Lymphocyte Infectivity and Characterization of *Theileria parva* strains isolated from Paddocks in Kiambu District.

By

Matete George Owour (BVM).

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Department of Veterinary Pathology and Microbiology

Faculty of Veterinary Medicine

University of Nairobi

Declaration.

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

Signed : 11/3/98

This thesis is submitted for examination with our approval as University supervisors.

Signed : _____ **Date:** MARCH 16TH 1998

Prof. P.W.N. Kanyari (BVM, MSc,PhD)

Department of Pathology and Microbiology

Signed : _____ **Date:** 16/3/98

Prof. T.A. Ngatia (BVM, MSc, Dip, PVM, PhD)

Department of Pathology and Microbiology

External Supervisor.

Signed : _____ **Date:** 16/3/98

Dr. D.P.Kariuki (BVM, MSc, PhD)

N.V.R.C. KARI

Dedication:

To My wife Achieng Owour.

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ABSTRACT

East Coast Fever, a disease of cattle that results in death by respiratory failure is caused by *Theileria parva*. Due to the widespread occurrence, the characterization of *Theileria* species and diagnosis of the disease has become increasingly difficult.

An attempt was made to study the infectivity and pathogenicity and to characterize various *T. parva* strains using observations of their clinical signs, parasitological, haematological and post-mortem findings as indicators. The clinical picture was found to be consistent to the findings of previous workers. However, when intra-lymphocytic theilerial parasites grown *in vitro* were assessed using antibodies specific to schizont epitopes, a marked antigenic diversity was noted between and within strains. Monoclonal antibodies were thus useful in characterizing extra- and intra- strain differences in *T. parva*. The Western blot technique further revealed polymorphism in the sizes of the Polymorphic Immunodorminant Molecule (PIM) ranging from 80 - 90 kilodaltons in isolates obtained from the same paddock and also when compared to *T. parva* Muguga.

Two batches of *Theileria* sporozoites were used for the comparison of the *in vitro* infectivity and early developmental stages of *Theileria* infection to peripheral blood mononuclear cells obtained from the same animal. Similarities were noted in the rates and periods of infectivity (3 - 6 days) regardless of the strain and sporozoite dilution. However, they showed different growth and cultural characteristics with *T. parva* Marikebuni producing grape-like clusters whereas the breakthrough parasite to *T. parva* Marikebuni immunization resulted in a uniform sheet of single cells in culture.

CHAPTER 1

1.0 INTRODUCTION

East Coast Fever (ECF), a disease of cattle caused by the protozoan parasite *Theileria parva* (Koch, 1903), is one of the most important causes of livestock losses in East and Central Africa. The main vector is the Ixodid tick *Rhipicephalus appendiculatus*. The parasite has a limited host range and attempts to adapt it into laboratory animals have not been successful (Purnell, 1977). *Theileria parva* causes three distinguishable syndromes in cattle. These are based on their clinical and parasitological manifestations, namely ECF, corridor disease and bovine malignant theileriosis (Lawrence, 1981). East Coast Fever has clinical manifestations which include acute, sub-acute, mild and inapparent syndromes (Neitz, 1957). These manifestations are influenced by age, immunological status, the genetic make up of the host, the infective dose and virulence of the strain involved.

Past work by Matson (1967) has indicated differences in schizont and piroplasm morphology and also the parasite levels in the blood between *T. parva parva* and *T. parva lawrencei*. Others however, report similar serological results

between the strains (Brocklesby and Barnett, 1966). In fatal cases Bwangamoi, Frank, Munyua and Wandera (1971) reported tumour of spleen and renal pseudo-infarcts, while Aruo (1977) noted only haemorrhages in the same organs. Differences in the haematological parameters between strains has also been reported by (Hill and Matson, 1970). Further studies using various strains may possibly throw more light on the pathogenesis of theilerial parasites in the cattle host.

Losses in ECF are either direct or indirect. Direct losses include the high mortalities and the decreased production attributed to the presence of disease in the host. These include reduced milk yield, weight loss in recovered animals and death in severe cases (Young, Groocork and Kariuki, 1988). Indirect losses are in the cost of tick control and treatment of diseased animals. The disease thus acts as a constraint to livestock production and improvement.

The characterization of *Theileria parva* has been necessitated by the presence of immunologically diverse strains. Characterization can be either *in vivo* or *in vitro*. Cross-immunity trials have been the most reliable methods of *in vivo* characterization. This involves the infection of cattle using low doses of stabilate and their simultaneous treatment using tetracyclines as a method of

control. The animals are subsequently challenged for immunity using different stocks. Parasites which break through the immunization are considered as distinct stocks (Morzaria, 1989). However these tests are time consuming and expensive since they involve the use of animals sero-negative to *Theileria* antibodies. This has necessitated the use of *in vitro* techniques which are able to relate parasite antigenicity to pathology. Such techniques include the use of Mabs profiles (Pinder and Howett, 1980) and Western blot (Toye, Goddeeries, Iams, Musoke and Morrison, 1991).

Monoclonal antibody profiles employ a battery of specific antibodies to characterize *T. parva* strains. These are useful in characterizing extra and intra strain differences. Monoclonal antibodies could thus identify similarities or differences between isolates of *T. parva* in a region. A study carried out using isolates from the same paddock, will test the usefulness of monoclonal antibodies in the characterization of *T. parva* strains. It would also attempt to show the profiles of the various parasite strains. Protein analysis by the Western blot method has been used in conjunction with Mabs to characterize different strains of *T. Parva*. There is a need to differentiate between isolates of the same strain and also to test isolates in the field.

Theileria parva Muguga sporozoites have been used to study the rate of infection of lymphocytes from different species of animals *in vitro* (Stagg, Young, Leitch, Grootenhuis and Dolan, 1983). Comparison of the rate of infection of lymphocytes obtained from a single steer by two different batches of tick sporozoites is yet to be tested. This could be a useful technique in distinguishing between *Theileria parva* strains.

1.1 OBJECTIVES

The objectives of this study were:-

1. To compare the clinical signs, parasitological, haematological and post-mortem findings of various *Theileria parva* strains namely *T. parva* Muranga Stabilate (St 232), *T. parva* Lanet (St 263), *T. parva lawrencei* (St 146) and a *Theileria parva* strain isolated from a highly infested paddock at the National Veterinary Research Centre - Muguga.

2. To characterize and compare various *Theileria parva* isolates and strains using monoclonal antibody profiles and Western blot techniques.

3. To compare the infection rates and observe the early developmental stages of two batches of *Theileria* sporozoites namely: *T. parva* Marikebuni (St 300A) and the breakthrough parasite to *T. parva* Marikebuni immunization.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 EAST COAST FEVER

East Coast Fever (ECF) was first described in 1898 by Koch in the then German East Africa now Tanzania. The disease was later described in Southern Rhodesia now Zimbabwe by Koch (1903). East Coast Fever is caused by *Theileria parva*, an intracellular protozoan parasite of the genus *Theileria*. The principal features of the disease are presence of schizonts in lymphoid cells, the presence of erythrocytic forms and the transmission by ticks (Koch, 1903). East Coast Fever in cattle probably originated from buffalo populations in eastern Africa. Following the introduction, it was disseminated from the southern African region to most parts of East and Central Africa hence the name ECF, (Young, 1981). Knowledge of the disease has been summarized in several reviews (Neitz, 1957; Wilde, 1967; Purnell, 1977) and symposia (Henson and Campbell, 1977; Irvin, Cunningham and Young 1981, Dolan, 1989).

2.1.1 AETIOLOGY

There are several species of *Theileria* recognized in Africa namely *T. parva*, *T. annulata*, *T. mutans*, *T. orientalis*, *T. taurotragi* and *T. velifera* (Norval, Perry and Young, 1992). *Theileria parva* is the most important due to its pathogenicity to cattle. It is subdivided into three sub-species based on clinical and epidemiological parameters. *Theileria parva parva* causes ECF, this is the classical disease with both schizonts and piroplasm being demonstrated in lymphocytes and erythrocytes respectively. *Theileria parva lawrencei* a buffalo-derived parasite produces few small sized schizonts and the piroplasm are rare, making their transmission by ticks inefficient. It causes corridor disease. *Theileria parva bovis*, however, produces many piroplasms. It causes January disease, a mild infection occurring in cattle of Zimbabwe (Young, Brown, Burridge, Grootenhuis, Kariuki, Punell and Stagg, 1978; Norval, Perry and Young, 1992).

2.1.2 LIFE CYCLE OF *Theileria parva*

2.1.2.1 Life cycle of *T. parva* in the tick

The life cycle of *T. parva* in the tick *R. appendiculatus* begins when infected erythrocytes are ingested by the ticks. These erythrocytes are lysed in the tick gut to release piroplasm which are infective. In two to four days, the piroplasm differentiate into ray bodies (microgametes) and later into spherical unnucleated macrogametes. Pairs of these gametes undergo syngamy to form a zygote within six days. The zygotes then invade the gut epithelium of the tick and later transform into motile club-shaped kinetes which invade the newly developing type III salivary gland acini. The kinetes further transform into sporonts, which as the tick feeds on a new host, develop into mature sporozoites within 5 days. The sporozoites are then ready for emission from the tick's salivary gland. It is estimated that a single infected salivary gland acini contains between 40,000 and 50,000 *T. parva* sporozoites. These can survive in the tick for up to 32 months (Purnell, Brown, Cunningham, Burridge, Kirimi and Ledger, 1973; Melhorn and Schein, 1984; Fawcett, Young and Leitch , 1985).

2.1.2.2 Life cycle in the mammalian host

The tick deposits sporozoites subcutaneously in the host during feeding. The sporozoites then enter into the host by an energy dependent mechanism (Shaw, Tilney and Musoke, 1991). Initially the sporozoite recognizes and binds to one or more sites on the host's lymphocyte cell surface, followed by formation of a close continual junction between the sporozoites and circulating lymphocyte membrane. A progressive circumferential zippering of the membrane internalizes the sporozoites in the host's cell. The surrounding membrane dissolves and the parasite escapes into the cytoplasm of the host's cell. Orderly array of host cell-derived microtubules are formed around the sporozoite. Antibodies raised against a 67 KDa antigen on the sporozoite surface prevent entry into the host cell, thus suggesting a relation to the receptor site. Penetration of the lymphocytes results into hyperplasia, characterized by increase in cytoplasm and enlarged golgi apparatus. Within 24 hours of entry, the sporozoite are located at the golgi. They undergo nuclear division forming multi-nucleated macroschizonts. The macroschizonts divide to give rise to microschizonts with small sized nuclei. Within a period of 14 days the schizonts develop into merozoites which penetrate erythrocytes to form piroplasm. These piroplasm are ingested by ticks during feeding and can subsequently be

introduced into a new host (Barnett and Brocklesy, 1966; Young *et al.*, 1978; Dobbelaere, Spooner, Barry and Irvin, 1984; Musoke, Nantulya, Rurangirwa and Buscher, 1984; Shaw, Tilney and Musoke, 1991).

Theileria schizonts induce lymphoblastogenesis and clonal expansion of infected cells. During host cell division, there is a synchronized division of schizonts (Hullinger, Wilder, Brown and Turner, 1964). There is always an equal number of schizont nuclei in each daughter cell. However, only lymphocytes infected with a single schizont exhibit unlimited clonal expansion (Stagg, Dolan, Leitch and Young, 1981).

2.1.3 DISTRIBUTION OF EAST COAST FEVER

Distribution of ECF is positively correlated to that of *R. appendiculatus* and that of suitable animal host. The disease is prevalent in areas of altitudes ranging from 0-5000 metres above sea level. In Kenya the disease occurs in most of the country except the arid North where the vector does not survive due to high temperatures (Kariuki, 1988, 1990; Lessard, L'Epllattenier, Norval, Kundert Dolan, Croze, Walker, Irvin and Perry, 1990; Walker, 1991).

2.1.3.1 Distribution of the tick vector

Theileria parva is mainly transmitted by the three host tick *R. appendiculatus*. Transmission is transtadial with both the larval and nymphal stages spreading the disease after moulting. The distribution of the tick is dependent on the amount of vegetation cover, rainfall, relative humidity, population density of suitable ruminant host and use of acaricide. High temperatures and low humidity limit the survival of the free-living stages of the tick. *Rhipicephalus appendiculatus* has been identified in fifteen countries of Africa, but occurs mostly in Kenya, Rwanda, Burundi, Tanzania, Malawi, Zimbabwe and South Africa (Lawrence and Mackenzie, 1980; Howell, De Vos, Bezuidenhout, Potgieter and Barrowman, 1981; Norval and Lightfoot, 1982; Lessard *et al.*, 1990).

Other ticks which have been incriminated include *R. duttoni* and *R. Zambenziensis*. These are the major vectors in the hot and dry areas of Central and Southern Africa (de Graca and Serrano, 1971; Lawrence, Norval and Uilenberg, 1983). The African buffalo acts as a constant reservoir for infection through the resident tick population (Young, Leitch and Newson, 1981).

2.1.4 PATHOGENESIS OF THEILERIOSIS

Theileria species are infective and virulent to various species of domestic ruminants (Barnett, 1977). *Theileria parva* is mainly a pathogen of cattle although buffaloes are also susceptible (Stagg *et al.*, 1983). Three broad phases of the disease process have been described. An initial incubation phase in which infection is not detected, a second phase associated with marked hyperplasia of lymphoid tissue and finally a period with high level of parasitism associated with cellular disorganization and depletion of lymphoid organs.

The prepatent period is characterized by slight lymphocytosis and increase in cellular efferent of the lymph nodes (Radley, Brown, Burridge, Cunningham, Pierce and Purnell, 1974). Schizonts are detected in the draining lymph nodes within 7 days of the tick inoculating sporozoites into the cattle. Infection is via the circulating lymphoid cells. Hyperplasia of lymphocytes is pronounced (DeMartini and Moulton, 1973b). Finally there is lymphoid disorganization and depletion characterized by involution of the germinal centres, lysis of lymphocytes and necrosis (Morrison, Buscher, Emery, Nelson and Murray, 1981; Morrison, Lalor, Goddeeris and Teale, 1986).

The infected lymphoid cells spread to all lymphoid organs like the thymus and bone marrow but more prominently to the lamina propria of the gastrointestinal tract and interstitial tissue of the lungs (De Kock, 1957; Bwangamoi *et al.*, 1971). The pattern of cellular changes in all lymphoid organs is similar to that of the regional lymph node. However, these changes are less pronounced and the increase in cellularity is more gradual than those of the regional lymph nodes (DeMartini and Moulton, 1973a).

2.1.5 CLINICAL MANIFESTATION

The severity of the clinical signs of ECF depends on the dose and strain of the parasite, and the immunological status of the host. The disease manifests itself in terms of severity of the symptoms as acute, sub-acute, mild and inapparent (Neitz, 1957). In the acute form, fever above 39.5°C is recorded. The incubation period ranges from 8-25 days with a mean of 14 (Neitz, 1957, Jura and Losos, 1980). Other signs include a gradual loss of appetite, excessive salivation and lacrimation, starring hair coat, dry muzzle, drooped ears, enlarged lymph node and ruminal stasis. Dyspnea characterized by a short cough occurs as the disease progresses. The animal becomes recumbent and dies due to

respiratory failure (Henning, 1956). Either diarrhoea or constipation can be observed with evacuations which are blood tinged and slimy.

The sub-acute form of the disease occurs in calves, adults in endemic areas and in partially immune animals (Neitz, 1957). The signs are less pronounced with recovery being a common sequelae. The mild form of the disease is characterized by a slight rise in temperature and presence of schizonts in the superficial lymph nodes. The inapparent form of the disease occurs in experimental animals which are solidly immune to parasites of challenge as observed in cross- immunity trials. A nervous form of the disease characterized by circling, head pressing, incoordination, blindness and death has been observed in Uganda among the young stock (Aruo, 1979).

Past work done by Brocklesby and Barnett (1966) and Young, Branagan, Brown, Burridge, Cunningham and Purnell (1973) in Kenya showed that buffalo-derived parasites when passaged in cattle developed morphologically similar schizonts and piroplasm parasitaemia and infectivity to ticks as *T. parva* of cattle origin. However Neitz (1957) in South Africa and Matson (1967) in Zimbabwe were able to show differences in the various isolates using cross-immunity trials. Burridge and Kimber (1972) did not find major differences in

East African strains of *T. parva parva* and *T. parva lawrencei* using serological techniques. The only differences noted were in the mean schizont index and absence of piroplasm in *T. parva lawrencei* infections.

2.1.6 POST-MORTEM FINDINGS

2.1.6.1 Gross pathology

In acute cases the condition of the carcass is good, whereas in protracted cases emaciation is evident. The pathology includes gelatinous or haemorrhagic sub-cutaneous fat and oedema. The lymph nodes are enlarged, hyperaemic and oedematous (Munyua, 1971). The abdominal cavity has a fair amount of straw coloured fluid and petechiations of the peritoneum. The abomasum is hyperaemic, oedematous and has mucosal erosions or ulcerations at the pylorus. Ecchymotic or petechial haemorrhages may be seen on the serosa and mucosa of the intestines. The Peyers patches are enlarged. The liver is enlarged, icteric and mottled with greyish white foci. The kidneys have a variegated appearance of greyish white pseudo-infarcts with haemorrhage of the pelvic epithelium. The perirenal fat is yellow, gelatinous and has petechial haemorrhages (Munyua, 1971). There is accumulation of straw coloured and in some cases blood-tinged fluid in the thoracic cavity. The lungs are enlarged, oedematous and

haemorrhagic. The trachea, bronchi and bronchioles are filled with variable amounts of froth (Munyua,1971).

In rare cases of cerebral theileriosis, meningeal haemorrhages are observed (Henning, 1956). Anaemia, muscle degeneration, oedema of the gall bladder mucosa, haemorrhage and ulceration of the intestines have also been noticed in some instances (De Kock, 1957; Bwangamoi *et al.*, 1971).

