweden) and it was the third (above) phosphate buffer with 0.25mol/L sodium chloride which eluted the optimally-labelled conjugate as verified by spectrometric readings as in section 2.1.10. This optimally-labelled conjugate (10ml) was used in subsequent experiments while the rest of the effluent was discarded.

2.1.17 PREPARATION OF UNCONJUGATED IMMUNE SERUM

(A) Serum was prepared from twelve swiss mice which had been inoculated intraperitoneally with a stabilate of C. ruminantium. All the mice had been inoculated with 2ml of stabilate on diverse days after separating the mice at random into two groups of six mice each. All the mice in the first group were inoculated on day one only, while group two mice were inoculated on day one and on day 15.

Three mice from each of the two groups were bled to death, as in section 2.1.6, by drawing blood from their hearts on day 30. Their blood was pooled then processed for serum as in section 2.1.6. The resulting serum was then preserved at 0°C in 5ml plastic vials and 2-3 crystals of salt added to prevent precipitation of immunoglobulins.

(B) Serum was also prepared from six mice which had been inoculated intraperitoneally with 2ml of C. ruminantium stabilate 70 days earlier. Their blood was pooled, processed for serum and the latter preserved as in section 2.1.17 (above) then used separately in the IFAT experiment.

(C) Serum from non-immunised mice was also prepared from five swiss mice as in section 2.1.17 (above) then preserved in separate vials as above until required for control purposes in the IFA test.

2.1.18 PREPARATION OF ANTIGEN (Cowdria ruminantium)

Infected mouse peritoneal macrophages (section 2.1.17 A&B) was the source of the antigen and was prepared according to the method of Du Plessis and Malan (1987a). This involved injecting 3ml PBS into the mouse abdominal cavity then gently massaging the abdominal contents with the needle in situ and maximum quantity of fluid withdrawn. The washings were pooled into two groups, centrifuged at 400G for 5 minutes and the supernatant was discarded. The cells were then resuspended with little buffer before being placed in wells on a slide. In this procedure, mice infected 70 days earlier had their peritoneal washings pooled, while those infected 30 days earlier (section 2.1.17) had their washings pooled separately and used as such.

2.1.19 INDIRECT STAINING (INCLUDING MOUNTING OF STAINED SLIDES)

This was done according to the method of Johnson et al (1978) which involves the use of unconjugated serum containing antibodies whose sites of reaction is demonstrated by means of labelled (conjugated) antiserum specific for immunoglobulin.

PROCEDURE

Two drops of the suspension of mice peritoneal macrophages containing the antigen (C. ruminantium) was placed in each well on IFAT glass slides (Wellcome PLC, England) and dried in an incubator at 37°C. The antigen in the wells was then fixed by flooding the

slide with acetone for 3 minutes. Serially diluted immune unconjugated serum, specific for the antigen, was put in the wells coated with the antigen. Sets of four wells received the serum either concentrated or diluted 1:1, 1:2, 1:3, 1:4, 1:5, 1:6 respectively then the slides were held for 30 minutes in a humid chamber maintained at 37°C in an incubator. The slides were then washed with phosphate buffered saline (PBS), pH 7.2 for 30 minutes by placing the slides vertically in a tray immersed in PBS on a rocking platform. After the washing, the wells were stained with the antiglobulin conjugate by placing it on the wells without allowing the wells to dry. The slides were then held once more for 30 minutes in a humid chamber at 37°C. The conjugate was not diluted prior to applying it on the wells. After 30 minutes, the conjugate was removed and the slides washed by being held vertically in a tray immersed in PBS on a rocking platform. The continuous washing lasted one hour. Control slides were treated in a similar manner except that the serum which was first applied on the antigen was from non-immunised animals.

MOUNTING OF STAINED SLIDES

After washing the slides, PBS was shaken off from them then three drops of glycine saline buffer, pH 8.6, 0.5M was placed on the slides to form a confluence over the wells. A glass cover slip, 22mm x 50mm, was then

mounted over the wells without forming air bubbles then observed with a fluorescence microscope (Leitz, Germany) at 495nm.

2.2.3 RESULTS OF IFAT EXPERIMENT

When the stained glass slides were observed in the fluorescence microscope at 495nm, the areas of specific fluorescence were bright-orange in a field which was dull as a result of the Evans Blue counter stain (Plate 33). The fluorescent material was closely associated with the mouse peritoneal cells (Plate 32) as revealed when the ultra violet (U.V) light is cut off and ordinary white light is used to view the fluorescing sites.

In this test, positive results were obtained only from mice inoculated intraperitoneally with the Tana River isolate of C. ruminantium for at least 70 days (post infection) when they served as the source of antigen.

Mice inoculated with the Transmara isolate were not used as a source of antigen in this experiment because no C. ruminantium parasites had been observed in the organs of mice inoculated with this parasites.

The positive results also confirm that the mice whose serum was used in this experiment had formed antibodies against the C. ruminantium parasites



PLATE 31: IFAT (positive) showing bright areas of specific fluorescence of C. ruminantium in a mouse peritoneal washing (fluorescein x 490).



PLATE 32: IFAT (positive) showing areas of specific fluorescence of C. ruminantium in mouse peritoneal macrophages. The top left shows the parasites in association with a monolayer cell while the top right is a congregation of the parasites (Fluorescein x 1000).

2.2.4 DISCUSSION AND CONCLUSION OF PART TWO

In this study, two Kenyan isolates of C.ruminantium from two geographically- distant places, one from Tana river district and the other one from Transmara region were used. Mice which were inoculated with the Tana River isolate were the only ones from which the rickettsial parasite could be recovered. This result is in agreement with the findings of Uilenberg (1983) who reported that there exists a wide spectrum of strains of the rickettsia with varied infectivity for mice and that some strains are non-infective and non-pathogenic to mice. It is possible that the Transmara isolate of C. ruminantium used in this experiment has these characteristics at least to the Swiss mice. On the contrary the Tana River isolate used was infective but non-pathogenic. This result is in agreement with the findings of Uilenberg (1983) who reported the existence of strains which are infectious but non-pathogenic to mice. The Tana river isolate could be recovered from the tissues of sacrificed mice for up to 196 days post infection but only from mice infected intraperitoneally. This finding contradicts the popular belief that the intravenous (i.v) route is better than the intraperitoneal (i.p) route of infecting mice with this parasite.