2.1.6.2 Histological changes

At the site of inoculation (tick bite) there is a raised nodule characterized by epidermal ulcers, haemorrhage , oedema and infiltration of inflammatory cells, mainly lymphocytes (Kimeto, 1978). However, the microscopic picture in lymphoid organs and tissues is dominated by proliferating lymphocytes. The lymph nodes show proliferation of lymphocytes and the presence of parasitised lymphoblasts. In protracted cases involuted germinal centres, necrosis and lympholysis are characteristic (DeMartini and Moulton 1973b; Morrison *et al.*, 1981).

The spleen and thymus exhibit lymphocytosis of parasitized cells and in some cases necrosis. Myeloid tissue shows increased lymphocytes, macrophages and phagocytized cell fragments. There are fewer cells of the erythroid and granulocytic series (De Kock, 1957). In the kidney tissue, pseudo-infarcts are observed. Lymphocytic infiltrations surround the glomeruli and extend from the Bowmans capsule to arcuate vessels. Pseudo-infarcts are large aggregates of lymphocytes. The lymphocytic infiltration is also intense in the tubular epithelium and lumen with haemorrhage and congestion also being observed. In the liver, infiltration of lymphocytes follows a centrilobular or periportal pattern. Fatty degeneration and necrosis are also observed in the same areas (Steck, 1928; De Kock, 1957). Lung tissues have pink staining oedema fluid in the alveolar spaces, infiltration of lymphocytes in the interstial tissue and a tendency of lymphocytes to occlude alveolar spaces in others. Some lobes have areas of emphysema (Bwangamoi *et al.*, 1971).

2.1.6.3 Haematological changes in *T. parva* infections.

Panleukopenia is the most common haematological abnormality in *T. parva* infections. It is usually as a result of concomitant neutropenia, lymphopenia and eosinopenia. The blood changes occur more rapidly in *T.*

parva parva than in *T. parva lawrencei* infections. The lymphopenia is due to depressed myelopoiesis although extra medullary lympholysis may play a role. Eosinopenia is caused by stress, and is mediated by an elevated cortisol and catecholamine concentration. Thrombocytopenia has been noted in *T. parva parva* infection, and is thought to be due to a disseminated intravascular coagulopathy (Muhammad, 1975; Maxie, Dolan, Flowers, Jura and Tabel, 1982).

2.1.7 IMMUNITY

Immune responses are directed against various *T. parva* life cycle stages occurring within the mammalian host. Solid immunity of up to 3 years is observed in cattle which have recovered spontaneously from infection with *T. parva*. This is a cell mediated response directed against schizont infected cells. The phenotype of cytotoxic killer cells (T- lymphocytes) which mediate this activity in cattle has been identified as CD 2+ CD 4+ CD 8+, the restricting elements being found in class 1 mass histocompatibility complex (Muhammed, Lauerman and Johnson, 1975; Goddeeris, Katende, Irvin and Chumo, 1982).

Antibodies are produced against all life cycle stages of the parasite. They do not play a key role in the protection in recovered animals (Burridge and Kimber, 1972; Muhammed *et al.*, 1975). However serum from animals which had been repeatedly exposed to challenge with infected ticks neutralized the infectivity of sporozoites in cattle (Musoke *et al.*, 1984). Recently a recombinant antigen (P67), derived from a plasmid expression vector (PMG1) was produced which prevents infection by inducing production of sporozoite neutralizing antibodies in experimental animals (Musoke, Nene and Morzaria, 1993). These antibodies produced *in vivo* however, are not significant because of the short duration of the various life cycle stages of *T. parva* in the host (Muhammad *et al.*, 1975; Goddeeris *et al.*, 1982).

2.1.8 DIAGNOSIS

Diagnosis of ECF in the field is based on a two pronged approach, namely, clinical signs and demonstration of schizonts in Giemsa-stained lymph node biopsy material. Lymph node biopsy smears are the most valuable and effective method of parasite detection in the mammalian host. The method is however limited in its ability to differentiate the various strains of *Theileria*, morphologically and quantitatively. Thin blood smears are useful in the

detection of piroplasm (erythrocytic forms). The detection of piroplasm however has no diagnostic value in the absence of clinical signs and schizonts in lymph node biopsy. This is because they occur even in carrier animals.

Schizont antigen indirect fluorescent antibody test is the serological technique used to assay serum antibodies. The technique is however, cumbersome, subjective and lacks specificity in some instances due to cross reactions. It is however suitable for epidemiological studies since antibodies can be detected up to six months post-infection (Burridge and Kimber, 1972; Cowan, 1981; Goddeeris *et al.*, 1982). Direct and indirect fluorescent antibody techniques can also detect piroplasm and schizont in blood smears and tissue sections respectively. Other serological tests that have been used include complement fixation, capillary agglutination and indirect haemaglutination, these however have low specificity (Duffus and Wagner, 1974). An enzyme linked immunosorbent assay (ELISA) kit for *T. parva* and *T. mutants* has been developed and is currently being tested for field use (Katende, Goddeeris, Morzaria, Nkonge and Musoke, 1990).

Deoxyribonucleic acid (DNA) probes utilize segments of parasite specific DNA cloned in plasmid or bacteriophage vector. These segments are radio-

labelled and used to detect parasite DNA sequences in samples. The probes distinguish between species, strains and stocks of *Theileria* (Williamson, Lesan and Awich, 1990). A repetitive DNA probe has been developed to distinguishes *T. parva* from other *Theileria* species. It discriminates between stocks of *T. parva* by detecting restriction fragment length polymorphism (Allsopp and Allsopp, 1988). The principle drawback of DNA technology is that radioactive probes have to be used to achieve high level of sensitivity. Polymerase Chain Reaction (PCR) is a method for *in vitro* amplification of specific DNA sequence using primers and thermostable DNA polymerase isolated from the bacteria *Thermus aquaticus*. The techniques is both sensitive and specific (Saiki, Gelfand, Stoffel, Sejart, Higuchi, Horn, Mullis and Erlichs, 1988).

A Ribosomal nucleic acid (RNA) probe has been developed which utilizes specific synthetic oligonucleotides to probe small subunit ribosomal RNA. It is able to probe naturally amplified RNA. The probe is sensitive, cheap and without the contamination problems of PCR (Allsopp, Baylis, Allsopp, Caraliersmith, Bishop, Cunningham, Sohampal and Spooner, 1993). However, by hybridization in a solution using a poly A- tailed specific centre probe reversibly captured on dT coated magnetic beads, together with a biotin-labelled probe targeted on a separate portion of the ribosomal RNA, the ribosomal targets

can be sensitively and specifically detected in tissue without the use of radioactive reagents (Hunsaker, Badri, Lombardo and Collins, 1989).

2.1.9 CONTROL

Measures in the control of ECF include :- (1) Those directed against the tick vector (2) Those directed against the parasite in the bovine host and (3) Immunization by infection and treatment method.

2.1.9.1 Measures directed against the tick vector

These include the use of acaricides, exploitation of host resistance to ticks and tick vaccines. Acaricides are the most extensively used method of tick control. They are commonly applied by dipping, spraying, spot-on's or use of slow release devices, like acaricide impregnated ear-tags. The acaricides in current use are the amidines and synthetic pyrethroids. Their disadvantages are in the rapid development of resistance and the build-up of acaricide residues in animal products and the environment (Young *et al.*, 1988).

Production of vaccines aimed at concealed tick antigens has been attempted. These concealed antigens are mainly of gut origin. Cattle have been effectively immunized against *Boophilus microplus* using antigens from whole tick homogenate (Willadsen and Kemp, 1988; Willadsen, 1990; Fivaz and Norval, 1990).

2.1.9.2 Measures directed against the parasite in the bovine host.

These measures involve the use of therapeutic drugs. The drugs of choice are parvaquone, buparvaquone and halofuginone. Parvaquone 993 (Clexon® ,Wellcome-UK) at 10 mg/kg body weight in two portions 48 hours apart and Buparvaquone (Butalex® , Coopers-Kenya Ltd.) at 2.5 mg/kg body weight as a single dose resulted in elimination of the parasite (McHardy, 1989). They also caused reduced fever, arrested leucopenia and eventual recovery. Their mode of action is the inhibition of the electron transport in the parasite. They were found efficacious in both experimental and field cases (Dolan, Young, Leitch, and Stagg, 1984; Chema, Weghala, James, Dolan, Young, Masiga, Irvin, Mulela and Wekesa, 1986; Dolan, Linyonyi, McHardy, Bond and Clampitt, 1988). Halofuginone (Terit® , Hoescht, England), an oral quinoxoline compound is

effective against theileriosis caused by *T. annulata* and *T. parva* at 1.2 mg/kg bodyweight (Schein and Voigt, 1981).

2.1.9.3 Immunization by infection and treatment

The infection and treatment method of immunization involves two simultaneous processes. One is the active infection of cattle with the *Theileria* parasites. Another is the treatment of recipient cattle with a chemoprophylactic agent. The chemoprophylactic agent acts to inhibit the early stages of the disease. This results in a mild or inapparent reaction and the subsequent production of immune status. The production of cryo-preserved sporozoite stabilate by Cunningham, Brown, Burridge and Purnell (1973) and the advent of tetracyclines has made this a feasible method. The major limitation to this method is the occurrence of many field strains which do not cross-protect and the need to monitor the animals for a long time (14 days) after immunization (Young *et al.*, 1988).

2.1.10 CHARACTERIZATION OF *T. parva* STRAINS

The research into the development of vaccines against *T. annulata* and *T. parva* has indicated the complexity of antigenic challenge in the field (Dolan, 1989). Cross-immunity trials have provided the only reliable method of characterizing parasites for use in immunization. However, these are expensive, time consuming and not very specific (Irvin, 1987). Therefore, there is need to develop *in vitro* techniques which are able to relate to results of cross-immunity trials.

2.1.10.1. Monoclonal antibody profiles (Mab) against *T. parva* strains.

Monoclonal antibodies are those antibodies which are raised against single specific epitopes (antigens) on the surface of the parasite (Pinder and Howett, 1980). The isolation and characterization of *Theileria* strains by Mab profiles is used to determine their distribution and to analyze their antigenic complexity. Monoclonal antibodies recognize schizont specific epitopes which may or may not be immunogenic. They are also able to detect contamination with other strains. A number of such Mabs have been raised for use in the indirect fluorescent antibody test (Minami, Spooner, Irvin, Ochama, Dobbeleare and

Fujinaga, 1983; Conrad, Stagg, Grootenhius, Irvin, Newson, Njamunggeh, Rossiter and Young, 1987; Shapiro, Fujisaki, Morzaria, Webster, Fujinaga, Spooner and Irvin, 1987; Morzaria, 1989).

Attempts to correlate Mab profiles to *in vivo* studies have shown disparities to a greater extent than previously thought. This is explainable on the basis of the heterogenicity of the studied stocks (Morzaria, 1989). Use of the monoclonal antibodies for the determination of antigenic profiles of parasite strains responsible for the disease outbreaks in the field would enable implementation of rapid prophylactic immunization with appropriate parasite stock (Irvin, 1987). An attempt to characterize field strains of *Theileria* has been made using isolates from Kiambu district (Lesan, Awich, Williamson, Linyonyi, Ondwasy and Stagg, 1989).

2.1.10.2 Protein analysis

Protein analysis by the Western blot technique is an assay for the detection of protein. It can identify particular proteins by using the specificity inherent in the antibody-antigen binding. The technique combines the electrophoretic separation of antigens with their immunological identification. After separation,

the antigens are further elucidated by Western blotting using either polyclonal or monoclonal antibodies. Western blots have been used in conjunction with Mabs to identify the immunogenic parasite proteins. These proteins can be used to identify specific stock markers (Shapiro *et al.*, 1987; Sugimoto, Mutharia, Conrad, Dolan, Brown, Goddeeris and Pearson, 1989).

The immunochemical characterization of schizonts and piroplasm proteins of *Theileria* was first described by Wagner, Brown, Duffus, Kimber, Graw and Lule (1974) and later by Creemers (1983). Characterization has been done using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-page). Separation of protein molecules depends on size and shape, since SDS changes the protein charge to negative. The method detects differences in *Theileria* strains through their protein molecular sizes. The method was further improved by transfer onto nitrocellulose paper and probing using either polyclonal or monoclonal antibodies.

Later using various monoclonal antibodies in the Western blot technique, Shapiro *et al.*, (1987) were able to detect a major schizont protein. This was the predominant antigen recognized in the serum from immune animals. The protein was given the name polymorphic immunodorminant molecule (PIM). The

molecule shows polymorphism in both size and expression of surface epitopes among the different stocks of *T. parva*. It was found to be present in schizonts and sporozoites. The PIM has a consistent molecular weight within *Theileria* strain regardless of the monoclonal antibodies used. It however varies in it's molecular weight size between stocks and strain of *T. parva*. The variation in molecular weight is due to the number of tandem repeats of specific nucleotide sequences. This is useful in differentiating between *T. parva* strains although the results did not necessarily correlate to the antibody profiles.

2.1.10.3 *In vitro* infectivity of lymphocytes

The *in vitro* infection of lymphocytes by sporozoites has been tested as a means of elucidating the infectivity of lymphocytes obtained from various animal species. Differences in the rate of infection have been noticed when *T. parva* Muguga sporozoites were used to infect peripheral blood mononuclear cells obtained from various animals. However, the use of lymphocytes from the same animal for this type of experiment has not been attempted (Stagg *et al.*, 1981; Stagg *et al.*, 1983).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 THE STUDY AREA

The study was done at the National Veterinary Research Centre in Kikuyu division of Kiambu district. The trial farm was located in KARI sublocation bordering Kerwa Jet Scheme. The area has an altitude of between 1600 and 2300 metres above sea level and has a mean annual rainfall of 1000 mm. The long rains fall between late March and June while the short rains come in October and November. The main occupation in the area is intensive small scale crop farming and keeping of dairy cattle.

The outlying paddocks of the National Veterinary Research Centre adjacent to the Kerwa Jet Scheme were used. These paddocks frequently house experimental cattle infected with *Theileria* parasites. The paddocks are also invaded by cattle from the farms adjacent to the station, especially during the dry months of the year. The invading animals bring in different types of disease-carrying vectors. Prior to the experiment the paddocks were suspected to be highly infested with *T. parva* infected ticks. Any animal introduced into the paddock invariably came down with ECF within two weeks.

3.2 SELECTION OF EXPERIMENTAL ANIMALS

Cattle free from ECF were selected for *Theileria* infection experiments. Briefly, twenty eight Friesian steers aged 9-12 months were acquired from farms known to practice good tick control and which had no recent history of ECF incidence. The animals were obtained from Marula and Manera farms in Naivasha. To confirm their ECF- free status, the animals were tested for theileria antibodies using the indirect fluorescent antibody test (IFAT) according to the method described by Burridge and Kimber (1972). The animals were ear-tagged for ease of identification, dewormed with Levamisole hydrochloride 2.5% (Levafas®, Norbrook Laboratories (G.B) Limited) and vaccinated against blackquarter, foot and mouth disease, rinderpest and leptospirosis. The animals were then left to acclimatize for two weeks.

3.3. EXPERIMENTAL DESIGN

The animals were randomly divided into two groups (A and B) in order to ensure an equal distribution by weight and also to avoid age bias. Twelve (Group A) of the steers were released to graze in the highly infested paddocks. These animals spent the nights in a reinforced wooden enclosure for protection against predators, with water provided *ad libitum*. The other sixteen (Group B)

steers were randomly assigned into four groups (B_1 - B_4) and placed into separate tick-proof barns. Three of the groups (B_1 - B_3) were infected with undiluted stabilates of *T. parva lawrencei*, *T. parva* Muranga and *T. parva* Lanet respectively. The site of inoculation with stabilate was subcutaneous in front and below the left parotid lymph node. The fourth group (B_4) was of uninfected controls.

In a separate experiment, attempts were made to titrate varying concentrations of sporozoites to a constant number of lymphocytes. The infection rates by sporozoites from two different strains of *T. parva* were also compared and observations made of the early developmental stages of *Theileria* species. The sporozoites were used to infect lymphocytes obtained from a single animal. Ticks previously fed as nymphs on *T. parva* Marikebuni infected cattle and those cattle coming down with E.C.F after *T. parva* Marikebuni immunization, were used as a source of sporozoites for the peripheral blood mononuclear cells infectivity experiment.

3.4 CLINICAL EXAMINATION OF EXPERIMENTAL ANIMALS

Clinical examination of experimental animals (Groups A and B) was carried out daily in order to monitor the progress of infection. The animals were

monitored daily for appearance, of clinical signs viz change in appetite, lacrimation, salivation, corneal opacity, nervous signs and diarrhoea. Rectal temperatures were recorded daily at 9.00 a.m. Rectal temperatures above 39.5°C were considered an indication of fever. Blood and lymph node samples from steers in Group A infected with *T. parva parva* were collected on the second day of fever. The animals were then treated using Buparvaquone (Butalex®, Cooper-Kenya Ltd.) at 2.5 mg/Kg bodyweight as a single injection on the same day. However, when schizonts persisted beyond three days after treatment a second injection was given.

3.5 BLOOD SAMPLE COLLECTION AND ANALYSIS

Ten millilitres of blood was drawn from the jugular vein of each experimental animal, three times a week. Gauge 16, 4cm long disposable needles were used to collect blood into vacutainer®, tubes (Becton Dickinson, U.K.) containing 0.05 ml of 20% dipotassium ethylene diamine tetraacetate (EDTA) and thoroughly mixed for determination of haematological values.

3.5.1. DETERMINATION OF PACKED CELL VOLUME (PCV%)

For each sample of EDTA blood, a capillary tube was filled for packed cell volume determination. The capillary tubes were sealed on one end using plasticine, then centrifuged for 5 minutes at 1000 rpm in a microhaematocrit centrifuge (Microfuge® , Heraeus Christ). The PCV percentage was determined using a haematocrit reader (Hawklesby and Sons Ltd. London).

3.5.2. DIFFERENTIAL LEUCOCYTE COUNT.

A single drop of EDTA blood (Groups A and B_1 to B_4) was placed on one end of the slide and a thin blood smear made for differential white blood cell counts. Blood smears were fixed for 3 minutes in absolute methanol and stained in Giemsa diluted 1:10 in distilled water for 30 minutes, rinsed in tap water and then allowed to dry. The differential leucocyte count was done under a microscope using the Battlement method (Schalm, 1986). Counting started at the thin end of the smear and a systematic meander of the slide made to avoid recounting of the same field. Fields selected for examination were those in which erythrocytes were well separated and the leucocytes thinly spread. Using high power (x 100) and oil immersion, at least 200 cells were counted in each side and the numbers of each type expressed as percentages.

3.5.3. TOTAL ERYTHROCYTE AND LEUCOCYTE COUNTS

The total erythrocyte (RBC) count and total leucocyte (WBC) count was done on a coulter counter (Coulter Electronics Inc. Hialeah FL., USA). The blood sample was diluted with Isoton II (Coulter Electronics Inc. Hialeah FL., USA). For total leucocyte count, three drops of Zap-o-globin was used to lyse the erythrocytes before the counts were done. Results were in 10^6 and 10^3 cells per microlitre (cells/μl) of blood for RBC and WBC respectively.

3.5.4. DETERMINATION OF HAEMOGLOBIN CONCENTRATION

Haemoglobin levels were determined spectrophotometrically on a coulter haemoglobinometer (Coultronics® , France S.A. Margency). A drop of the sample lysed with Zap-o-globin was poured into an inbuilt 1 ml cuvette. The haemoglobin level was read directly in grams per decilitre (g/dl).

3.6 POST-MORTEM AND HISTOPATHOLOGY PROCEDURES

A complete post-mortem was done on all animals dying during the course of the experiment. The gross pathological lesions were recorded organ by organ. Tissues for histopathology were taken from all major organs. The tissue

samples included a portion of the gross lesion and part of normal tissue where possible. These were preserved in 10% buffered formalin awaiting routine processing. Tissues were processed by dehydration through increasing concentrations of isopropyl alcohol. They were cleared in xylene. The tissue sections were then embedded in paraffin wax (Paraplast, Sigma-Kenya Ltd.) before being sectioned into 5 mm pieces, then mounted onto glass slides and stained in Harris haematoxylin for 4 minutes. The slides were washed under running water and destained in 1% acid alcohol. Counter-staining was by use of Putt's eosin stain for 2 minutes.

3.7. ISOLATION AND CULTURE OF *Theileria parva*

Isolation and culture of schizont-infected lymphocytes was done in order to obtain material for the characterization and comparison of various *Theileria parva* strains, using monoclonal antibody profile and Western blot technique. This was done after the animals were confirmed to be infected with ECF, by schizont demonstration in lymphocytes.

3.7.1 PARASITOLOGY

Monitoring of steers (Groups A and B_1 to B_4) for *Theileria* schizonts was done from needle biopsies. These were taken from the left parotid lymph nodes between the seventh and the thirty fourth day. Briefly, the skin was shaved and sterilized using alcohol. While immobilizing the node with one hand, a gauge 16, 4 centimetres (cm) needle was inserted and moved clockwise to trap lymphoid cells. Lymph node smears were made on glass slides by smearing lymph node tissue with another slide. The slides were fixed in methanol and then stained in Giemsa (1:10) for 30 minutes. The lymphocytes were also assessed for hyperplasia. From day 10 of experiment, both the right parotid gland and the left prescapular lymph nodes were also examined for schizonts in the same manner. Peripheral blood smears for the detection of piroplasm parasitaemia were made daily starting from the day schizonts were detected until death or when no piroplasms were detected for seven consecutive days.

3.7.2 PASSAGE OF BOVINE EMBRYONIC SPLEEN (BESP) MONOLAYERS:

The bovine embryonic spleen acted as a feeder layer on which lymphocyte tissue culture grew and were established. A flask containing the bovine

embryonic spleen monolayer (Primary culture) passage 28 was removed from the incubator. The flask was assessed for viability of cells and contamination by bacteria or fungi. This was done both visually and microscopically. The culture media was poured off and replaced by 2 ml of antibiotic, trypsin and versene (ATV). The culture flask was then shaken and the ATV poured off. Using a pipette 4 ml of fresh ATV was added. The flask was then incubated at 37°C for 10 minutes until the monolayer detached. The monolayer cell suspension was pipetted out and dispensed into a centrifuge tube containing 10 ml of culture medium. The suspension was centrifuged at 1000 rpm for 10 minutes in a cryo-centrifuge (Megafuge®, Hereaus, GMBH Lab Division, 1563-63405, Hanau, Germany). The supernatant was pipetted out and the pellet resuspended in fresh media. The suspension was distributed into 25 cm^3 culture flasks (Sterilin Ltd. U.K) and also in 100 µl aliquots into the 96 well plates. These were incubated at 37°C for 2-3 days until they formed a confluent layer of spindle shaped cells.

3.7.3. ESTABLISHMENT OF TISSUE CULTURE FOR MONOCLONAL ANTIBODY PROFILES AND WESTERN BLOTS.

Establishment of tissue culture from naturally infected animals in the National Veterinary Research Centre paddock was done on the second day of fever. The samples obtained were peripheral blood and lymph node biopsies.

3.7.3.1 Bovine lymphocyte tissue culture from blood of infected steers

Tissue cultures were raised from peripheral blood mononuclear cells according to the method described by Brown (1987). Briefly 10 ml of blood was collected from the jugular vein into an EDTA bottle, then transferred into centrifuge tubes. Centrifugation was done at 2500 rpm for 20 minutes at 4°C in a cryo-centrifuge (Megafuge®, Hereaus, GMBH Flow labs Division, Hanau, Germany). This was in order to separate out the buffy coat which is rich in lymphocytes. Most of the supernatant plasma was removed and discarded using a 10 ml pipette. A Pasteur pipette was used to remove the remaining plasma to within 1-2 mm of the buffy coat.

Cells in the buffy coat were collected using a pasteur pipette and then resuspended in an equal volume of phosphate buffered saline (PBS). The suspension was layered onto 3 ml lymphoprep (Ficoll plaque®, Pharmacia- Fine Chemicals AB Uppsala, Sweden) and centrifuged at 2500 rpm for 20 minutes to wash and separate out the lymphocyte cells. Mononuclear cells were then recovered at and below the interphase using a 5 ml pipette. The cells were resuspended in 9 ml phosphate buffered saline (PBS) and centrifuged at 1000 rpm for 10 minutes. They were rinsed twice in the same way. The pellet was suspended in 10 ml Roswell Park Memorial Institute Medium (RPM1) 1640

(Gibco, Oxbridge England) supplemented with 2 ml heat inactivated Foetal Calf Serum (FCS) (Flow Lab Irvine, Scotland). The mononuclear cells were introduced into culture flask with the Bovine embryonic spleen monolayer and incubated at 37°C with 5% CO_2 (Appendix 2).

3.7.3.2 Bovine lymphocyte tissue culture from lymph node biopsies.

Establishment of tissue culture was done according to the method described by Brown (1987). Briefly, the area of the lymph node was shaved and scrubbed. It was then sterilized using 70% alcohol. Using a 10 ml syringe and gauge 14, 4 cm needle, 5 ml of heparinized RPMI 1640 media was introduced into the immobilized lymph node. The needle was then turned round while applying negative pressure with the plunger. The needle was then withdrawn and the biopsy material discharged into a universal bottle with 5 ml of double distilled water. In the laboratory the cell suspension was centrifuged at 1000 rpm for 5 minutes and the supernatant discarded. The cell pellet was then resuspended in 5 ml of PBS and washed. Lymphocytes were separated using Ficoll and finally seeded into feeder layers in tissue culture flasks (Appendix 2).

3.7.4 RESUSCITATION OF CULTURES CRYO-PRESERVED IN DIMETHYL SULPHOXIDE (DMSO)

Resuscitation of cultures of *T. parva* Muguga, *T. parva lawrencei*, *T. taurotragi* and *T. parva* Lanet cryo-preserved in DMSO was done in the laboratory according to the method described by Brown (1987). Each vial of culture cells was removed from the liquid nitrogen canister and thawed in warm water at 37°C . The culture cells were added to 8 ml of warm complete culture medium. The suspended cells were centrifuged at 1000 rpm for 5 minutes. The pellet was resuspended in 5 ml of fresh culture medium and then transferred into 25 ml culture flask. Incubation of the flasks was done at 37°C with 5% CO_2 (Appendix 2).

3.7.5 ASSESSMENT OF CELL CULTURES

Cell growth and behaviour of *Theileria* infected cells were assessed routinely to ensure the cultures developed normally. Cultures were monitored at each subculture. Two sets of observations were made (Appendix 2).

3.7.5.1 Determination of cell number and viability.

Cell concentration in culture was assessed by the direct haemocytometer count method described by Brown, (1987). Specific counts of viable cells was done by the dye exclusion method using 2% Trypan blue prepared in normal saline. The samples were prepared by gently pipetting to mix clumps of the culture. One millilitre of the culture was sampled into a Bijou bottle. The cover slip was fixed onto the haemocytometer by moistening the edges of both coverslip and haemocytometer with distilled water. With the haemocytometer placed on the flat bench top and the coverslip in position, its sides were gently but firmly pressed using both thumbs. A rainbow effect (Newton's rings) appeared when coverslip was properly fixed. Using a pasteur pipette, the sample was lightly touched against the edge of the cover slip and slide. The fluid flowed out to fill the square. For viable counts, the culture sample was mixed with an equal amount of dye and placed on the other side of the haemocytometer slide. Counting of cells was done in all the four corner squares of the nought and crosses grid. Cells on the right and top lines were however disregarded, all clumps were counted as single cells. Since the volume of the sample was 0.1 ml, the number counted was multiplied by 1000 to give concentration per ml.

3.7.5.2 Giemsa-stained cytospin smears

The characteristics of cells and schizonts within the cultures were assessed on thin, air-dried Giemsa-stained smear preparations. The cytospin smears were made by centrifuging 50 ml aliquots of culture suspensions onto clean glass slides at 1000 rpm for 5 minutes in a centrifuge (Cytospin®, Shandon, Southern Sewickley, PA., USA). The slides were initially made wet by doing a preliminary centrifuge of tissue culture medium. Cytospins thus prepared were air dried, fixed in methanol and stained for 30 minutes in Giemsa (1:10) buffered at pH 7.2.

3.8 CHARACTERIZATION OF *T. parva* STRAINS BY MAB PROFILES

Characterization of *Theileria* species using their monoclonal antibody profiles compared the different *T. parva* strains using the affinity of Mabs to schizont surface epitopes.

3.8.1 PREPARATION OF ANTIGEN SPOT SLIDES FOR MONOCLONAL ANTIBODY PROFILES.

Preparation of antigen spot slides was done using 200 ml of microscopically assessed healthy looking cell cultures according to the method of Minami *et al.*, (1983) (Appendix 2). The cultures were pipetted vigorously to break the clumps. They were divided out into 20 ml plastic centrifuge tubes. The cell suspension was centrifuged at 1500 rpm for 10 minutes at 4°C. (Megafuge®, Hereaus, GMBH Lab Division, 1563-63405, Hanau, Germany). They were then washed twice in ice cold PBS and spun down each time at 1000 rpm for 10 minutes at 4°C. The cells were then resuspended in 4 ml of cold PBS. Viability of the cells was determined by the Trypan blue exclusion method as described in section 3.7.5.1. The cell concentration was adjusted to 1 x 10^7 cells/ml in ice cold PBS. The cells were then fixed for 10 minutes with an equal volume of 3.7% ice cold formaldehyde (40% v/w in PBS pH 7.2), added dropwise while constantly shaking. The suspension of cells was then spun down at 1000 rpm for 5 minutes at 4°C. The supernatant was discarded. Cells were washed three times in cold PBS and pelleted by centrifuging at 1000 rpm for 5 minutes in between. The cells were then resuspended again in cold PBS, counted and the volume adjusted to 1x10^7 cells/ml. Antigen slides were spotted by placing a drop of cell suspension into each well and immediately sucking it up

using a Pasteur pipette. This gave a thin and uniform coat of infected lymphocytes. The slides were then air dried before use.

3.8.2 PROCEDURE FOR MONOCLONAL ANTIBODY PROFILES

Antigen spot slides were tested for their profiles using Mabs (provided by ILRI) and following the method described by Pearson, Pinder, Roelants, Karsklundin, Mayor-Withey and Hewett (1980). The Mabs used were numbers 1-4, 7, 10, 12, 15, 20-23. Drops of the respective monoclonal antibodies in PBS were placed in alternating wells of the antigen slides. The slides were incubated for 30 minutes in a humid chamber at a temperature of 18°C to avoid evaporation of PBS. They were then washed once and rinsed twice in PBS. Each wash or rinse took 10 minutes. Goat antimouse IgG Fluorescein Isothiocyanate (FITC) labelled conjugate diluted to a strength of 1/80 v/w (i.e. 50 ml labelled conjugate + 3.95 ml PBS and 0.01% Evans blue dye) was added to the antigen slides. The slides were further incubated and washed as mentioned above. After the wash, the slides were dried and mounted using 2:1 glycerol to PBS. They were overlaid with coverslips. The slides were observed for schizont fluorescence using a fluorescent microscope (Orthoplan®, Leitz, Germany) under oil immersion.

3.9 CHARACTERIZATION OF *T. parva* BY PROTEIN ANALYSIS

This was done by sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western blot techniques, following the method described by Toye *et al.*, (1991).

3.9.1 PREPARATION OF INFECTED CELL LYSATE FOR PROTEIN ANALYSIS.

Four hundred millilitres of healthy culture material was removed from the incubator and centrifuged at 1500 rpm for 10 minutes in order to pellet them. The cells were washed once and rinsed in PBS as previously described for antigen spot slides (Section 3.8.1). The cells were subsequently prepared for electrophoresis by lysing in sodium dodecyl sulphate sample buffer (SDS-SB). Lysis was at the rate of 10^6 cells/ml, within a cytofuge with a pierced top and boiled for 5 minutes in water (Appendix 3).

3.9.2 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS Page).

The SDS page was used to separate protein antigens, first the vertical slab gel assembly was set using 1.5 mm spacers. Separation gel of 10% polymer was added. The gel had been made from a 23.3 gm mixture of acrylamide and Bisacrylamide, 0.7 gm of 20% SDS and 0.7 gm EDTA in 31.3 ml of double distilled water. These were mixed with 14 grams Tris-Hcl, pH 8.8 and degassed in a vacuum pump. Thirtyfive ml of ammonium persulphate and 0.7 gm of Tetramethylene diamine (TEMED, BDH, U.K.) was added to the mixture just before use. Twenty eight ml of the 10% polymer was pipetted into the slab sandwich to a level of 4 cm from the top. A water layer was subsequently introduced by adding it gently from one side. This removed the meniscus and allowed the gel to set evenly for two hours. The water was then poured out and a 5% polymer of stacking gel was added. This consisted of 5 gm of acrylamide/bisacrylamide mixture, 3.8 gm Tris Hcl pH 6.8, 0.3 gm EDTA, 0.3 gm 20% SDS, 20.6 gm of double distilled water, 16ml ammonium persulphate and 0.3 gm TEMED.

The gel was then stood overnight after introducing a 3 mm combs to form sample slots of 3 mm width. The vertical slab assembly was introduced into the

electrophoresis tank. Enough electrophoresis buffer was added to flow across the slots and cover the top of the gel. Fifty µl aliquots of lysed samples prepared as previously described in section 3.9.1 were applied. Empty wells were maintained alternate to those wells with sample. The standard molecular weight markers from Amersham was first introduced and two empty well spaces left before other samples were applied. Electrophoresis was carried out at 90 mA for 12 hours until the samples were completely separated out. This was indicated by the level of Bromophenol blue dye. The gel was removed and one half stained in Coomassie blue dye for 2 hour while gently shaking in a rocker. The gel was then destained overnight using 25% methanol: 12.5% acetic acid in PBS to reveal the protein bands.

3.9.3 WESTERN BLOT TECHNIQUE

This technique utilized the molecular weight of polymorphic immunodominant molecule of various *T. parva* isolates when probed with monoclonal antibody number 4 to characterize and compare field isolates.

Half of the gel not stained in Coomassie blue in section 3.9.2 was blotted onto nitrocellulose paper according to the method described by Toye *et al.*, (1991). The side with the markers was indicated by cutting out a small triangle.

A sandwich of nitrocellulose membrane, gel, blotting paper, sponge and plastic grid was made and inserted into the transfer tank for 90 minutes in blotting buffer at 200 mA, 12 volts. After the transfer was complete the portion with markers was cut out and visualized by staining in 0.1 % amido black in 25 % isopropanol containing 10 % acetic acid and destained for 30 minutes in the same solution. The non-specific binding sites of the nitrocellulose were blocked using 10% w/v fat free powdered milk in blocking buffer pH 7.4. The nitrocellulose was then rinsed in Tris buffered saline pH 7.5 containing 20 ml EDTA and 500 mM NaCl (Appendix 3).