The Tana river isolate may be infectious to mice when inoculated in high doses only as occurs during i.p infection. The i.v. route allows the administration of

only a little amount of infected blood and therefore few organisms which in turn fail to initiate an infection. The failure to isolate the Transmara isolates from mice unlike the Tana river isolate suggest that the two isolates of the parasite vary in their ability to survive in mice. This being the case, further research should be undertaken to study their immunological characteristics and their ability to infect various domestic ruminants and laboratory animals. This is important because they may have undergone an alteration in their virulence and antigenic identity as was reported by Jongejan et al (1988) after they undertook a study on the cross protection between different stocks of C. ruminantium

The route of infection and the nature of stabilate used are important in determining whether infection will occur or not. Jongejan et al (1988) successfully infected Balb/c mice with C.ruminantium (Senegal) by the intravenous injection of tick derived stabilate whereas blood stabilate via the same route failed to initiate an infection. This report leads this author to recommend that tick derived stabilates of both the Tana river and Transmara isolates should be administered into mice by the intra-venous route. This is especially so because the author failed to isolate any C. ruminantium parasites from all the mice infected by the intravenous injection of infected goats blood.

The mice from which the rickettsial parasites were recovered showed piloerection as the only sign of disease. The subclinical reaction may be as a result of the isolate's characteristic of being infective but non-pathogenic to Swiss mice. This is in agreement with the findings of Uilenberg (1983) who reported that goat blood infected with some two strains of C. ruminantium did not cause symptoms or mortality in Swiss mice injected intravenously. In these experiments, the presence of C. ruminantium parasites in the chronically infected mice was confirmed by immunofluorescence test. This test also confirmed that those mice which harboured the rickettsias for many months had specific antibodies in their sera which failed to eliminate the rickettsial parasites.

The Kenyan isolate of C. ruminantium persisted in the mouse tissues for at least 196 days. One would expect the immune defenses of the host mice to eliminate the rickettsia during that period but this did not happen. Under natural conditions, some parasites have evolved effective evasive mechanisms which allow them to survive while under a full immunological attack of a mammalian host. Parasites can survive the immune attack of the host by occupying sites where they cannot be reached by the host's antibodies. According to Greenwood and Whittle (1981), micro-organisms within host cells, e.g. macrophages are

safe from the effects of antibodies and can survive for prolonged periods causing chronic and latent infections. The rickettsia used in this experiment may have survived in the host in this manner. Another possible method which the rickettsia may have used to evade the immune reaction of the host is by undergoing antigenic change. This aspect of immune evasion was studied by Wassink et al (1986) who demonstrated that no antigenic change occurred in the isolates which they used. This does not rule out the existence of antigenic change in other isolates of C. ruminantium including the Kenyan isolates. It was recently reported by Jongejan et al (1988) that stocks of C. ruminantium from geographically distant areas have been compared and shown that some cross-protect fully, others partially while others do not cross-protect at all. This emphasizes the existence of immunologically different isolates of C. ruminantium each of which ought to be studied individually for their antigenic identity. This is an important step in our search for a suitable isolate for use as a vaccine. Any antigenic differences between the parasite isolates are likely to complicate immunisation against the disease and, according to Uilenberg (1983) the existence of such a variation may explain some of the disappointing vaccination results reported by some authors from the field.

The grey-white lesions which occurred on the surface of the liver and kidney were shown histologically to be perivascular infiltrations of lymphocytes which had also spread to the lung and cardiac muscle. The histological preparations of the mouse organs also revealed degenerative changes in the kidney, lung and heart mainly. These changes included swelling, cast deposits, haemorrhage and necrosis. The kidneys also showed interstitial and periglomerular cellular infiltration as earlier reported by Uilenberg (1983). The cellular infiltration seen in this experiment were similar to the infiltrations which occur in cattle with East Coast Fever (Munyua, 1971). This disturbance of the lymphocytes was reflected in the spleens of some of the mice where there was often depletion of the lymphocytes and these were replaced by a homogenous fibrin material. The lymphocytes play a role in the immune response of the host mice but this author has no explanation on how lymphocyte cells become disturbed leading to their infiltration of organs of animals with heartwater. This author appreciates that these infiltrating cells can cause other pathological changes in the long run when they destroy the liver, heart and kidney. Their extensive destruction of these organs could lead to oedema as a result of ensuing hypoproteinaemia and renal dysfunction.

The nephrosis observed in mice used in this experiment confirmed the findings of Prozesky (1987c) who reported that nephrosis is a common change in animals that die of heartwater. Work by previous researchers may provide the explanation of how the degenerative changes come about. Allison and Houba (1976) summarised the findings of other researchers who demonstrated that glomerulonephritis in mice infected with Trypanosomes and in humans with malaria is a result of the formation in circulation of immune complexes which become trapped in the glomerular capillary walls. They also stated that thrombi consisting of platelets and leucocytes form in such affected blood vessels. This author suggests that a similar situation is true of heartwater. Under those circumstances, the blood capillaries with thrombi and immune complex deposits suffer localized ischaemia leading to the observed necrosis and haemorrhage. Likewise, the thrombi could cause increased hydrostatic pressure and subsequently to exudation and the oedema observed. The pathological changes observed were well developed because the mice were chronically infected, which means there was sufficient time for the changes to be expressed.

In heartwater infection, the induction of autoimmune antibodies directed against renal glomerular basement membrane is another possibility explaining the glomerulonephritis and this ought to be investigated experimentally.

According to Roitt (1986) antigen-antibody complexes cause aggregation of platelets which form microthrombi and vasoactive amine release. It is this author's conviction that the increased capillary permeability in heartwater is a result of similar antigen-antibody complexes which stimulate vasoactive amine release. This leads to hydropericardium, lung oedema and effusion of the body cavities. It was reported by Cowdry(1925a) and confirmed by subsequent researchers that C. ruminantium organisms cause minimal damage to endothelial cells with the result that no plausible explanation was given on how the increased vascular permeability is caused. Recently, Du Plessis et al (1987) suggested that the increased vascular permeability was an immune complex type of hypersensitivity reaction triggered by the release of pharmacologically active substances and that the mechanism of release is still to be determined. This author suggests that the mechanism of release is as explained above by Roitt (1986).