Parasite proteins were detected by incubation of the blots with a 1:200 dilution of monoclonal antibody No 4 in PBS mixed with 1% w/v fat free powdered milk and 0.02% Sodium Nitrite. This was done overnight while shaking on a rocker. They were subsequently incubated for 1 hour in goat antimouse IgG conjugated to horse radish peroxidase (Cappel, Organon, Teknika Corp Westchester) at 1:1000 dilution in 1% skimmed milk in Tris Buffered Saline (TBS). Blots were developed by insertion into TBS containing 50 mg diamino benzidine tetrahydrochloride (Sigma, UK) and 0.06% hydrogen peroxide (Appendix 3).

The molecular weight of the bands was estimated using the following molecular weight markers myosin, 200 KDa; phosphorylase a, 92.5 KDa; bovine serum albumin KDa, 69 KDa; ovalbumin, 46 KDa; carbonic anhydrase, 30 KDa and lysozyme, 14.5 KDa (Amersham-UK). The size of the polymorphic immunodorminant molecule (PIM) was estimated by drawing a graph of logarithm of distance moved by known molecular weights against distance moved by PIM of sample in each case. The corresponding molecular weight in kilodaltons was read off from the graph.

3.10. *IN VITRO* INFECTIVITY OF LYMPHOCYTE BY SPOROZOITES OF TWO *T. parva* STRAINS.

A comparison was made between *T. parva* Marikebuni and the breakthrough to Marikebuni immunization. The infectivity of the two sporozoites *in vitro* was done using a modification of the technique described by Stagg *et al.*, (1981). Assessment of the differences was by the microscopic examination of the cultures, followed by Giemsa-stained cytospins of transformed cultures.

3.10.1 ISOLATION OF *T. parva* SPOROZOITES.

Nymphs were put in ear bags and allowed to feed on infected animals, drop, moult and harden. They were further fed on rabbits for five days to enhance sporozoite maturation. Isolation of *T. parva* sporozoites from ticks began by the demonstration of *T. parva* infection in the salivary glands of ticks. Five female and five male ticks obtained from among the batches infected with *T. parva* Marikebuni and breakthrough parasite had their salivary glands dissected. These glands were then stained with Feulgen's stain and examined under a microscope for infection, according to the method described by Blewett and Branagan (1973) and Young *et al.*, (1981).

The number of adult ticks required to give 400 infected acini was calculated as 20 for *T. parva* Marikebuni and 14 for the breakthrough parasite. In order to isolate the sporozoites, the ticks were placed in a large sieve and washed under running sterile distilled water while shaking continuously. The ticks were then rinsed in 70% ethanol and embedded in paraffin wax block in a petri dish. The petri dish was then flooded with culture medium. Using iridectomy scissors and a fine pair of forceps the dorsal integument of each tick was removed to expose the salivary glands. The salivary glands were then removed and homogenized in incomplete culture medium using a mortar and

pestle. This released sporozoites into the culture medium. The suspension was then aspirated into sterile 10 ml centrifuge tubes. The aspirates were centrifuged at 1000 rpm for 10 minutes to sediment salivary gland debris. The supernatant containing sporozoites was placed in sterile universal bottles. Fifty microlitres of aspirate were deposited onto a microscope slide using a cytocentrifuge (Cytospin™, Shardon) at 600 rpm for 10 minutes, the cells were then fixed in methanol and stained in Giemsa for sporozoite examination.

3.10.2. *IN VITRO* INFECTION OF LYMPHOCYTES USING *T. parva* SPOROZOITES

From the supernatant mentioned in section 3.10 1. above, three fold dilutions were made beginning with the initial sporozoite concentration. Fifty microlitres aliquots of each dilution was added to the 96 well plates (Costar, Cambridge, MA, USA) previously seeded with 100 microlitres BESP monolayer as described in section 3.7.2. Then 50 microlitres of peripheral blood mononuclear cells (PBM) containing 5 x 10^5 cells, collected from a *Theileria* free animal following the procedure described in 3.7.3.1 was added. The boundary wells on each edge of the plate were filled with 200 microlitres of sterile distilled water. Twenty wells were filled at each sporozoite dilution. Each 96 well plate therefore contained two dilutions of *T. parva* Marikebuni and

the breakthrough respectively. Culture plates were then incubated at 37°C in an incubator under 5% Carbon dioxide The plates were visually examined daily for transformation using the inverted microscope as described in section 3.7.5.1 and culture stained cytospins as described in section 3.7.5.2. The cytospins were made from every well that showed signs of transformation.

3.11. STATISTICAL ANALYSIS OF HAEMATOLOGICAL PARAMETERS

The design was a two factorial experiment with repeated measures 1. The parameters PCV, Hb, WBC and RBC were analyzed by Analysis of Variance (ANOVA).

CHAPTER 4

4.0 RESULTS

4.1 CLINICAL AND HAEMATOLOGICAL RESPONSE TO INFECTION

The response to infection and chemotherapy are summarized and presented in Table 1.

4.1.1. CLINICAL SIGNS OBSERVED IN EXPERIMENTAL ANIMALS

All the twelve steers (Group A) introduced into the outlying paddock developed fever in 13 to 21 days (mean 16.3. ± 2.4) after exposure. Their temperatures ranged from 40.6 to 42°C with an average of 40.9 ± 0.5°C. Peak fever occurred between days 14 and 21 (mean 17.6 ± 2.2 days). This was usually 1 to 2 days after the onset of fever. Three of the twelve steers manifested an undulating fever. One of the steers in the group however, did not show fever despite the presence of schizonts in it's prescapular lymph nodes. Most of the major clinical signs were seen after fever had set in. In four of the twelve steers in group A the left prescapular was slightly swollen before the onset of fever. All the twelve steers developed a dull and rough hair coat. Their demeanour was dull and had a gradual

reduction of appetite. A serous nasal discharge was also seen in some. The animals had increased salivation and lacrimation. Pulse and respiratory rates were elevated, while respiration became harsh. Dyspnea was severe terminally. A short dry cough which lasted 2 to 4 days, was evident in four of the twelve steers (Group. A). Enlargement of the lymph nodes occurred between days 14 and 19. This corresponded to the presence of schizonts within the nodes. A mild unilateral corneal opacity was seen in one steer in the group. Mild subcutaneous and submandibular oedema was observed in six of the twelve steers. Six steers in group A died during the experiment. Two of these steers became recumbent before death, while the other four were found dead in pasture. In two of these, death occurred on days 18 and 19 respectively, while the others died between days 21 and 45 post-infection.

Among group B steers, the response to infection with *T. parva* strains was variable. Animals infected with *T. parva* Muranga stabilate (Grp. B_1), showed fever (mean 40.6°C) between days 2 and 12 (mean 4), dullness, rough hair coat, dyspnea, salivation and lacrimation. The animals had grossly enlarged superficial lymph nodes. Three animals of the four infected with *T. parva* Muranga died between days 13 and 21 (mean 17). *Theileria parva lawrencei* (Grp. B_2) infected steers developed

fever of 40.3°C. between days 10 and 11. The clinical signs observed were dullness, anorexia, lacrimation and swollen lymph node. One animal in the group showed nervous sign of incoordination of movement and hind limb paralysis before death on day 17. Cattle infected with *T. parva* Lanet stabilate (Grp. B_3) and the uninfected controls (Grp. B_4) did not show any clinical signs of disease.

4.1.2. PARASITOLOGY

The parasitological results of the various groups are summarized in Table 1. The incubation period for animals exposed to theileriosis in the paddock (Grp. A) ranged from 15 to 20 days (mean 16.3 ± 1.8). Schizonts (Fig. 1) were detected a day earlier in the left prescapular, than in the right prescapular lymph node in most cases. Schizonts were detected for a period of 3 to 8 days (mean 5.3 ± 2.1). Only two of the twelve animals in group A showed a piroplasm parasitaemia. The piroplasm were observed only on day 17. East Coast Fever in animals infected with *T. parva* Muranga (Grp. B_1) had an incubation period of 4 days indicated by fever., Schizont parasitosis was evident from the same day and persisted for 5 days. Piroplasm were seen between days 11 and 19. In animals infected with *T. parva lawrencei* (Grp. B_2) had an incubation period of 10 days indicated by a rise in body temperature was

observed. Piroplasm parasitaemia was seen in two animals. This lasted for 4 and
day, respectively, beginning at day 17. The steers infected with *T. parva* Lanet (Gr
B_3) showed no fever although schizonts were detected between days 8 and 12. Th
duration of schizonts in the right prescapular was 6 days except in one animal i
which it lasted 2 days.

4.1.3. HAEMATOLOGY

The haematological results for group A and groups B_1 - B_4 are presented i
Fig 2 - 4.

(n.b. The control Group B_4 is not included in Table 1.)

4.1.3.1. Packed cell volume

Changes in the mean PCV values in the groups of experimental cattle ar
presented in Fig. 2. The pre-infection values ranged from 32-36% for all the groups
On days 4 and 10 the values fell slightly to 30. All the groups however, constantl
showed values between the normal PCV range of 24 to 46 observed in cattle (P for
test = 3.94).

4.1.3.2. Haemoglobin concentration

Changes in the mean values of haemoglobin concentration in the groups of experimental cattle are presented in Fig. 3. The haemoglobin concentration values initially ranged from 11 to 13 g/dl for all the experimental animals. The values of animals infected with *T. parva* Muranga (Grp. B_1) declined to 9 g/dl on day 21 when three out of four cattle within the group had died. Values beyond day 21 were therefore those of one remaining animal within the group. The values for *T. parva* Lanet infected animals (Grp. B_3) declined to 9 g/dl by day 25 before rising again to 12 g/dl on day 27, while those of animals infected with *T. parva lawrencei* and of the controls (Grp. B_4) did not change significantly from the normal range of 10 to 14 g/dl.

4.1.3.3. Red blood cell (RBC) counts

Changes in the mean values of red blood cell counts in the groups of experimental cattle are presented in Fig. 4. Red blood cell values ranged between 5.7 to 7.5 x 10^6 /µl. Groups B_1 - B_3 indicated a decline to day 14 after which blood values began to peak again. Values of animals infected with *T. parva* Muranga were

in many cases lower than those of animals infected with other strains. Group B_4 values did not vary from the pre-infection levels.

4.1.3.4. White blood cell (WBC) counts

Changes in the mean values of white blood cell counts in the groups of experimental cattle are presented in Fig. 5. White blood cell counts of all groups (Grps. B_1 to B_4) ranged from 6-9 x $10^3/\mu l$ in the pre-infection stages. These values rose slightly to between 8.5 and 12.25 x 10^3 on the third day. The values fell to pre-infection levels on day 8. Leucocyte counts were higher in *T. parva lawrencei* infected animals (Grp. B_2) being 14.2 x 10^3 on day 12 than the controls (Grp. B_4) of 8.1 x 10^3. The other strains also had a slight peak to 8.5 in *T. parva* Lanet (Grp. B_3), while those of *T. parva* Muranga infected animals (Grp. B_1) continued to fall. Lower leucocyte levels was evident on day 14 in group B_3 when values fell to 5.6 x 10^3 after which they rose again to pre-infection levels.

4.1.4. STATISTICAL FINDINGS ON HAEMATOLOGICAL DATA

There was no symmetry for all the parameters considered with each valu being dependent of the others. There was interaction between strain and days pos infection for PCV. This meant that the rate of change of PCV depended on both th strain *T. parva* and its duration within the host. Rate of change for the othe parameters did not differ between strains (no significant interaction). Haemoglobi concentration and red blood cell counts showed significant changes over tim irrespective of strain while white blood cell counts did not show any significar variation either by time or by strain for this experiment. The P for F test for H concentration and packed cell volume was 0.0009^2 and 0.0056^2, 0.4106 and 0.508 for strain and the strain versus day interaction was 0.2206^2 and 0.5365^2 respectively This means time was more significant than the infective strain.

4.1.4.1. Effect of time and strain on packed cell volume

One way analysis of variance at each level of strain revealed no differenc between days and the PCV of steers infected with the *T. parva* Muranga strai (P=0.8973). Steers infected with *T. parva lawrencei* (P=0.0086) and *T. parv*

Lanet strains (P=0.0019) and those in the control group (P=0.063) showed decline in PCV. *Theileria parva* Lanet strain was associated with the highe rate of decline.

4.1.4.2. Effect of time on haemoglobin concentration and red blood cell counts

Haemoglobin concentration and Packed cell volume showed a similar trend logarithmic decline over time irrespective of strain. Both parameters had hig rate of decline with the most significant occurring before day 14. There wa no difference in the PCV of animals affected with *T. parva* Muranga since day 14, three of the animals in the group were dead. The only one remainin was mildly affected.

Table 1: Clinical response of steers to infection by various *Theileria parva* strains and their subsequent treatment.

Mean Parameters	Group A Paddock n=12	Group B1 *T.p.*Muranga n=4	Group B2 *T.p.*lawrencei n=4	Group B3 *T.p.*Lanet n=4
Days to onset of fever	16.3	4	10	-
Mean fever ^{0}C	40.9	40.6	40.7	-
Days to peak fever	17.6	4.7	11.5	-
Days to schizont	17.3	9.75	10	9.5
Duration of schizonts	5.3	5.5	5.5	4
Days to piroplasm	17	17	-	-
Duration of piroplasm	1	2.5	-	-
Disease score	++(+)	+++	+	-
Days to death	27(6)	17.5(3)	17(1)	-
Days to recovery post treatment	25(1)	14(1)	13(3)	-
Mortality Rate	6/12=50%	3/4=75%	1/4=25%	0=0%

Key:

- No data
+ Mild
++ Moderate
+++ Severe

* Numbers in brackets indicate the numbers of cattle involved in each case.

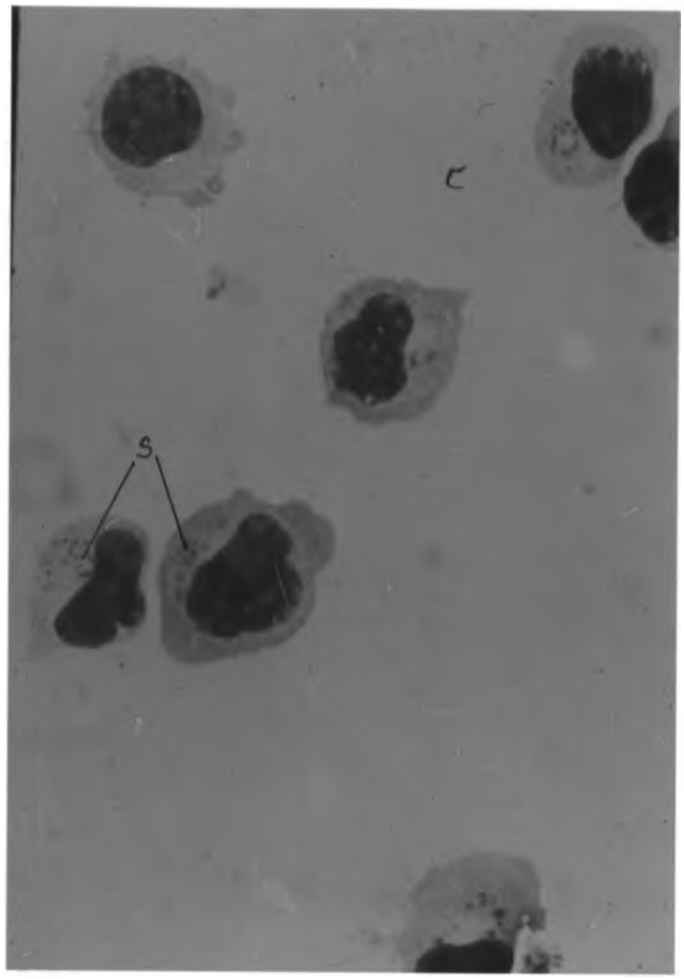


Fig. 1. Cytospin smear of lymph node biopsy obtained from a steer in group A, infected with *T. parva* and showing schizonts (S) in the cytoplasm (Giemsa x 1000).