This author's experimental mice harboured the heartwater parasites for many months without showing any signs of oedema. This may have been because the parasites remained intracellular and out of reach of the host's antibodies which would have otherwise formed complexes with the parasites to cause the release of the vasoactive amines in sufficient quantities to cause increased vascular permeability. Once in a while host cells harbouring the parasites may rupture to release the organisms into the circulation where some become eliminated by the mouse immune defenses. Meanwhile those parasites which enter host cells before being eliminated remain intracellular and multiply. In such a case, the number of organisms present in the mouse body is controlled and the mouse lives in balance with this infection.

The lesions in the brains of animals with heartwater are considered mild and insufficiently constant to explain the pathogenesis of the nervous symptoms but some researchers have invoked toxins without evidence, Uilenberg (1983). According to Roitt (1986), the choroid plexus is favoured for immune complex deposition by virtue of its being a major filtration site. This could explain the frequent central nervous disorders in heartwater when antigens released by C. ruminantium parasites form complexes with antibodies which in turn become deposited in

various sites including the choroid plexus. The deposit alone could be responsible for the nervous symptoms observed in some animals with heartwater. Any oedema which might develop in the brain as a result of a release of vasoactive amines could worsen the nervous symptoms and cause rapid death. Animals which die but do not exhibit nervous symptoms may have died without having formed immune - complex deposits in their choroid plexus. Research to verify if animals dying of heartwater have immune-complex deposits in their choroid plexus ought to be carried out.

The persistence of C. ruminantium in laboratory mice implies that similar situation could occur in wild mice which would therefore act as reservoirs of heartwater. If the guinea fowl and the tortoise are known to harbour these parasites then pass the infection to ticks (Oberem and Bezuidenhout, 1987), then mice could play a similar role. The result also shows that swiss mice can be infected with the Tana river isolate then used to transport the organism and at the same time, to "store" the parasite for many months.

A laboratory model of heartwater would obviously be of major potential importance for studies on aspects such as chemotherapy, immunology and pathogenesis of the disease. However, the mouse infective isolates have some variations in their pathogenicity to mice

therefore findings obtained from studies in mice are not necessarily always representative for the field strain in ruminants. This variation of adoption to mice together with the recent findings of Munene (1987) who reported the existence of an intranuclear Kenyan isolate of C. ruminantium are interesting. His report is in contradiction to previous literature which insisted that this parasite occurs exclusively intracytoplasmically. These findings show that intensive research on the heartwater parasites isolated in Kenya and in other countries should be carried out as this may eventually lead to the possible discovery of another species and a re-examination of the parasite's taxonomy.

In this experiment, the observation of C. ruminantium within lymphoid cells infiltrating the kidneys, liver and lung is in general agreement with the findings of Munene (1987) who observed the parasites in the lymph nodes of infected goats. These findings show that it is possible to make a diagnosis of heartwater from the lymph node smears of live affected animals after which such an animal can benefit from a specific treatment of the disease.

The isolate of C. ruminantium used in this experiment infected the laboratory mice and multiplied in sites which include the lymphoid tissue thus inducing a marked proliferation of the lymphoid tissue. The foci of proliferation gave rise to nodula grey-white formations on the kidney, liver and lungs. The organisms were observed in lymphocytes of the formed nodules and in extracellular spaces. The presence of C. ruminantium in mice tissues was confirmed by indirect fluorescent antibody test (IFAT).

PART THREE

3.0 ADAPTATION OF C. RUMINANTIUM TO CELL CULTURES

3.1 MATERIALS AND METHODS

3.1.1 PREPARATION OF CELL CULTURES

A gravid uterus was obtained intact from Dagoretti abattoir and transported in a bucket by vehicle to the department of veterinary pathology and microbiology. After washing the surface of the uterus, it was incised to expose the foetus which was then removed and suspended by the legs. The foetal abdomen and thorax were then dissected open aseptically to expose the internal organs. The kidneys were dissected out along with the lungs and the two organs put in separate sterile petri dishes. The pulmonary arteries and major

veins were dissected out and pooled with the jugular veins together with the major blood vessels of the heart in a third petri dish.

The primary cell cultures were initiated from kidney, lung and endothelial cells from the same foetus but in separate culture vessels. All the cell lines developed were used in the experimental culture of C. ruminantium in vitro.

3.1.1.1 PREPARATION OF LUNG CELLS FOR CULTURE

After trimming away the connective tissues and bronchi, the lungs were cut into small pieces then transferred aseptically into a conical flask containing a magnet. Phosphate buffered saline (PBS), pH 7.2 was added to submerge the magnet and float the lung pieces, then stirred gently for five minutes over a magnetic stirrer. This washing process was repeated with fresh PBS which was similarly discarded after the second washing.

After the second washing, 10ml of 0.25% warm trypsin was introduced into the flask and the organ stirred by a magnetic stirrer for 15 minutes to dissociate the lung cells. The supernatant was decanted into universal bottles and a fresh 0.25% trypsin added to the large organ pieces still in the flask for further stirring.

The contents of the universal bottles were centrifuged at 1660G for five minutes. The supernatant, consisting of trypsin, was decanted and discarded while the cell sediment was resuspended in warm Eagle minimum essential medium (MEM) and re-centrifuged to wash away trypsin. The supernatant was discarded once more while the cell sediment was resuspended in a little MEM and transferred to a tissue culture bottle. The cells arising from the second trypsinisation procedure was similarly processed and pooled with the previous harvest of cells in the tissue culture bottle. Warm 20ml of MEM was added onto the cells and immediately supplemented with 2ml (10%) foetal bovine serum (Gibco Ltd., Paisley, Scotland) and sodium benzylpenicillin (200 i.u/ml) and streptomycin sulphate (200ug/ml) of culture medium.

After a thorough mixing by aspiration and forceful expulsion using a 10ml pipette and a rubber bulb, 2ml aliquots of the cell suspension was dispensed into eight (8) Leighton tubes. The tubes, which are fitted with glass cover slips (10mm x 30mm), were corked with rubber bungs and incubated at 37°C. The Leighton tubes and tissue culture flasks containing cultured cells were only removed from the incubator during media change or microscopic observation.

1.2 PREPARATION OF KIDNEY CELLS FOR CULTURE

The renal capsules were removed along with adhering extrarenal connective tissues and discarded. A scalpel blade was then used to separate the cortex from the medulla by trimming the kidney superficially and retaining the cortical material. This was then cut into small pieces and transferred aseptically into a conical flask containing a magnet then washed and subjected to the cell dissociation process of trypsinisation as in section 3.1.1.1. The resulting cells were processed and dispensed into eight 2ml Leighton tubes and the tissue culture flasks and then incubated as done under section 3.1.1.1.

1.1.3 PREPARATION OF ENDOTHELIAL CELLS FOR CULTURE

After removing as much connective tissue as possible, the blood vessels were slit open and cut into small pieces. The resulting pieces were transferred aseptically into a conical flask containing a magnet then washed and subjected to trypsinisation to dissociate the cells as explained under section 3.1.1.1. In this case, the stirring process was very vigorous and the trypsinisation process lasted 20 minutes per session. This was because of the difficulty of dissociating the cells from blood vessels unlike in the earlier processes involving either the lung or kidney.

The resulting cells were resuspended in 20ml MEM containing 10% foetal bovine serum (FBS), mixed thoroughly, then aliquots of 2ml was dispensed into eight Leighton tubes. The remaining cell suspension was dispensed into two plastic tissue culture flasks (250ml) and then the volume of medium (MEM) increased to 30ml in each flask. Penicillin (200 i.u/ml) and streptomycin (200ug/ml) were added then the cells were incubated at 37°C as in section 3.1.1.1.

3.1.2 CARE OF THE CELL CULTURES

The culture media in all the Leighton tubes and tissue culture flasks were changed aseptically on the second day of culture in order to remove dead and non-attached cells. The fresh replacement MEM contained 10% FBS and antibiotics as in section 3.1.1.1., and it was warmed to 37°C in a water bath prior to being introduced into the tubes. As regards subsequent changes of culture medium (MEM), a change of colour of the medium over the cultured cells was used as an indicator whereby a change from pink to yellow (increased acidity) signalled the time to introduce fresh culture medium. The cultured kidney cells changed the colour of their culture medium to yellow much faster than cells from either lung or blood vessels.

3.1.3 CELL PASSAGE

This involved cultured lung, kidney or endothelial cells. The cells which were passaged had been cultured in plastic tissue culture flasks (250ml) and had formed a thick monolayer of uninfected cells. This stage of cell growth which necessitated passage was reached in twelve to fifteen days of culture and the cells were passaged either into Leighton tubes or other tissue culture flasks.

The culture medium in the tissue culture flask was decanted and discarded then 5ml of a warm mixture of 0.25% trypsin and versene (1:4 respectively) introduced into the flask. After swirling the flask's contents briefly, it was examined using an inverted microscope (Leitz, Germany). The swirling procedure was repeated and when many cells had detached from the flask, 10ml of prewarmed (37°C) MEM was introduced into the flask and swirled around then decanted into a clean sterile universal bottle. Fresh culture medium (MEM) was then introduced into the initial tissue culture flask and incubated at 37°C so that the remaining viable cells in it could grow and multiply.

The universal bottles containing cells detached from the tissue culture flask were centrifuged at 1700G for 5 minutes then the supernatant was decanted and discarded. The sediment was resuspended in 10ml of fresh MEM and the centrifugation process repeated to

wash away any residual trypsin. The supernatant was then decanted to leave a sediment of cells which was resuspended in 5ml of fresh culture medium and transferred into a clean sterile tissue culture flask or into a new set of Leighton tubes depending on requirement. The contents of the flasks was then raised to 30ml by the addition of fresh MEM with 10% FBS. Streptomycin and Benzylpenicillin G were added to the final volume at 200 ug/ml and 200 i.u/ml respectively. Likewise, where the cells were passaged into Leighton tubes, the volume of the final culture medium was raised to 2ml. All the flasks and Leighton tubes containing cells were incubated at 37°C during the duration of the experiment except during microscopic observation and when changing the culture medium.

3.1.4 INOCULA AND INOCULUM PREPARATION

3.1.4.1 INOCULUM ONE (Designated S₃₀)

This was a heartwater blood stabilate (Tana River isolate) prepared forty days earlier from an experimentally infected goat then preserved with ten per cent (10%) dimethylsulfoxide in liquid nitrogen (-196°C). The donor goat had a temperature of 41.5°C when its venous blood was drawn and preserved in sterile 10ml plastic vials containing EDTA. The stabilate was allowed to thaw at room temperature before being used in the inoculation experiments.

3.1.4.2 INOCULUM TWO (Designated GA/B30)

This inoculum was prepared by homogenising organs from two mice inoculated 70 days earlier with heartwater blood stabilate (Tana River isolate). Both mice had been inoculated intraperitoneally with 2ml and 5ml respectively, and were sacrificed within 10 minutes of each other. From each of the mice, the spleen, one kidney and a lobe of the liver were removed and pooled in a sterile petri dish. These organs were transferred into a sterile frozen (-20°C) mortar and ground with pestle by hand.

When the organs froze, 2ml of cold (4°C) culture medium (MEM) was added into it and the grinding continued. Four more 2ml aliquots of culture medium was added at intervals during grinding. When the grinding was completed, the contents of the mortar were transferred into sterile universal bottles and centrifuged at 700G for 3 minutes to sediment large tissue particles.

The supernatant, containing suspended cells, was decanted into a clean sterile universal bottle while the sediment was discarded. Streptomycin (1800ug) and Benzylpenicillin G (1800 i.u) were added to the 9ml of supernate collected.

3.1.4.3 INOCULUM THREE (designated S31)

This is the Transmara isolate of C. ruminantium which was prepared and preserved as described under section 1.3.2. The inoculum was prepared by thawing the stabilate at room temperature then mixed two parts (8ml) of it with one part (4ml) of cold culture medium (MEM) containing 10% FBS and antibiotics as in section 3.1.1.1.

3.1.5 INOCULATION PROCEDURES

Two different inocula of Cowdria ruminantium were used separately to inoculate the cell cultures on the third day of their culture. This was when the cultured cells had grown to form a fairly uniform monolayer.

3.1.5.1 INOCULATION EXPERIMENT WITH INOCULUM ONE (S₃₀)

As soon as inoculum one (S₃₀) was fully thawed, two parts of the stabilate (10ml) were diluted with one part (5ml) of cold culture medium consisting of MEM with 10% FBS and antibiotics as in section 3.1.1.1.

Fifteen (15) Leighton tubes containing cultured cells either from the lung (5), kidney (5) or endothelial cells (5) were removed from the incubator when the cells had grown to form monolayers.

The culture medium in each of these tubes was decanted and discarded. Each tube was then inoculated aseptically with 0.8ml of the diluted inoculum and incubated at 37°C for 105 minutes before most of the inoculum was drained out of the tubes and discarded.