Fig 2. Packed cell volume (percent) of blood obtained from steers infected with various strains of *T. parva*

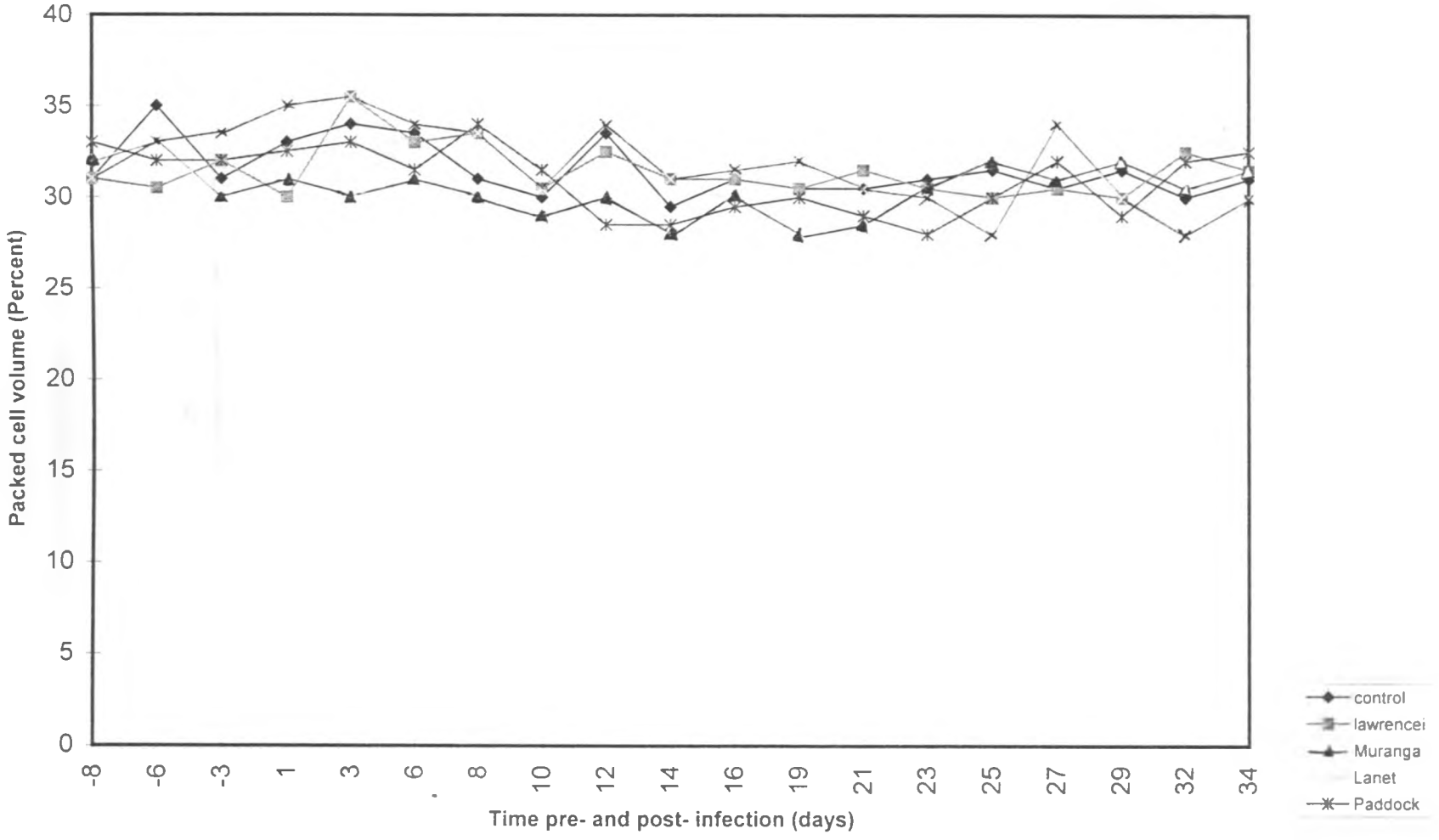


Fig 3. Haemoglobin concentration values (g/dl) of steers infected with various strains of T. parva

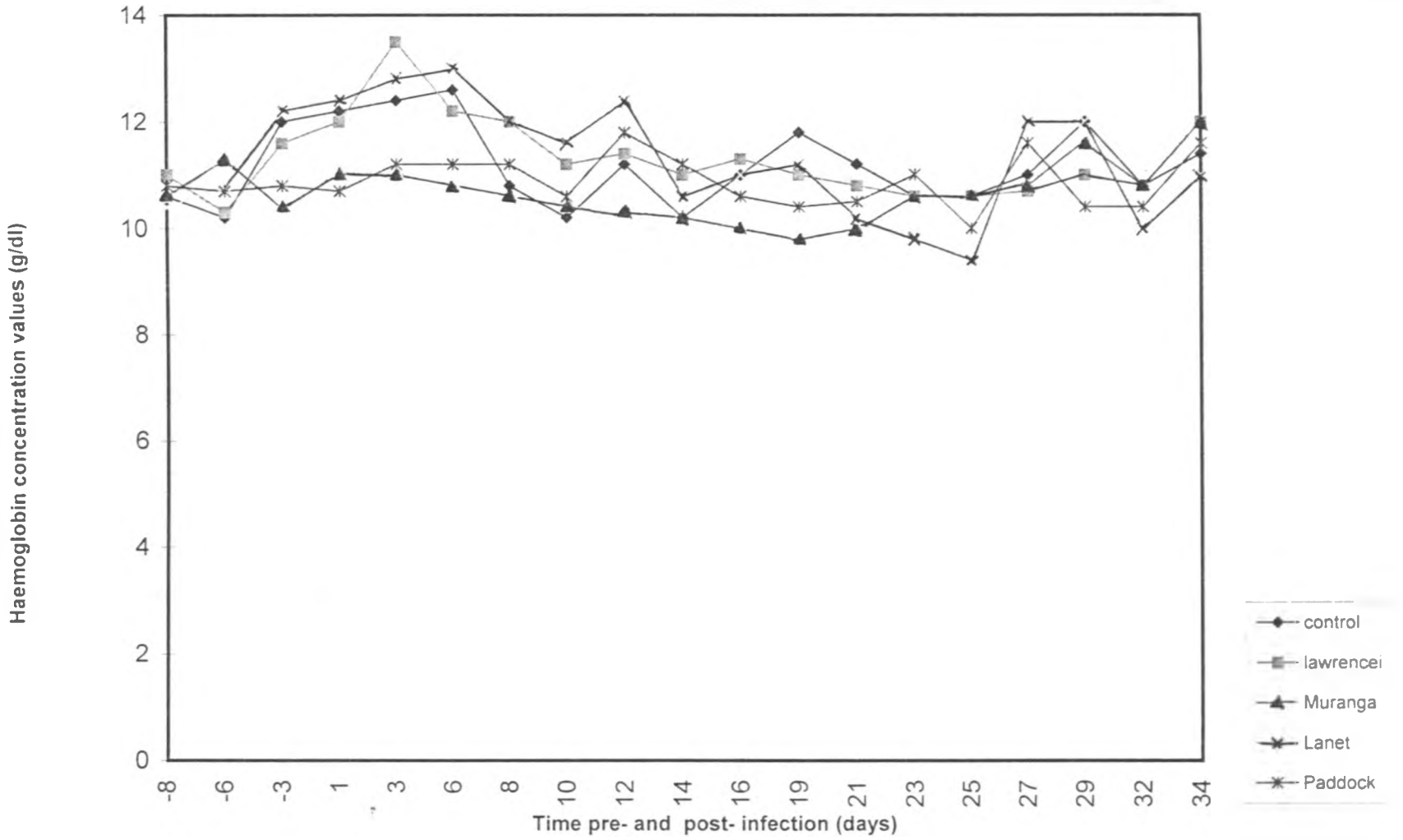


Fig 4. Red blood cell counts in Millions/ µl of steers infected with various strains of T.parva.

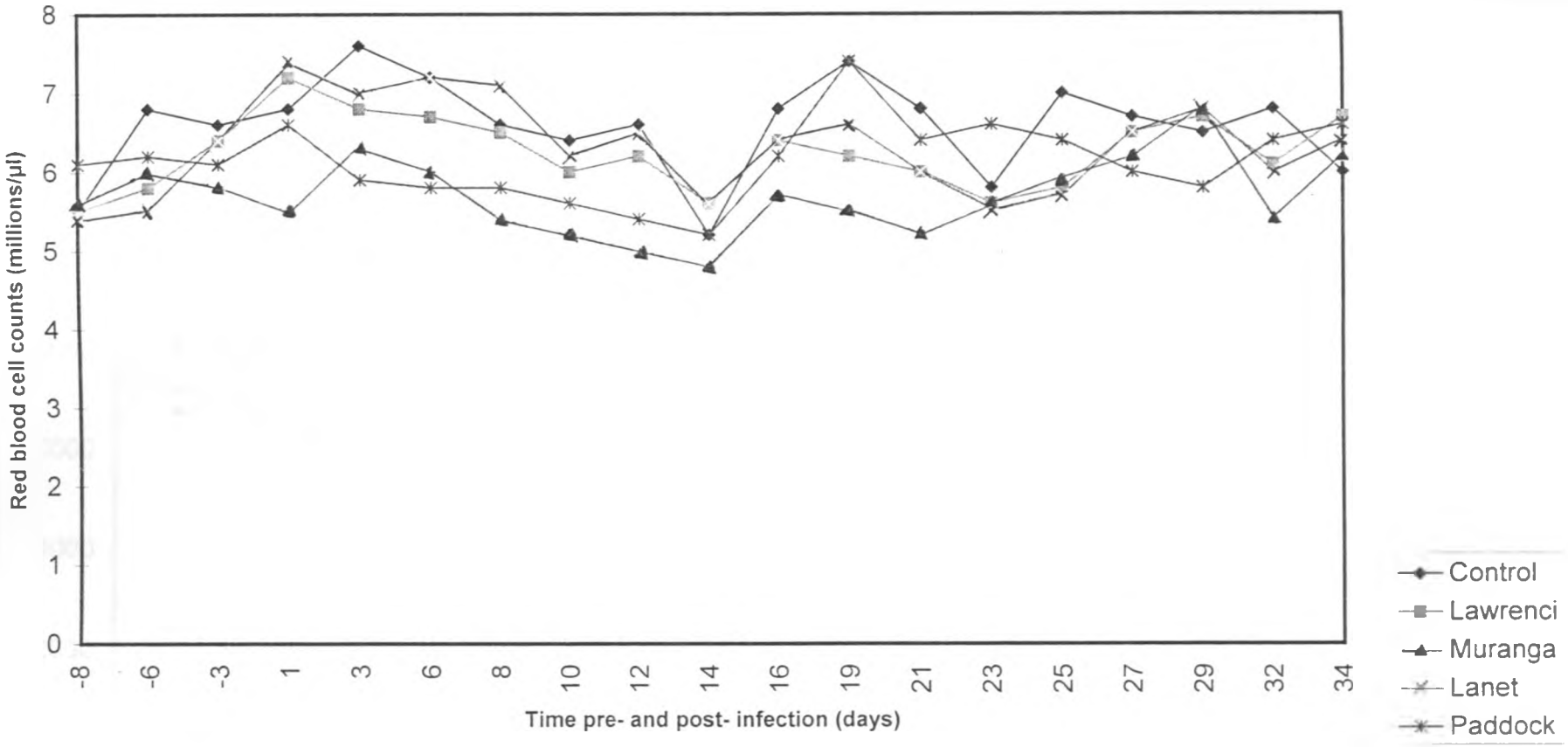
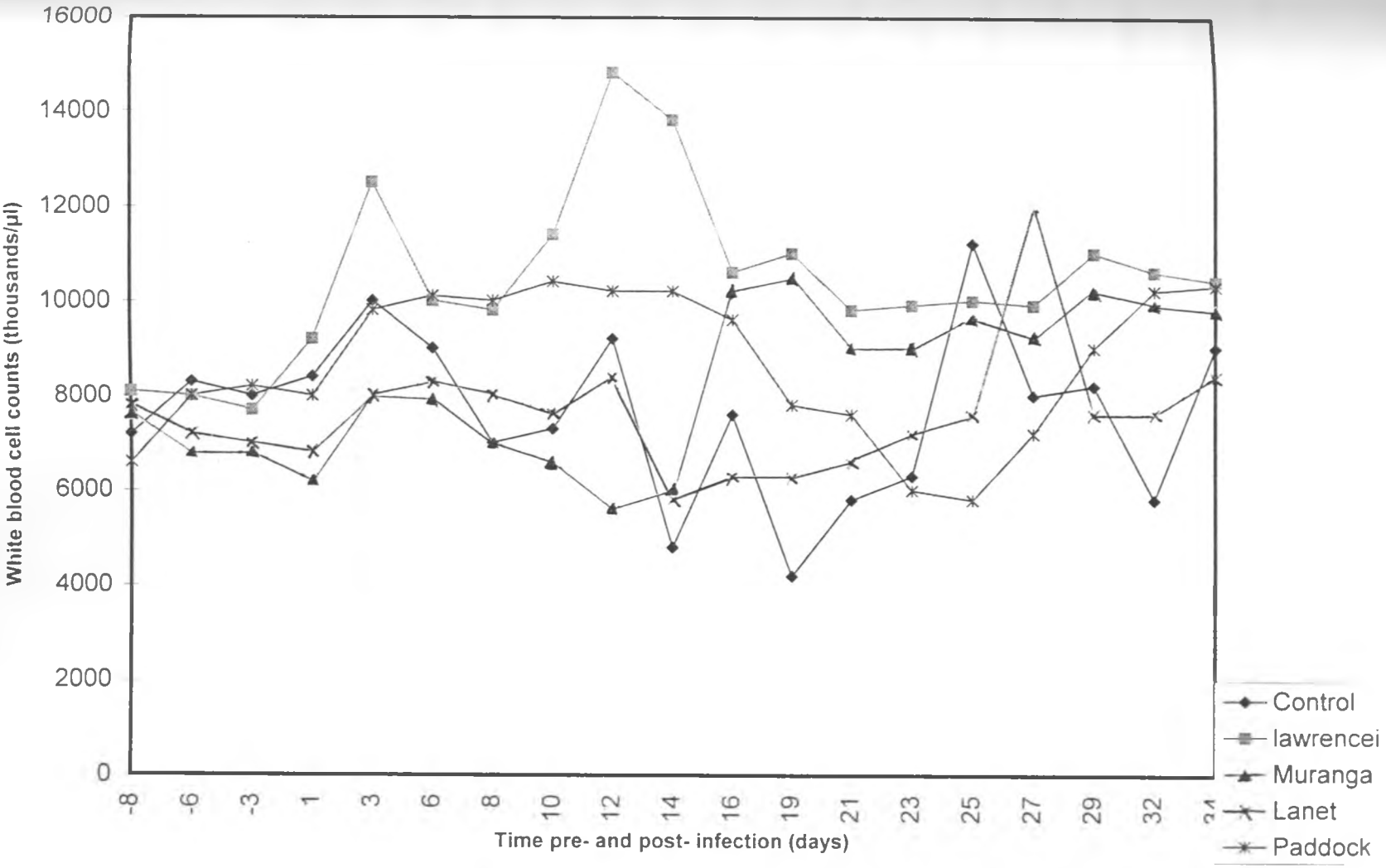


Fig 5. White blood cell counts (thousands/ µl) of steers infected with various strains of T. parva.



4.2. PATHOLOGICAL OBSERVATIONS

4.2.1. GROSS FINDINGS

Pathological observations in the various organs of steers (Grps. A and B_1 and B_3) are summarized on Table 2. Three steers which died from infection with *Theileria parva* derived from the paddock (Grp. A) had a poor body condition at post-mortem. However, the body condition was good in two animals and fair in one. All the six steers had pale mucous membranes. Froth was present in the nostrils and trachea. The ventral aspects of the abdomen and the intermandibular space in two steers in group A had gelatinized subcutaneous and intramuscular fat. The subcutaneous blood vessels were engorged in all the six cases. Petechial and ecchymotic haemorrhages were observed on the subcutis, intercostal muscles and omentum of three animals. All the six animals showed ecchymotic haemorrhages varying in degrees of severity on the peritoneum. One animal in group B_2 which died after *T. parva lawrencei* stabilate infection had a poor body condition and little subcutaneous fat. Two animals infected with *T. parva* Muranga (Grp. B_1), that died of disease also had a poor body condition but one died while in good condition. Two animals in this group had ecchymotic haemorrhages on the subcutis, intercostal muscles and omentum.

The thoracic cavity of the animals had varying amounts of either straw coloured or in some cases blood tinged fluid, except for four animals from the paddock (group A) and one animal infected with *T. p*arva Muranga (Grp. B_1) which did not show any pathology.

The urinary bladder was only affected in two cases from the paddock (Grp. A). One steer had petechial haemorrhages while another had hyperaemia of the mucosa. Two animals in group B_1 infected with *T. parva* Muranga stabilate had urinary bladder haemorrhages, while another infected with *T. parva lawrencei* (Grp. B_2) showed congestion of blood vessels. The spleen was enlarged and pulpy with prominent corpuscles in three animals from the paddock (Grp. A), one of which was also congested. The animals infected with *T. parva* Muranga (Grp. B_1) had haemorrhages in the splenic tissue. Hepatomegaly was observed in all the six steers in group A arising from the paddock infection. Two of them showed congestion, while another had a yellow tinge indicative of icterus on cut surface. The liver of one animal infected with *T.* parva *lawrencei* (Grp. B_2) was enlarged, and mottled with greyish white patches. Two animals infected with *T. parva* Muranga had enlarged and mottled liver. Kidney of three steers (Grp. A) from the paddock showed petechial haemorrhages, while in another one icterus was observed. All the

steers which died after stabilate infection (Grps. B_1 and B_2) had haemorrhage on cut section of the renal pelvis.

The abomasal folds in three of six fatal cases arising from the paddock derived *T. parva* (Grp. A) were oedematous. This was marked in two animals and mild in one. Two of the fatal cases in group A had *Trichostrongylus axei* and *Haemonchus placei* worms in the abomasum while one animal had no detectable abnormality. Five of the fatal cases had ulceration of the mucosa especially at the pylorus (Fig. 6). In some cases these ulcers were multiple, irregular in shape and surrounded by a haemorrhagic zone. The three fatal cases which died of infection with *T. parva* Muranga (Grp. B_1) had oedema of abomasal folds, pyloric ulcers and few patches of petechial haemorrhage. The fatal case infected with *T. parva lawrencei* (Grp. B_2) showed mild oedema of the abomasal folds. The mucosa of the small intestines was thickened in one animal from the paddock (Grp. A), while streaks of haemorrhages were observed on both serosal and mucosal surfaces of the intestines. Two animals infected with *T. parva* Muranga (Grp. B_1) showed both serosal and mucosal haemorrhages of the small intestines. Congestion and hyperaemia of the serosa was seen in one animal. The fatal case of *T. parva lawrencei* (Grp. B_2) showed mild hyperaemia of the mucosa.

One fatal case from the paddock, had mucosal haemorrhages present in th large intestines (Fig. 7). These haemorrhages were in the colon, caecum and rectu

Another steer showed a patchy distribution of hyperaemia in the large intestin (Fig. 8) while one had ulcers and desquamation of the epithelium in the colon Hyperaemia was the major observation in the large intestines of all the fatal cas infected with stabilate. Three animals in group B_1 infected with *T. parva* Murang and the animal infected with *T. parva lawrencei* (Grp. B_2) had hyperaemia of th serosal surface. Two animals, in each of groups B_1 and B_2 showed petechial an ecchymotic haemorrhages. Lymph node enlargement involving especially th parotid, prescapular and precrural nodes was observed in all the six fatal cases group A obtained from the paddock. Haemorrhage was present in these anima except in one animal in which the lymph nodes were hyperaemic, oedematous an necrotic (Fig.9). The lymph nodes were both enlarged and congested in all th animals dying of stabilate derived infection.