The Leighton tubes were then refilled with 2ml of fresh warm culture medium and re-incubated at 37°C throughout their culture period except when the culture medium (MEM) had to be changed or when screening the tubes microscopically once each day using an inverted microscope (Leitz, Germany).

3.1.5.2 INOCULATION EXPERIMENT WITH INOCULUM TWO (GA/B30)

Twelve Leighton tubes of cultured cells were removed from the incubator on day three of culture. There were four tubes containing cultured cells from either kidney, lung or endothelial cells respectively. The culture medium from each tube was discarded and replaced with 0.7ml of the supernatant obtained from organ homogenate. The cultured cells were then re-incubated at 37°C for 105 minutes. After the incubation, most of the supernatant over the cultured cells was decanted and discarded then each Leighton tube was replenished with 2ml of fresh culture medium containing FBS and antibiotics as in section 3.2. All the tubes were then incubated at 37°C throughout the duration of the experiment except during their microscopic observation and when changing the culture medium. Microscopic observation of the cultured cells was done using an inverted microscope (Leitz, Germany). The culture medium in the tubes was changed either when microscopic observation revealed too many dead floating cells or when the medium over the cultured cells turned

to yellow. This colour change is indicative of high acid content from metabolic activity of the cells.

3.1.5.3 INOCULATION EXPERIMENT WITH INOCULUM THREE (S31)

Fifteen (15) Leighton tubes containing cultured cells either from the lung (5), kidney (5) or endothelial cells (5) were removed from the incubator on day three of culture when the cells had grown to form a uniform monolayer. The cultured cells in each tube were inoculated with 0.8ml of diluted S31 then handled and incubated as in section 3.1.5.1.

3.1.6 FIXATION AND STAINING OF INOCULATED AND CONTROL CELL CULTURES

3.1.6.1 FIXATION AND STAINING OF CELLS INOCULATED WITH S₃₀

One Leighton tube for each cell type (lung, kidney and endothelial cells), was removed from the incubator on day two following inoculation and, after draining the culture medium in it, methanol (70%) was introduced into the tubes to fix the cultured cells in situ. After a fixation of five minutes, the methanol was drained and replaced with 10% Giemsa stain which stained the cells for 20 minutes.

The cover slips on which the cultured cells were growing in the Leighton tubes were then removed, dried and mounted upside down on microscope glass slides ready for microscopic observation. One tube from each group was treated in a similar manner at intervals of every three days.

3.1.6.2 FIXATION AND STAINING OF CELLS INOCULATED WITH GA/B30

One Leighton tube for each cell type (lung, kidney and endothelial) was removed from the incubator on day two and subsequently every after three days then the cells therein are fixed, stained and mounted as under section 3.1.6.1.

3.1.6.3 FIXATION AND STAINING OF CELLS INOCULATED WITH S31

One Leighton tube for each cell type (kidney, lung and endothelial cells), was removed from the incubator on day two of inoculation then handled, fixed and stained as in section 3.1.6.1. More tubes had their contents fixed and stained every after three days until the tubes were exhausted.

3.2 RESULTS OF PART THREE

ADAPTATION OF C. RUMINANTIUM TO CELL CULTURES

Cultured lung, kidney and endothelial cells grew and multiplied very well under the conditions of their culture. Under similar conditions, the kidney cells altered their culture medium to acidic much faster than the other two cell types, thus implying that the medium had to be changed more often. Likewise, kidney cells formed a dense monolayer of cells in the Leighton tubes or tissue culture flasks faster than the lung or endothelial cells could do. This necessitated fixation and staining of the cells cultured in Leighton tubes or passaging of the cells cultured in tissue culture

flasks within twelve days of their culture before they died and peeled off from the vessel. On the other hand, lung and endothelial cells could be passaged after a maximum of sixteen days of culture when they started dying.

3.2.1 RESULTS OF STAINED INOCULATED CELL CULTURES

(A) LUNG CELLS

Under the light microscope, lung cells inoculated separately with either inoculum one (S30), two (GA/B30) or three (S31) exhibited intracytoplasmic dense forms of C. ruminantium which at times appeared in their typical elliptical morula form (plate 30).

(B) KIDNEY CELLS

Intracytoplasmic dense forms of C. ruminantium were revealed by the light microscope in cultured kidney cells inoculated separately with either inoculum one (S30), (GA/B30) or three (S31) (Plate 29).

(C) ENDOTHELIAL CELLS

Dense forms of C. ruminantium were observed in the cytoplasm of the endothelial cells but they were very few.

It was more difficult to observe cells inoculated with the organ homogenate (GA/B30) because of tissue debris from the homogenised organs.

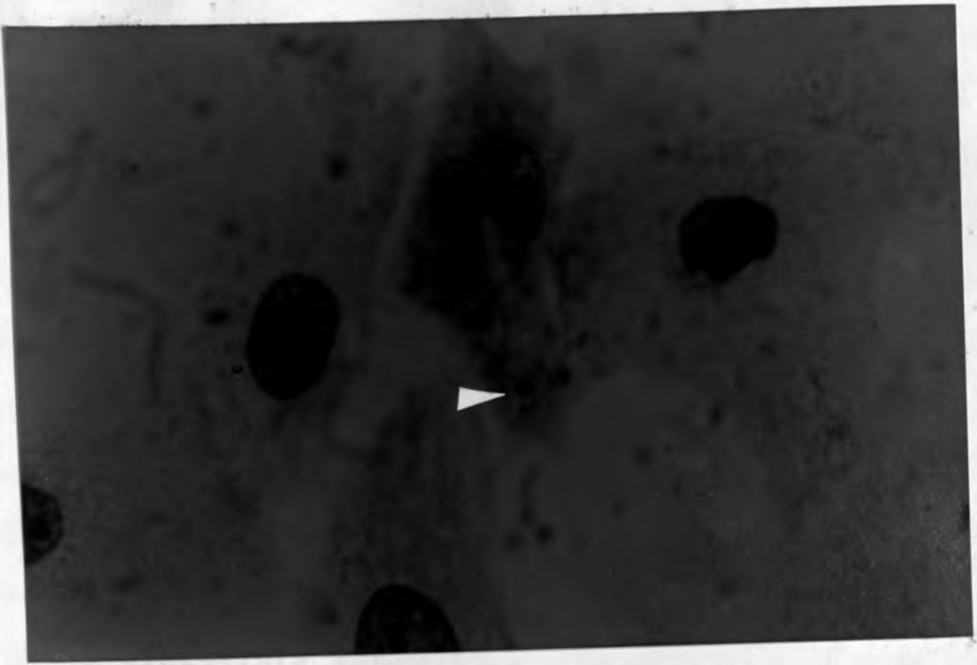


PLATE 29: Kidney cell culture (day 7) showing intracytoplasmic dense forms of C. ruminantium (Giemsa x 1000).