The most significant lesions observed in the respiratory tract of anima included distended, heavy and friable lungs in group A (Fig. 10). Two fatal cas had emphysema of some lobes, while the other four were oedematous. Three cas in this group had froth in both trachea and bronchi. Yellow gelatinous exudate wa

seen in the interstitial spaces on the cut surface. The same picture of a heavy, distended, oedematous and congested lungs was seen in the fatal case infected with *T. parva lawrencei* (Grp. B_2) and in two animals infected with *T. parva* Muranga (Grp. B_1). Copious amounts of froth was seen in the trachea and bronchi of four out of six fatal cases from the paddock (Group A). Three animals infected with *T. parva* Muranga stabilate (Grp. B_1) also had froth and hyperaemia of the bronchioles.

TABLE 2: Observations of pathology in organs from steers infected with various strains of T. parva

	STRAIN OF *THEILERIA PARVA*		
Appearance of organ/tissue	Paddock n=6 (Group A)	*T.p* Muranga n=3 (Group B1)	*T.p* lawrencei n=1 (Group B2)
Body condition at death	Poor(3), Fair(2), Good (1)	Poor(2), Good(1)	Poor(1),
Abomasum	Oedema(3), Haemorrhage(2), Ulceration(5)	Oedema(3), Ulceration(3)	Oedema(1), Ulceration(1)
Small intestines	Haemorrhage(6), Ulcers(3), Hyperaemia(2)	Haemorrhage(2), Hyperaemia(1)	Hyperemia1)
Large intestines	Haemorrhage(1), Ulcers(1), Hyperaemia(1)	Haemorrhage(2), Hyperaemia(3)	Hyperemia(1)
Liver	Enlarged(6), Congestation(2), Icterus(1)	Enlarged(2),	Enlarged(1), Mottled(1)
Gall bladder	Full(5)	Full(3)	Full(1)
Lymph nodes	Oedema(1), Enlarged(6), Hyperaemia(1), Haemorrhage(5), Necrotic(1)	Oedema(1), Enlarged(3), Haemorrhage(2),	Enlarged(1), Haemorrhage(1),
Spleen	Enlarged(3), Pulpy(2)	Enlarged(2),	Enlarged(1)
Kidney	Haemorrhage(3)	Haemorrhage(3)	Haemorrhage(1)
Urinary bladder	Haemorrhage(4), Hyperaemia(2)	Haemorrhage(2), Hyperaemia(2)	Hyperemia(1)
Trachea	Congestation(6), Froth(6), Hyperaemia(6)	Froth(3), Hyperaemia(2)	Froth(1), Hyperemia(1)
Bronchi	Froth(6)	Froth(3)	Froth(1)
Lungs	Emphysema(3), Oedema(5), Congestion(2), Interstitial pneumonia(6)	Oedema(2), Congestion(2), Interstitial pneumonia(3)	Oedema(1), Congestion(1), Interstitial pneumonia(1)
Brain	-	-	Haemorrhage(1), Congestion(1)
Heart	Haemorrhage(5)	Haemorrhage(2)	Haemorrhage(1)
Thoracic cavity	Hydrothorax(6)	Hydrothorax(2)	Hydrothorax(1)

* Numbers in brackets indicate the number of steers involved in each case.

Fig. 6. Abomasum: Mucosal surface of a steer (Grp. A) which died of ECF on day 32 of experiment, showing petechial haemorrhages (H) and epithelial erosions (E) of the pyloric region.

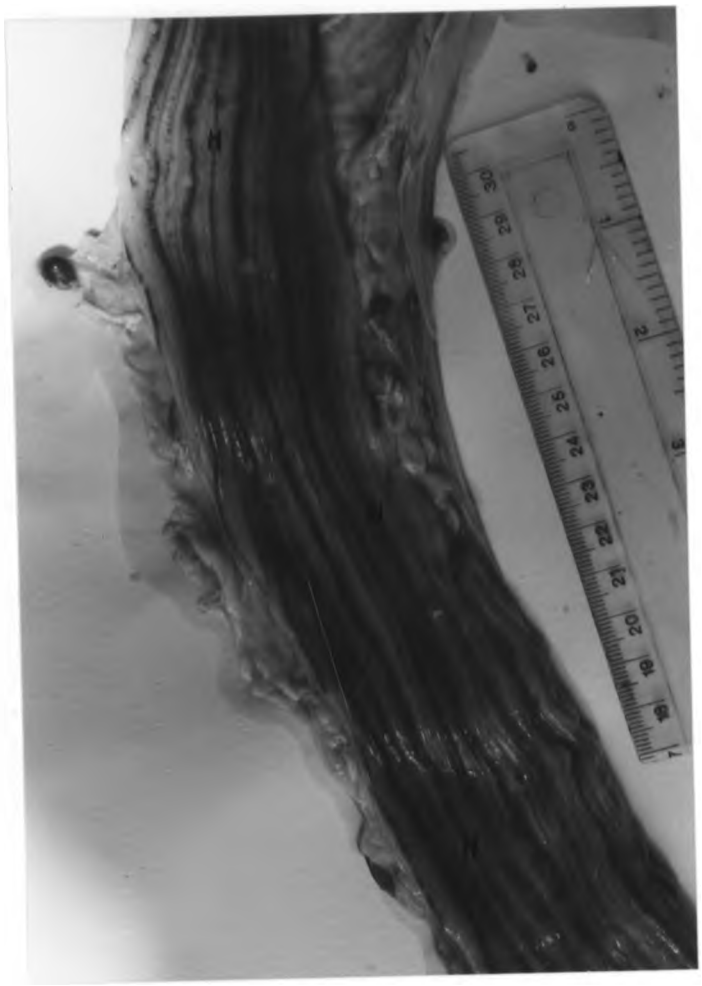


Fig. 7. Longitudinal section of large intestines from a steer (Grp. A) which died of ECF on day 32 of experiment, showing linear haemorrhages (H) of the mucosa.

Fig. 8. Large intestines of steer No. 790 in Group A which died of ECF on day 18 of experiment showing haemorrhages (H), hyperaemia (Hy) and necrosis (N) of the serosa.

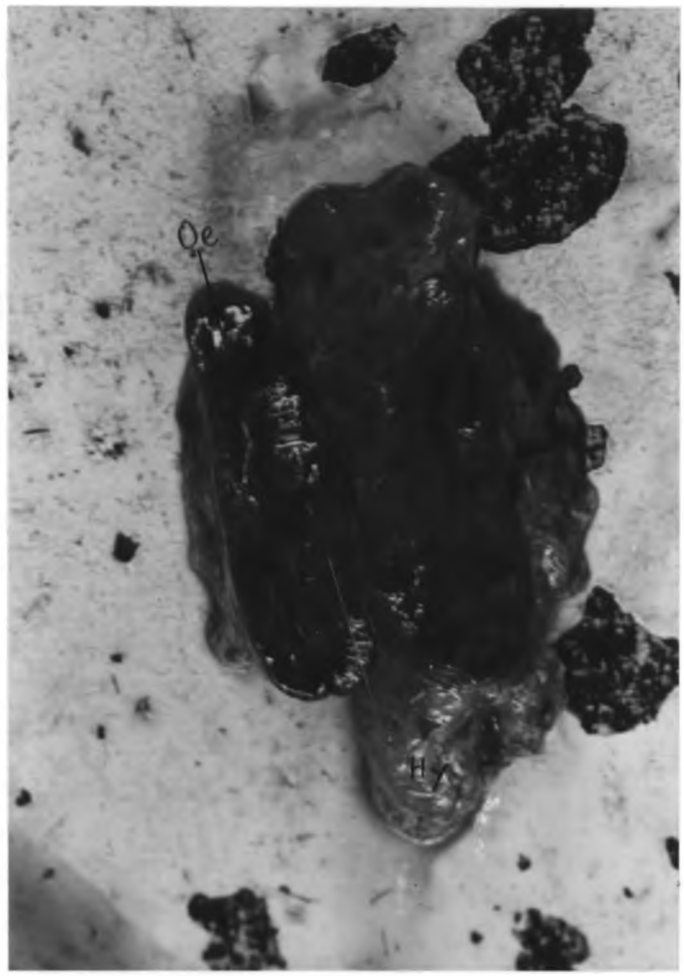


Fig. 9. Enlarged, hyperaemic (Hy) and oedematous (Oe) lymph node of steer No. 790 which died of ECF on day 18 of experiment.

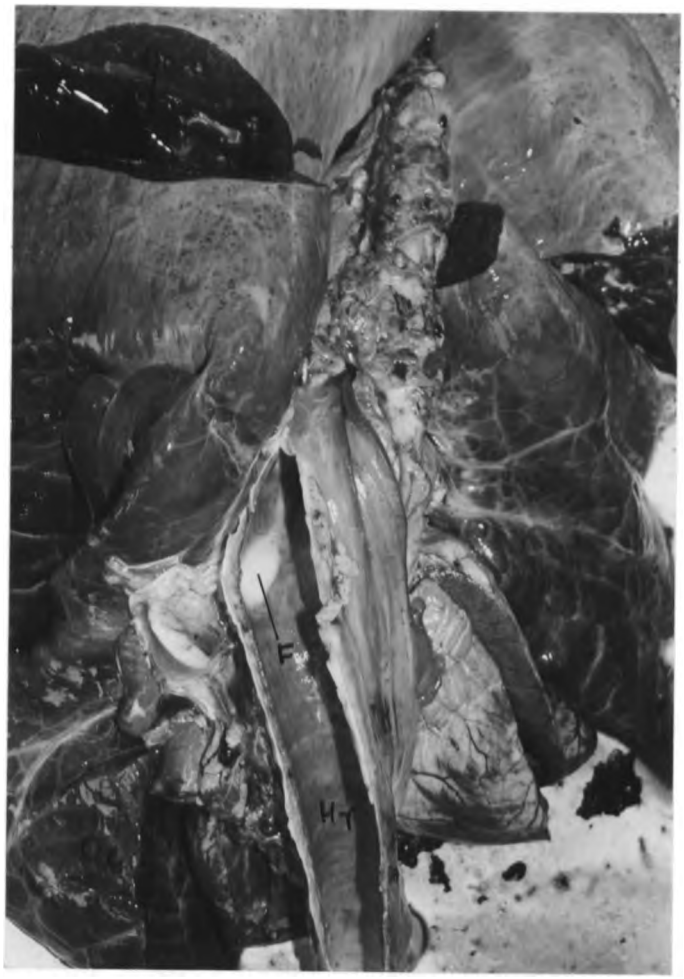


Fig. 10. Trachea and lungs of steer (Grp. A) which died of ECF on day 18 of experiment, showing froth (F) and hyperaemia (Hy) of the tracheal mucosa and oedema (Oe) of the lungs.

4.2.2. HISTOPATHOLOGY

The results of histopathological findings from various organs are presented on Figs 11-15. The liver from fatal cases arising from the paddock (Grp. A) and naturally infested with ECF transmitting ticks, showed a predominantly patchy infiltration of lymphocytes into the centrilobular zones (Fig. 11). This was mild to moderate in intensity. The sinusoids contained low numbers of lymphocytes. Slightly congested blood vessels were seen in two animals, while one had bilirubin deposits in the hepatic cells. The centrilobular zones showed a varying degree of fatty degeneration and necrosis.

The livers of the three animals that died of *T. parva* Muranga stabilate infection (Grp. B_1) had centrilobular necrosis of hepatocytes following a pattern similar to those from the paddock. Two animals had mild to moderate lymphocytic infiltrations, while another showed severe lymphocytic infiltration in the centrilobular zones. The hepatic blood vessels were moderately congested and few bilirubin deposits were scattered over the hepatic tissue. The gall bladder had petechial haemorrhage on its mucosa. The liver of one steer in group B_2 infected with *T. parva lawrencei* was extensively damaged. It had moderate lymphocytic infiltration in the centrilobular zones, congested blood vessels with bilirubin deposits surrounding them.

The kidneys of animals which were infected by *T. parva* derived from the paddock (Grp. A) showed a mild to moderate infiltration of lymphocytes in the renal parenchyma in three cases (Fig. 12). The infiltrations were mainly in the cortex with foci around glomerulus, Bowmans capsule and their associated blood vessels. In one steer of the same group the lymphocytic infiltrations also extended into the medulla to involve the renal tubules, while another had bilirubin deposits at the pelvis. Haemorrhage was common in all the six dead animals in the group. Kidneys of the animal which died of *T. parva lawrencei* (Grp. B_2) stabilate infection had no visible lesion, while two that died of *T. parva* Muranga stabilate infection (Grp. B_1) had moderate infiltration of lymphocytes in a pattern similar to those seen in the paddock (Grp. A). One animal showed slight lymphocytic infiltration and haemorrhage in the renal parenchyma and perirenal fat.

The abomasum of three animals in group A, showed varying degree of lymphocytic infiltration into the lamina propria (Fig. 13). All these steers, also showed epithelial erosions at the pyloric region. All the animals infected with stabilate showed moderate lymphocytic infiltrations in the lamina propria and epithelial erosions of the abomasum. The lymph nodes had a similar histopathological pattern in all categories of dead animals. They showed moderate to severe areas of necrosis in the cortical and central zones of the node,

and loss of tissue. Necrosis was diffuse, patchy or focal in distribution. Two cases showed proliferation of lymphocytes in lymph nodes while in the more protracted cases these nodes were less dense (Fig. 14).

The lungs showed major histopathological changes. Those of animals in group A that died from the paddock derived ECF showed moderate lymphocytic infiltration in the lung interstitium, walls of blood vessels, bronchi and bronchioles. The alveoli spaces were filled with oedema fluid (Fig. 15). The infiltration of lymphocytes into the alveolar septa gave an impression of alveolar thickening to occlude alveolar spaces in all the six steers. The steers infected with stabilates (Grp. B_1 and B_2) which died showed the same picture as above.

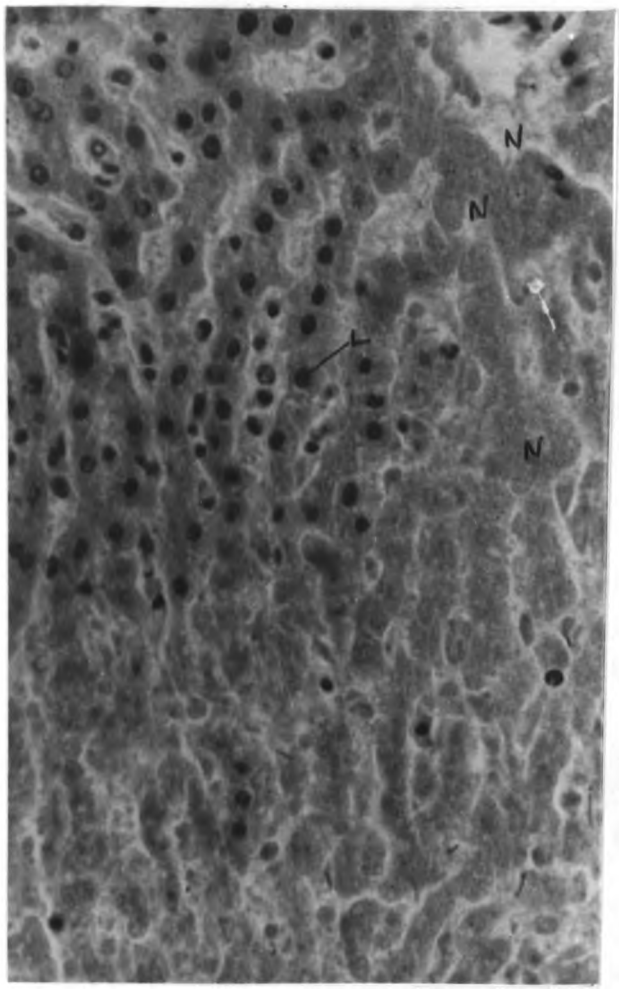


Fig. 11. Liver section of steer that died of ECF on day 32 showing mild infiltration of lymphocytes (L) and centrilobular necrosis (N) (H&E x 400).

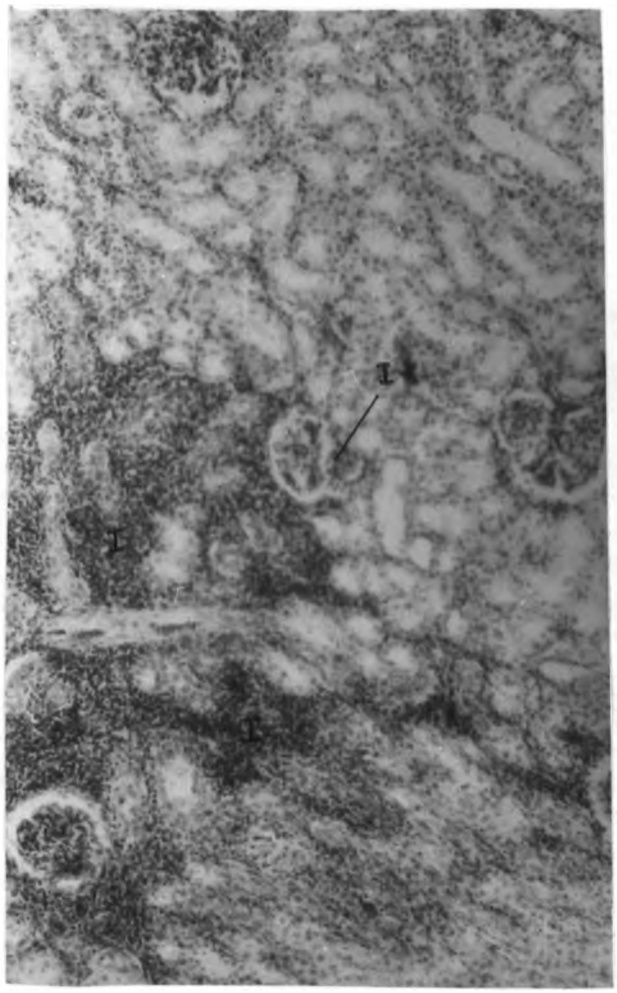


Fig. 12. Patchy infiltration (I) of lymphocytes into the renal cortex of steer which died of ECF on day 32 and involving the Bowman's capsule, glomerulus and associated blood vessels (H&E x 100).