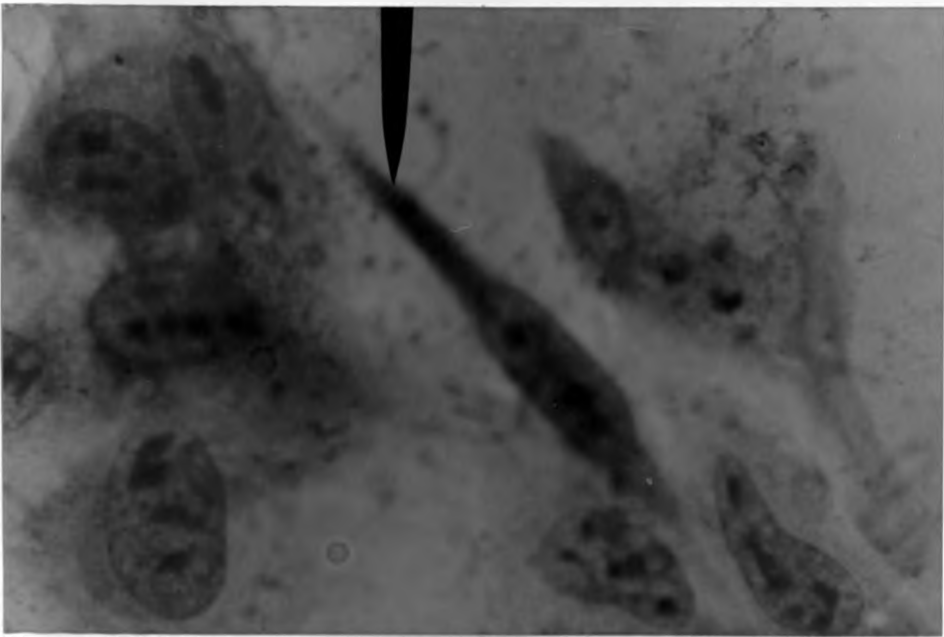


PLATE 30: Lung cell culture showing an elliptical intracytoplasmic congregation of C. ruminantium (Giemsa x 630).

DISCUSSION AND CONCLUSION OF PART THREE

There has been a report of successful propagation of C. ruminantium in cell cultures (Bezuidenhout, 1987a) but all the organisms used had been isolated in South Africa. The existence of antigenically variable organisms which differ in their ability to adapt to mice (Logan et al 1987) lends credit to the possibility of their having different abilities to be propagated in vitro. This kind of experiment hasn't been carried out using rickettsia isolated in Kenya hence the author's attempt to do so.

The results obtained in this experiment showed that the Tana River isolate of C. ruminantium was similar to the Transmara isolate in their ability to be propagated in cell cultures. In both cases, dense intracytoplasmic particles were observed microscopically. The observation that the cells harbouring them were intact and viable implies that the parasites must have entered the cells without damaging the cell membranes appreciably. The author has no explanation of the mechanism by which the parasites penetrate the cultured cells.

In this experiment, lung, kidney and endothelial cells were investigated for their ability to support the propagation of C. ruminantium in vitro. The result shows that any of these cell types can be used in the

propagation of C. ruminantium. Although it is acknowledged that an animal is most resistant to heartwater at birth (Du plessis and Malan 1987b), foetal cells were cultured in this experiment. This was done with the knowledge that no effort has been made to study if cultured cells are intrinsically resistant to infection by the rickettsia by virtue of their being foetal cells. This implies that if foetal cells are naturally resistant to infection by heartwater agents then the few cultured cells which were infected in this experiment somehow confirms the reports that foetal resistance to heartwater is not absolute (Du Plessis and Malan, 1987b).

The result in this experiment was obtained after only a few days of culture thus being in general agreement with the findings of Uilenberg (1983) whose cultures lasted a maximum of 14 days. This short culture period is disadvantageous. The ideal situation is still to be achieved so that the C. ruminantium parasites can be maintained indefinitely in vitro by being passaged in freshly cultured cells every after few days. The successful propagation of C. ruminantium would be of great research potential because it could facilitate the study of the parasite's biological characteristics, antigens and the screening of new chemotherapeutic agents. This could lead to the development of vaccine against heartwater. Under those

circumstances, it will be important to know whether one strain can be used in all regions where the disease occurs or whether significant antigenic differences would preclude this.

In this experiment, the cultured cells with granules similar to C. ruminantium should have been tested for their infectivity to susceptible ruminants but economic constraints precluded this important confirmatory test. Likewise, the non-availability of electron-microscope facilities also precluded the ultra-structural studies of the intracellular granules.

The results in this experiment affirms that all in vitro attempts to propagate C. ruminantium isolated from Kenya and from other parts of the world should be continued. This is because of their immunological variation which makes it possible that the parasites may be having varying capabilities of being propagated in cultured cells. This may be the explanation of the encouraging results obtained from the Kenyan isolates cultured in vitro in this experiment.

REFERENCE

- ALLISON A.C. and HOUBA V. (1976). Immunopathology due to Complexes of Antigen and Antibody (Type III Reactions) in parasitic infections. IN Immunology of Parasitic Infections; Sydney Cohen and Elvio H. Sadun editors. Blackwell Scientific Publications, Oxford pp. 436 - 447.
- BARRE N. and CAMUS E. (1987). The reservoir status of goats recovered from heartwater. Onderstepoort J. Vet. Res. 54: 435-437
- BARRE N., UILENBERG G., MOREL P.C., and CAMUS E. (1987). Danger of introducing heartwater onto the American mainland: Potential role of indigenous and exotic Amblyomma ticks. Onderstepoort J. Vet. Res. 54: 405-417
- BEZUIDENHOUT, J.D. (1981). The development of a new heartwater vaccine using Amblyomma hebraeum nymphae infected with Cowdria ruminantium. Proceedings of an International Symposium on Tick Biology and Control; Rhodes University, Grahamstown, 41-45.
- BEZUIDENHOUT, J.D. (1987a). The present state of Cowdria ruminantium cultivation in cell lines. Onderstepoort J. Vet. Res. 54:205-210
- BEZUIDENHOUT, J.D. (1987b). Natural transmission of heartwater. Onderstepoort J. Vet. Res. 54: 349-351.
- BEZUIDENHOUT, J.D. and BIGALKE R.D. (1987). The control of heartwater by means of tick control. Onderstepoort J. Vet. Res. 54: 525-528.