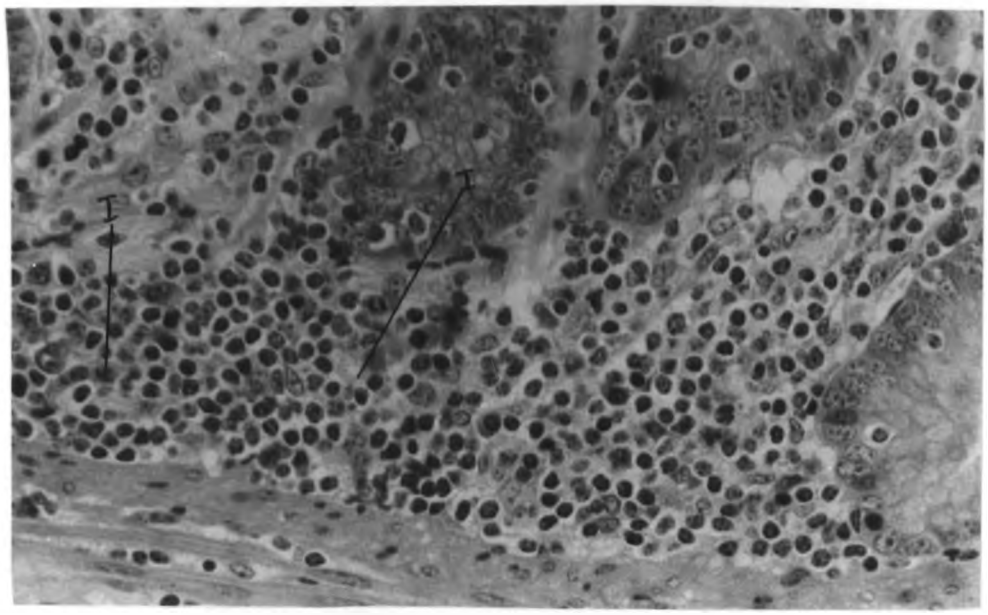


Fig. 13. Abomasal section obtained from steer (Grp. A) which died of ECF on day 18, showing a mild lymphocytic infiltration (I) into the lamina propria and mucosa (H&E x 400).

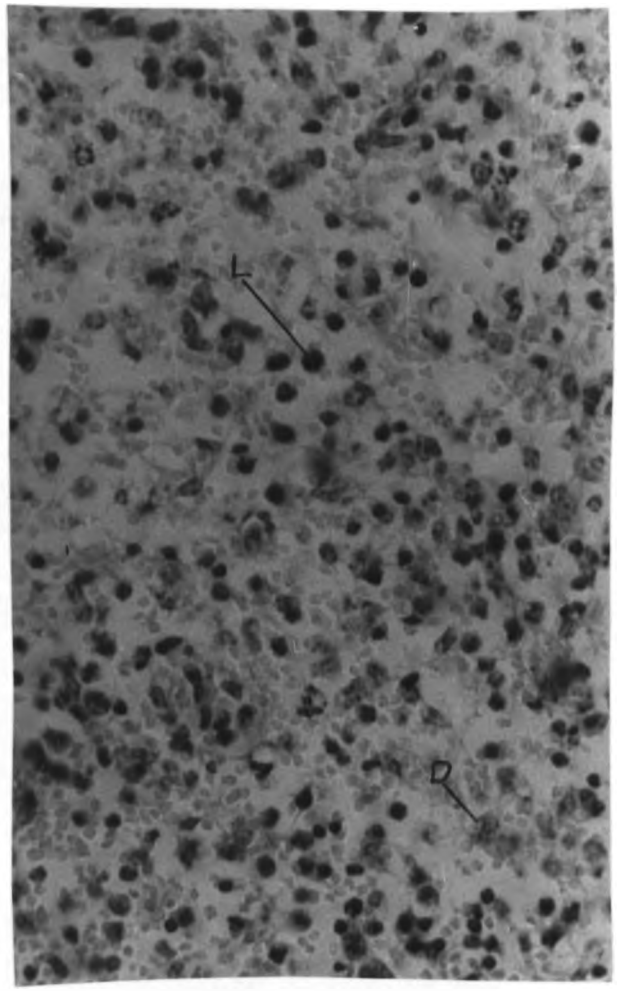


Fig. 14. Lymph node section of a steer that died of ECF on day 25, showing reduced density of lymphocytes (L) and degeneration (D) of the same (H&E x 400).

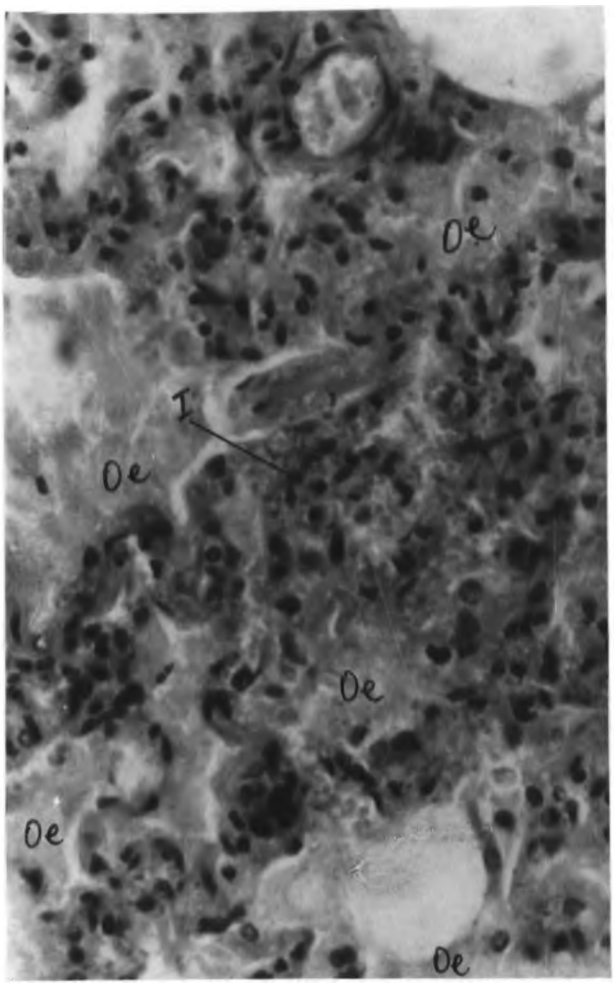


Fig. 15. Lung section of steer No. 790 which died of ECF on day 18,, showing interstitial pneumonia, lymphocyte infiltration (I) into the interstitium and oedema (Oe) fluid in the alveolar spaces (H&E x 400).

4.3. MONOCLONAL ANTIBODY PROFILES OF VARIOUS *T. parva* STRAINS.

Five lymphocyte tissue culture isolates were successfully raised. These were 790, 795, 759, 769 isolated from group A and NDA/12 (Fig. 16) as named according to their ear-tag number. The Mab profiles of these and other rescucitated cultures are summarized in Table 3. *Theileria parva* Muguga reacted to monoclonal antibodies 1 - 4, 7, 10, 12, 21 - 22. The degree of fluorescence was highly intense for Mabs 1, 3, 4, 7, and 10, moderate in 2 and 22 and variable using Mab 21. *Theileria parva* Marikebuni reacted to Mab 1, 4, 10, 12, and 21. The degree of fluorescence was highly intense for Mabs 1, 4 and 7, moderate in 10 and 12 and variable using Mab 21. *Theileria parva* Lanet reacted to Mab 1, 4, 7, 10 and 12. The degree of fluorescence was highly intense for Mabs 1, 4, 7 and 10 and moderate for Mab 12. *Theileria taurotragi* reacted to Mab 10, 12, 15, 21 - 23. The degree of fluorescence was highly intense in 12 and 23, moderate in 10, 21 and 22 and slight for Mab 5. *Theileria parva lawrencei* reacted to Mab 4, 7, 10, 12 and 20, The fluorescence was highly intense for Mabs in 7, 10 and 20, moderate for Mabs 4 and 12 and of low intensity for Mab 22.

Isolate from animal number 795 reacted to Mabs 4, 7, 10 and 12. It also showed surface fluorescence with Mab 21. The degree of fluorescence was highly intense for Mabs 7 and 12 and moderate for 4 and 10. Isolate from animal number 790 reacted to Mab 1 , variably to 2 and 3 and also to 4, 7, 10, 12, 20 and 21. The degree of fluorescence was highly intense for Mab 10 (Fig. 17), moderate for Mabs 4 and 7 and slight for the remainder. Isolate from animal number 759 reacted to Mab 4, 7, 10 and 12. The degree of fluorescence was highly intense for Mab 7, moderate for Mabs 10 and 12 and slight for Mab 4. The isolate from steer number 769 reacted to Mabs 1, 4, 7, 10, 12, 21 and 23. The degree of fluorescence was highly intense for Mabs 7, 10 and 12, moderate for Mab 21 and slight for Mabs 1, 4 and 23. NDa/12 reacted to all Mabs except for 23. The degree of fluorescence was intense for Mabs 2, 4, 7 and 12, moderate for Mabs 1, 3, 20 and 22 and slight for Mabs 10, 15 and 21. All the observations indicate that various *T. parva* isolates have differences in their monoclonal antibody profiles. This is true even for isolates obtained from the same area.

4.4. RESULTS OF WESTERN BLOT

The lysates used for protein analysis by the western blot technique were *T. parva* Muguga, 759, 769 and 790 (Fig. 18). The molecular weights were 86, 82, 84 and 90 kilodaltons (kDa) for *T. parva* Muguga, 759, 769, and 790 respectively.

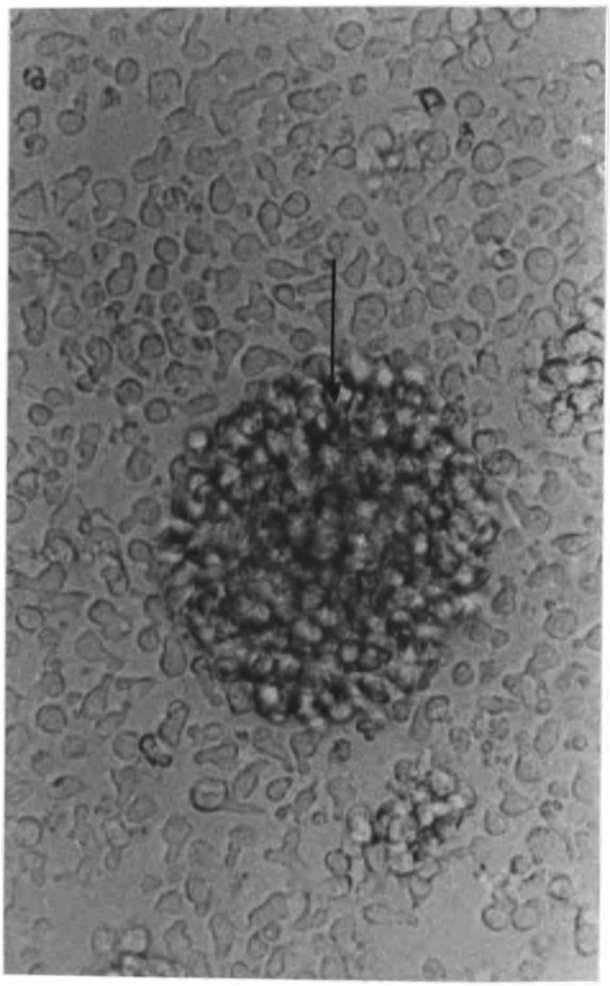


Fig 16. Lymphocyte tissue culture passage 4 of a steer (Grp. A) showing a clump (arrow) and cells undergoing clonal expansion (x 400).

Antigen patterns of bovine lymphocyte cultures raised from steers infected with various strains field isolates tested against 12 monoclonal antibodies at 1/200 dilution.

Mab	Is.795	Is.769	Is.759	Is.790	Muguga	NDa/12	Marikebuni
1	-	-	+	-	+++	++	+++
2	-	-	-	+	++	+++	-
3	-	-	-	+	+++	++	-
4	++	+	+	++	+++	+++	+++
7	+++	+++	+++	++	+++	+++	+++
10	++	+++	++	+++	+++	+	++
12	+++	+++	++	-	+++	+++	++
15	-	-	-	-	-	+	-
20	-	-	-	-	-	-	-
21	+ (SF)	+++ (SF)	+ (SF)	+	±	±	±
22	-	-	-	+	++	-	-
23	+	+	+	-	-	-	-

Key:

+	Low intensity of fluorescence	±	Variable reaction of fluorescence
++	Moderate intensity of fluorescence	-	No fluorescence
+++	High intensity of fluorescence	SF	Surface fluorenscence

Fig. 17. Indirect Fluorescent Antibody Test of 790 when tested using monoclonal antibody number 1 (x 400).

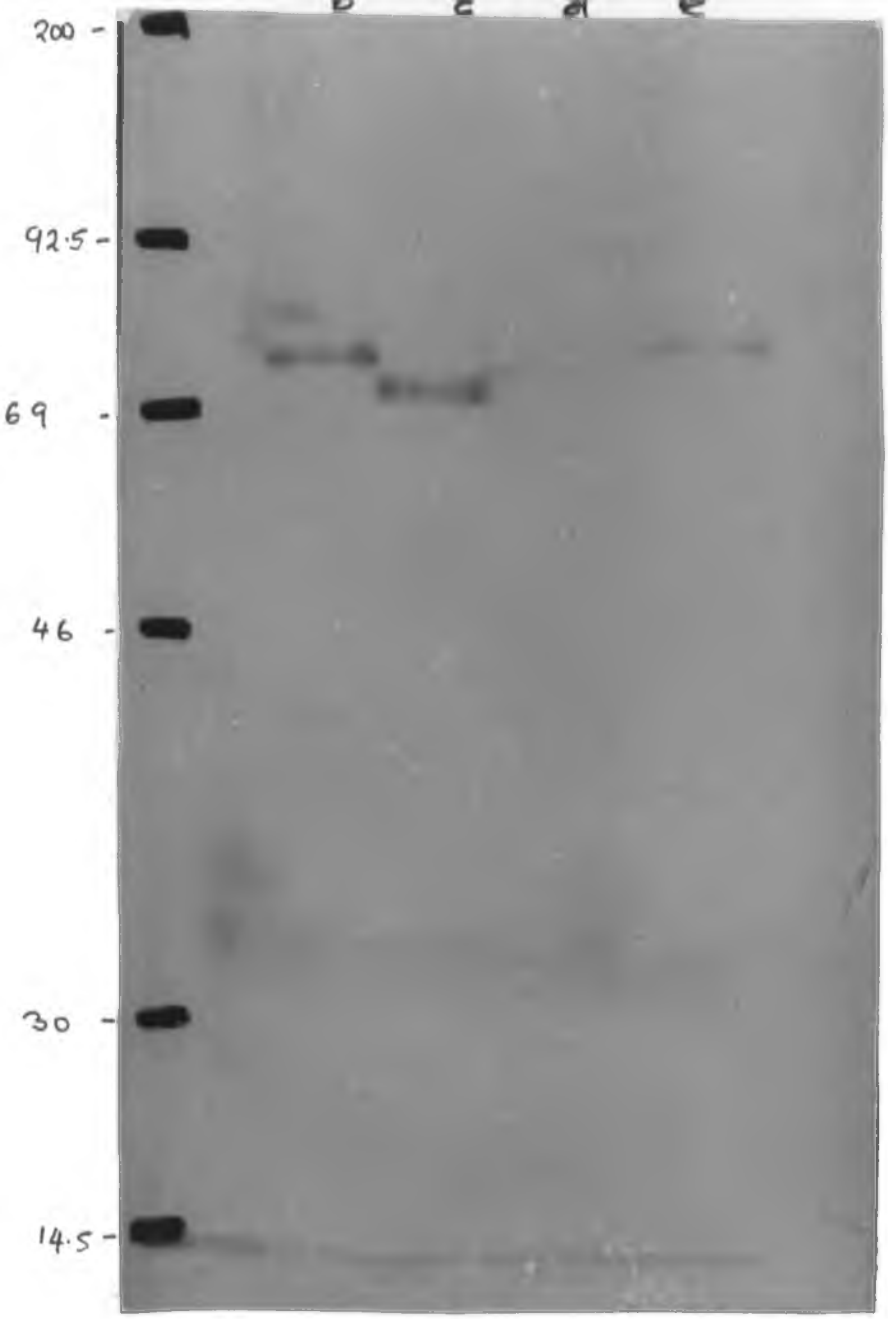


Fig 18. Nitrocellulose blots of whole cell lysate of isolates made from *T. parva* infected steers in a highly infested paddock showing within species variation in the polymorphic immunodorminant molecule. Lane a, molecular weight markers; lane b, (*T. parva* Muguga, 86kDa); lane c, (759, 82kDa); lane d, (769, 84kDa) and lane e, (790, 90kDa) (x 10).