- REZUIDENHOUT, J.D. OLIVER J.A., GRUSS B. and RADENHORST J.V. (1987). The efficacy of alternative route for the infection or vaccination of animals with Cowdria ruminantium. Onderspoort J. Vet. Res. 54: 497-506.
- CAMUS E. and BARRE N. (1987). Epidemiology of heartwater in Guadeloupe and in the Caribbean. Onderspoort J. Vet. Res. 54:419-426.
- COWDRY, E.V. (1925). Studies on the aetiology of heartwater. I. Observation of a rickettsia, Rickettsia ruminantium (n. sp.) in the tissues of infected animals. J. Experimental Medicine 42: 231-252.
- DU PLESSIS, J.L. (1970). Pathogenesis of heartwater. I. Cowdria ruminantium in the lymph nodes of ruminants. Onderspoort J. Vet. Res. 37: 89-96
- DU PLESSIS, J.L. and MALAN, LETITIA (1987a). The application of the Indirect Fluorescent antibody test in research on heartwater. Onderspoort J. Vet. Res. 54: 319-325
- DU PLESSIS, J.L. and MALAN, LETITIA (1987b). The non-specific resistance of cattle to heartwater. Onderspoort J. Vet. Res. 54: 333-336
- DU PLESSIS, J.L. and MALAN LETITIA (1987c). The block method of vaccination against heartwater. Onderstepoort J. Vet. Res. 54: 493-495
- DU PLESSIS J.L., MALAN LETITIA and KOWALSKI Z.E. (1987). The pathogenesis of heartwater. Onderstepoort J. Vet. Res. 54: 313-318

- FAHEY J.L. and TERRY E.W. (1978). Ion exchange chromatography and gel filtration IN. Hand book of experimental immunology; D.M. Weir(ed.) 3rd edition, Blackwell scientific publications, Oxford pp.8.1-8.15
- GRADWELL D.V., VAN NIEKERK C.A.W.J. and JOUBERT D.C. (1976). Attempted artificial infection of impala, blue wildebeest, buffalo, kudu, giraffe and warthog with heartwater. J. South African Vet. Med. assoc. 47:209-210
- GREENWOOD B.M. and WHITTLE H.C. (1981). Evasion of the immune response to infection. IN. Current Topics in immunology. Turk J. (ed.). pp. 62-86 Spottiswoode Ballantyne Ltd, Colchester. U.K.
- ILEMOBADE, A.A. (1977). Heartwater in Nigeria. I. The susceptibility of different local breeds and species of domestic ruminants to heartwater. Tropical Animal Health and Production 9:177-180
- ILEMOBADE, A.A. (1978). The persistence of Cowdria ruminantium in the blood of recovered animals. Tropical Animal Health and Production 10:170
- ILEMOBADE A.A. and BLOTKAMP J. (1978). Preservation of Cowdria ruminantium at low temperatures. Research in Veterinary Science 19:337-338
- JOHNSON G.D., HOLBOROW E.J and DORLING J. (1978). Immunofluorescence and Immunoenzyme techniques. IN: Weir D.M. (ed.). Handbook of experimental immunology, 3rd edition, Blackwell Scientific Publications, Oxford.

- JONGEJAN F., VAN WINKELHOFF A.J. and UILENBERG G. (1980).
Cowdria ruminantium (Rickettsiales) in primary goat
 kidney cell cultures. Research in Vet. Science
 29:392-393
- JONGEJAN F., UILENBERG G., FRANSSEN F.F.J. (1988).
 Antigenic differences between stocks of C.
ruminantium. Res. Vet. Sci. 44: 186 - 189.
- KARRAR G. (1960). Rickettsial infection (heartwater) in
 sheep and goats in Sudan. British Vet. Journal
 116:105-114
- KIERNAN J.A., (1983). Histological Staining in one or two
 colours IN: Histological and Histochemical methods:
 Theory and Practice pp. 77-80.
- KOCAN K.M., BEZUIDENHOUT J.D. and HART A. (1987).
 Demonstration and ultrastructural features of Cowdria
ruminantium in midgut epithelial cells and salivary
 glands of nymphal Amblyoma hebraeum. Onderstepoort J.
 Vet. Res. 54:87-92
- LOGAN, L.L. (1987). Cowdria ruminantium: Stability and
 preservation of the Organism. Onderstepoort J. Vet.
 Res. 54: 187 - 191.
- LOGAN L.L., BIRNIE E.F. AND MEBUS C.A. (1987). Cross-
 immunity between isolates of Cowdria ruminantium.
 Onderstepoort J. Vet. Res. 54: 345.
- MARE C.J. (1972). The effect of prolonged oral
 administration of Oxytetracycline on the course of
 heartwater (Cowdria ruminantium) infection in sheep.
 Tropical Animal Health and Production 4: 69 - 73.

- MATHERON G; BARRE N; CAMUS E; and GOGUE J. (1987). Genetic resistance of Guadeloupe native goats to heartwater. Onderstepoort J. Vet. Res. 54: 337 - 340.
- MEBUS C.A. and LOGAN L.L. (1988). Heartwater disease of domestic and wild ruminants: Review Article. J. Amer. Vet. Med. Assoc. 192: 950 - 952.
- MUNENE N. JOHN (1987). Pathogenesis and pathology of heartwater (Cowdriosis) in sheep and goats. M.sc. Thesis. University of Nairobi, 276pp.
- MUNYUA W.K. (1971). Pathogenesis and Pathology of East Coast Fever in Cattle. M.Sc. Thesis, University of Nairobi IIIp.
- NEITZ W.C. and ALEXANDER R.A. (1941). The immunization of calves against heartwater. Journal of the South African Vet. Med. Assoc. 12: 103 - 111.
- NOEL, KRIEG and HOLTS (1984) (eds). Bergeys Manual of Taxonomic Bacteriology, Vol. 1. Williams Wilkins Co. Baltimore, USA.
- OBEREM P.T. and BEZUIDENHOUT J.D. (1987a). Heartwater in hosts other than domestic ruminants. Onderstepoort J. Vet. Res. 54: 271 - 275.
- OBEREM P.T. and BEZUIDENHOUT J.D. (1987b). The production of heartwater vaccine. Onderstepoort J. Vet. Res. 54: 485 - 488.
- OUCHTERLONY O. and NILSSON L.A. (1978). Immunodiffusion and immunoelectrophoresis. IN: Handbook of experimental immunology; 3rd edition, pp 19.1 - 19.44. D.M. Weir (ed). Blackwell Scientific Publications, Oxford, U.K.

- PETNEY T.N.; HORAK I.G. and RECHAV Y. (1987). The ecology of the African vectors of heartwater, with particular reference to Amblyoma hebraeum and A. variegatum. Onderstepoort J. Vet. Res. 54: 381 - 395.
- PIENAAR J.G. (1970). Electron microscopy of Cowdria (Rickettsia) ruminantium (Cowdry 1926) in the endothelial cells of the vertebrate host. Onderstepoort J. Vet. Res. 37: 67 - 78.
- PROVOST A. and BEZUIDENHOUT J.D. (1987). The historical background and global importance of heartwater. Onderstepoort J. Vet. Res. 54: 165 - 169.
- PROZESKY L. (1987a). Heartwater: The morphology of Cowdria ruminantium and its staining characteristics in the vertebrate host and in vitro. Onderstepoort J. Vet. Res. 54: 173 - 176.
- PROZESKY L. (1987b). Heartwater: The artificial transmission of Cowdria ruminantium in domestic ruminants and mice. Onderstepoort J. Vet. Res. 54: 277 - 279.
- PROZESKY L. (1987c). The pathology of heartwater. III. A review. Onderstepoort J. Vet. Res. 54: 281 - 286.
- PROZESKY L. (1987d). The diagnosis of heartwater at post-mortem in ruminants and the confirmation of Cowdria ruminantium in mice. Onderstepoort J. Vet. Res. 54: 301 - 303.

- PROZESKY L., BEZUIDENHOUT, J.D. and PATERSON C.L. (1986).
Heartwater: An in vitro study of the ultrastructure
of Cowdria ruminantium. Onderstepoort J. Vet. Res.
54: 153 - 159.
- PROZESKY L and DU PLESSIS J.L. (1985). The pathology of
heartwater. I. A study of mice infected with the
Welgevonden strain of Cowdria ruminantium.
Onderstepoort J. Vet. Res. 52: 71 - 79.
- PROZESKY L. and DU PLESSIS J.L. (1987). Heartwater: The
development and life cycle of Cowdria ruminantium in
the vertebrate host, ticks and cultured endothelial
cells. Onderstepoort J. Vet. Res. 54: 193 - 196.
- PURCHASE H.S. (1945). A simple and rapid method for
demonstrating Rickettsia ruminantium (Cowdry, 1925)
in heartwater brains. The Veterinary Record 57: 413
- 414.
- RIGGS J.L., LOH P.C. and EVELAND W.C. (1960). A simple
fraction method for preparation of fluorescein-
labelled gamma globulin. Proc. Soc. Exp. Biol.
(N.Y.), 110: 833.
- ROITT IVAN, (1986). Hypersensitivity. IN: Essential
immunology 5th edition, pp. 233 - 267, PG Publishing
Pte Ltd., Singapore.
- SCHREUDER B.E.C. (1980). A simple technique for the
collection of brain samples for the diagnosis of
heartwater. Tropical Animal Health and Production
12: 25 - 29.

- STEWART C.G. (1987a). Specific immunity in farm animals to heartwater. Onderstepoort J. Vet. Res. 54: 341 - 342.
- STEWART C.G. (1987b). Specific immunity in mice to heartwater. Onderstepoort J. Vet. Res. 54:343- 344.
- TRINGALI G.R; MONTENEGRO, M.R; WALKER, D.H. and MANSUETO S. (1983). Immunity and Rickettsial infection. Acta Tropica 40: 311 - 320.
- UILENBERG G. (1983). Heartwater (Cowdria ruminantium infection): Current status. Advances in Veterinary Science and Comparative Medicine 27: 427 - 479.
- UILENBERG G.; BARRE N.; CAMUS E.; BURRIDGE M.J. and GARRIS G.J. (1984). Heartwater in the Carribean. Preventive Veterinary Medicine 2: 255 - 267.
- UILENBERG G., ZIVKOVIC D., DWINGER R.H. et. al (1983). Cross Immunity between strains of Cowdria ruminantium. Rest. Vet. Sci. 35: 200 - 205.
- VAN DER MERWE, L. (1987). The infection-and-treatment method of vaccination against heartwater. Onderstepoort J. Vet. Res. 54: 489 - 491.
- VAN AMSTEL S.R.; GUTHRIE A.J.; REYERS F.; BERTSCHINGER H; OBEREM P.T., KILLEEN V.M; and MATHEE O. (1987). The clinical pathology and pathophysiology of heartwater: A review. Onderstepoort J. Vet. Res. 54: 287 - 290.
- VAN DE PYPEKAMP H.E. and PROZESKY L. (1987). Heartwater. An overview of the clinical signs, susceptibility and differential diagnosis of the disease in domestic ruminants. Onderstepoort J. Vet. Res. 54:263 - 266.

- WILJOEN G.J.; VERMEULEN N.M.J and NEITZ A.W.H. (1987).
Theoretical aspects of the enzyme-linked
immunosorbent assay technique and its use in the
detection of Cowdria ruminantium antigen and antibody
in reacting animals. Onderstepoort J. Vet. Res. 54:
305 - 312.
- WALKER, JANE B. (1987). The tick vectors of Cowdria
ruminantium (Ixodoidea, Ixodidae, genus Amblyoma) and
their distribution. Onderstepoort J. Vet. Res. 54:
353 - 379.
- WASSINK L.A., FRANSSEN F.F.J. and UILENBERG G. (1986).
Observations on mouse-infective stocks of Cowdria
ruminantium: Attempts to demonstrate antigenic
changes of the Kwanyanga Stock in mice. Res. Vet.
Sci. 41: 98 - 101.
- WILLIAMS C.A. and CHASE M.A. (1967). Preparation of
antigens and antibodies. IN: Methods in Immunology
and Immunochemistry pp. 345 - 347.