4.5. LYMPHOCYTE INFECTIVITY BY *T. parva.* MARIKEBUNI AND BREAKTHROUGH PARASITE SPOROZOITES *IN VITRO.*

The experiment compared the rates of infection of lymphocytes by sporozoites from two batches of ticks carrying Marikebuni and a parasite breaking through Marikebuni immunization. During the first three days after *in vitro* infection in both cases there was slight increase in the size of cells. These became rounded and by the 5th to the 7th day, cytospins showed marked increase in the cytoplasm of lymphocytes. Mitotic figures were visible in both groups of infected cells, with no visible difference between the two batches. By the seventh day, differences in their cultural characteristics began to be noticed. When observed under the inverted microscope, cells infected with *T. parva* Marikebuni began to form clumps (Fig. 19) or grape-like clusters. Those infected with parasite breaking through Marikebuni immunization were spread out and occurred singly.

Cytospins made on the seventh day showed lymphocytes with more extensive cytoplasm, mitotic figures and elongated cells in both cases. Schizonts were seen from day 6. They ranged in number from 3 to 8 per lymphocyte. Occasionally only one was seen. Lymphocytes at both the highest and lowest concentration of sporozoites were transformed at the same rate. Between days 9

and 12 the lymphocytes were more rapidly transformed. Cell division was extensive (Fig. 20) and most wells showed transformation of cells. However, by day 12 the presence of macrophage like cells was noted. These tended to coalesce to form giant cells. Between days 12 and 14 the transformation of cells ceased. There was inhibition of infection by sporozoites and the already transformed cells began to granulate, degenerate and lyse. Cell death was extensive by day 14.

From the experiment there was no difference between the rate of infection and transformation of lymphocytes by the two batches of sporozoites. However, the cultural and growth characteristics described above could be used to distinguish between the two strains.

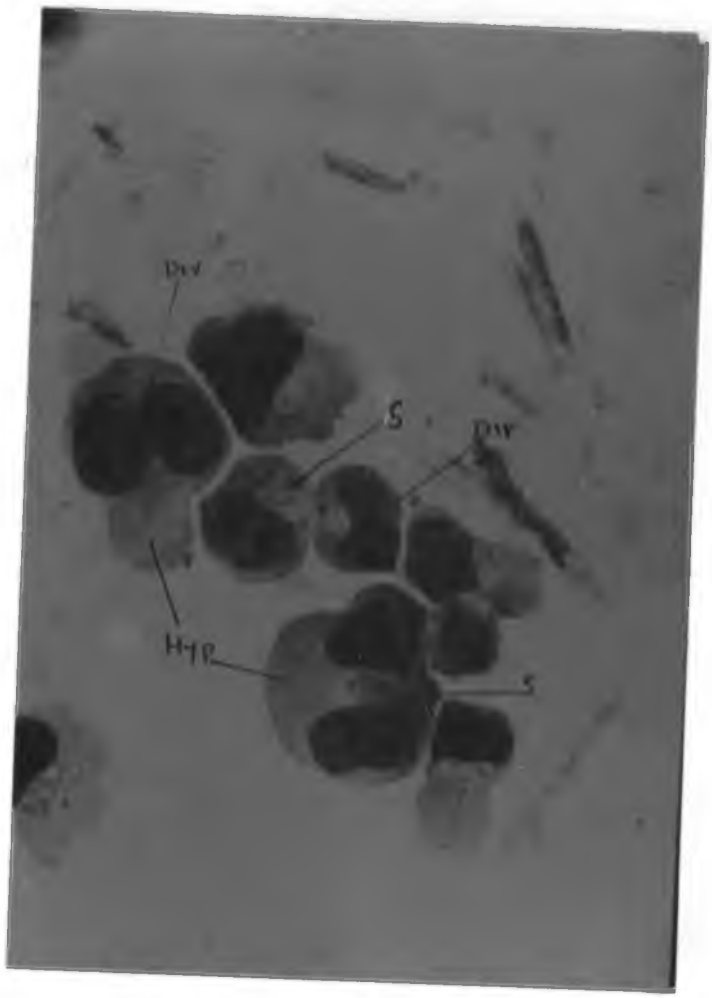


Fig 19. Cytospin of lymphocytes infected using *T. parva* Marikebuni sporozoites *in vitro* on day 7 of infection showing Hyperplasia (Hyp), schizonts (S) and cell division (Div) (Giemsa x 1000).

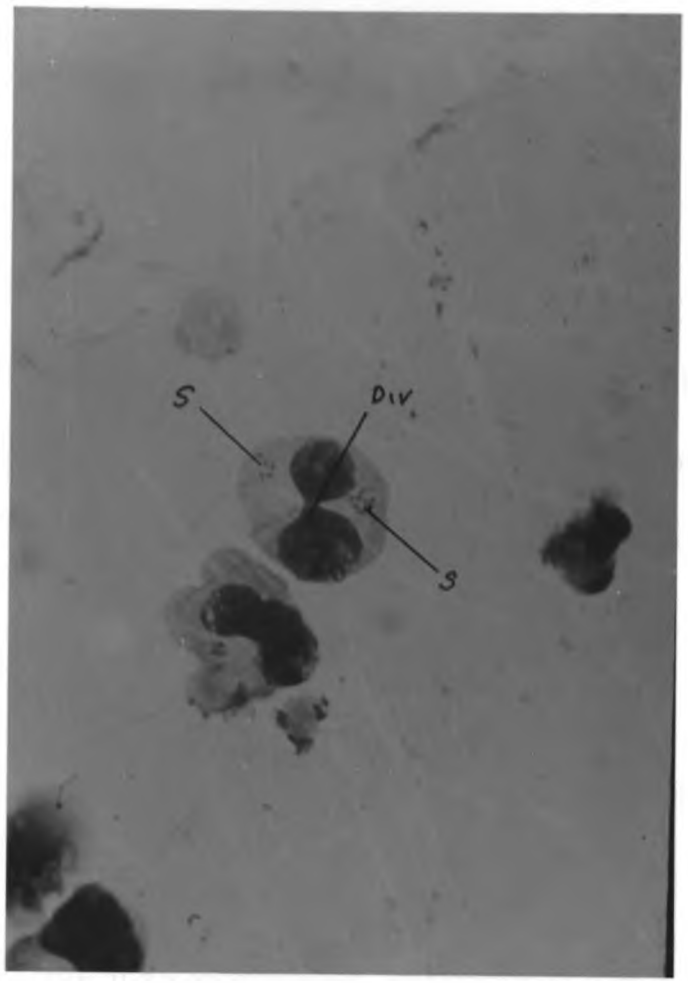


Fig 20. Cytospin of lymphocytes infected with the breakthrough parasite to *T. parva* Marikebuni immunization showing synchronized division (Div) of schizonts (S) and host lymphocytic cells (Giemsa x 1000).

CHAPTER 5

5.0 DISCUSSION AND CONCLUSIONS

The clinical signs observed in this study are largely in agreement with the findings of previous workers, but more closely resembled the acute disease described by Neitz (1957) and Maribei (1974). The incubation period for ECF in the naturally infected steers agreed with those described by Henning (1956) of 8-24 days; Brocklesby (1962) of 10 to 17 days; but not with 8-10 days obtained by Neitz (1957).

Fever was the first sign of disease in this study. This finding agreed with the observations of Henning (1956) and Neitz (1957) when working with naturally infected cattle in the Southern African region. Fever lasted 2 to 9 days of infection, an observation which is in agreement with the work of Brocklesby (1962). The disease conditions observed could be categorized as acute to subacute according to the description of Neitz (1957). Dysentery was observed by Neitz (1957), but was however not seen in this study. Hind quarter weakness described by Henning (1956) was observed in one steer infected with *T. parva* lawrencei. A mild subcutaneous swelling of both the intermandibular region and

the ventral aspects described by both Neitz (1957) and Maribei (1974) was also observed in this study.

The incubation period of 2-8 (mean 4.5) days for *T. parva* Muranga (Group B_1) infection and day 10 for *T. parva lawrencei* (Group B_2) was similar to that of 5 to 6 days obtained by Maribei (1974) when working with *T. parva* Muguga and *T. parva* Aitong respectively and that of Dolan, Young, Losos, McMillan, Minder and Soulsby (1984) while working with *T. parva lawrencei*. The disease occurring due to *T. parva* Muranga infection could be considered acute due to it's high mortality of 75 %, and *T. parva* Lanet as inapparent according to the category of Neitz (1957) since there were no mortalities.

The mortality rate varied among the strains depending on their pathogenicity. *Theileria parva* Muranga was the most pathogenic with 75% deaths followed by the paddock derived strain with 50% and *T. parva lawrencei* with 25% mortality. *Theileria parva* Lanet had no mortalities, hence could be useful in the infection and treatment method of control.

Piroplasm were observed from day 17 in the paddock (Group A) for between 1 and 3 days after the appearance of schizonts in the regional lymph

nodes. In *T. parva* Lanet there were no clinical signs observed inspite of the presence of schizonts in the lymph nodes.

The major haematological sign of leucopenia commonly seen was not observed in all animal groups in this experiment. This finding differed with that of Wilde (1967), Munyua (1971) and Maribei (1974). This could have been as a result of treatment with Butalex on the second day of fever for steers in group A, and low doses of infective sporozoites in Groups B_1 - B_3) .

A mild normocytic normochromic anaemia indicated by pale mucous membranes with no change in neither the mean corpuscular volume nor the mean corpuscular haemoglobin concentration, was present in groups A and B_1 and B_2). This was similar to the finding of Munyua (1971) and Maribei (1974) when working with *T. parva* Muguga and *T. parva* Aitong stabilates while in contrast to those of Wilde (1967) who considered anaemia an insignificant observation in the course of disease. It is therefore possible that the icterus noted at post-mortem could have been as a result of concurrent infection with *Anaplasma marginale* seen in few of the blood smears.

The gross and histopathological observations for the stabilate infections (Groups B_{1-2}) were similar to those seen among steers in the paddock (Group A). Grey white lesions (pseudo-infarcts) described on the surface of the liver and kidney by Henning (1956), Neitz (1957) and Bwangamoi *et al.*, (1971) were not seen grossly in these organs when examined during this study. However, histologically the lesions were visible as perivascular infiltrations of lymphocytes. This was in agreement with the work of both Munyua (1971) and Aruo (1977). The main cause of death was the histaminic reaction in the lungs. This resulted in oedema and the animals drowned in their own fluids.

The major histological findings in this study was infiltration of lymphocytes into various organs. This was in agreement with the work of Munyua (1971) that in early stages proliferation of lymphocytes occurred, while in latter stages they regressed. Both Steck (1928) and Munyua (1971) observed that infiltrating lymphocytes were retained in the various organs while simultaneously undergoing clonal expansion.

The monoclonal antibodies profiles of the known stains *Theileria parva* Muguga, *T. parva* Marikebuni, *T. taurotragi*, *T. parva lawrencei* and *T. parva* Lanet that were rescusitated were similar to those obtained by Minami *et al.*,

(1983), because they were clones. They exhibited a similar pattern of reaction to epitopes located on the surface of schizonts. The Mabs profiles of the field isolates were similar to those of *T. parva* strains but more closely resembled in Mab reaction and profile configuration those of *T. parva* Kiambu isolates by Lesan *et al.*, (1989). However, the variable level of fluorescence with Mab 23 could be an indication of mixed infection with *T. taurotragi* which could have been selected out *in vitro*. The field isolates were similar although they showed slight variations in their reactions to Mabs, in both intensity and percentage of schizont fluorescence. Similar results have been obtained by Shield, McDougall, Tait and Brown (1986) when working with *T. annulata*. They showed intra-species difference in *T. annulata*, that was to a lesser extent than seen between species. Minami *et al.*, (1983) and Conrad *et al.*, (1987) obtained similar results while working with *T. parva* strains. Absence of reaction to Mab 20 suggest that there was no incident of buffalo involvement. The results of monoclonal antibody profiles observed in this study could have been due to genetic recombination, somatic mutation or mixed infections. Some of these strains could be selected out *in vitro*. Hybridization between *T. parva* Muguga, *T. parva* Marikebuni and *T. taurotragi* may have occurred to a varying degree.

Mab profiles is an important technique for the rapid diagnosis and screening of *T. parva* infections. The irregular binding of Mabs to schizonts may be evidence of specific schizont surface determinants that are related to cyclic changes during parasite DNA synthesis. Minami *et al.*, (1983) suggested that the profiles were phenotypic expressions (finger prints) of different antigenic determinants. They did not provide information on the genetic properties (genotypes) of these determinants. The shortfall of this study was the inability to prove the presence of mixed infection in lymphocyte cultures of the cattle naturally infected in the paddock (Group A). A high portion of infected lymphoblastoid cells died during processing. Schizonts in such cells lost their antigenic properties and did not fluorescence when tested with Mabs.

The field isolates from the paddock (Group A) showed differences in the size of their polymorphic immunodominant molecules (PIM) by the western blot techniques. The PIM sizes ranged from 70 to 90 KDa. This was similar to the findings of Toye *et al.*, (1991) when working with various strains of *T. parva* at 10% polyacrylamide gel concentration. Their results showed that *T. parva* Muguga (C_2) had a molecular weight of 80/90 KDa. In this study only *T. parva* Muguga and isolate 759 showed the double banding of PIM. Tissue cultures raised from isolates of steer No. 769 and 790 were faint indicating lower

amounts of antigens. Molecular weights of 82 KDa for isolate 759 and 84 KDa for isolate 769 were similar to 82 KDa obtained by Shapiro *et al.*, (1987) for *T. parva* isolates.

The technique was sensitive because when using solubilized antigens, the molecular weight of PIM varied considerably between isolates obtained from the same paddock. The results were however inconclusive as to whether or not the isolates were exactly the same parasite strain. The variation in monoclonal antibody profiles was however to a lesser extent than assessed by Mab profiles reported by Minami *et al.*, (1983) and obtained in this study. The variation in the molecular weight of PIM is an indication of divergence between and within strains. Since the biological function of PIM is in species conservation, the variation seen could be due to hybridization or the evolution of new substrains in the area.

The technique of sporozoites infection and transformation of lymphocytes is sensitive and reproducible. Stagg *et al.*, (1983) used it to test infectivity of *T. parva parva*, *T. parva lawrencei* and *T. taurotragi* to lymphocyte of various animal species. This was the first attempt to compare the rates of infection of

different strains of *T. parva*, to PBM from the same animal in order to remove any bias in the response caused by the idiosyncratic nature of each steer.

The rate of sporozoites infection of lymphocytes in this instance was similar for both *T. parva* Marikebuni and the breakthrough to the Marikebuni immunization. This rate was not dose dependent since transformation took place at the same rate at both high and low sporozoite concentrations. This was however in disagreement to what is known *in vivo* infections where severity of disease is dependent on the infective dose. It can be interpreted that at the dilutions used here, the rate of infection of lymphocytes was independent of sporozoites concentration or that the breakthrough was as a result of an improper administration of the stabilate. The conclusion therefore, is that at a high concentration of sporozoites, the rate of infection of lymphocytes *in vitro* is constant. The two batches however, showed differences in their culture and growth characteristics. This could be used as a means of differentiating between cultures of *T. parva*. Multiple infections with 3-8 schizont per cell were common in both batches, a further pointer to the influence of sporozoite dilutions. The schizonts developed in 3-6 days, a result consistent with 3-5 days seen by Stagg *et al.*, (1983). However, any lymphocytes with multiple infections failed to establish in culture when passaged (transferred) into tissue

culture flasks. Macrophage like cell clusters (giant cells) which were probably cytotoxic (killer cells) were seen from day 7. They probably contributed to early cell death.

The infection of lymphocytes by *Theileria parva* sporozoites is a useful technique for predicting the course of the disease East Coast Fever. It can be used as a measure of both infectivity and pathogenicity by using the cultural and growth characteristics. The breakthrough in immunity observed in the previous experiment could have been due to a greater dose of sporozoites which broke through the drug regimen or the inability of *T. parva* Marikebuni to protect on cross challenge with *T. parva* Kiambu 5.

SUMMARY OF CONCLUSIONS

1. Clinical, parasitological, haematological and pathological picture of *Theileria* infection was consistent with observations that have been made over the years.

2. Monoclonal antibody profiles and Western blot method are both important technique for the rapid diagnosis and characterization of *Theileria* and are useful in screening of E.C.F. infections in the field. However, the Western blot technique is more superior since it showed more definite differences than Mab profiles.

3. The infection of lymphocytes by *Theileria parva* sporozoites is a useful technique for predicting the course of the disease East Coast Fever. It can be used to determine both infectivity and pathogenicity of *T. parva* by their cultural and growth characteristics.

6.0 REFERENCES

Allsopp, B.A. and Allsopp, N.T.E.P. (1988). *Theileria parva* Genomic DNA studies reveal intra-specific sequence diversity. Molecular and Biomedical Parasitology, 28: 77-84.

Allsopp, B.A., Baylis, H.A., Allsopp, M.T.E.P. Caraliersmith, T., Bishop, R.P., Cunningham, P.M., Sohampal, B., Spooner, P. (1993). Discrimination between 6 species of *Theileria* using oligonacleotide probes which detect small subunit Ribosomal RNA sequences. Parasitology, 107: 157-165.

Aruo, S.K (1977). The clinical and pathological picture of *T. parva* (East Coast Fever) in European breeds of cattle in Uganda. Bulletin of Animal Health and Production Africa, 25: 223 - 227.

Barnett, S.F. (1977). *Theileria.* In Parasitic Protozoa. (Ed, J.P., Krier), Academic Press, New York .Vol.IV, pp. 77-113.

Barnett, S.F. and Brocklesby, D.W. (1966). Recent investigation into theileridae of cattle and buffalo in Africa: A mild form of East Coast fever (*Theileria parva)* with persistence of infection. British Veterinary Journal, 122: 361-370.

Blewett, D.A. and Branagan, D. (1973). The demonstration of *T. parva* infection in intact *R. appendiculatus* salivary glands. Tropical Animal Health and Production, 5: 22-25.

Brocklesby, D.W.(1962). *Cytauxzoon taurotragi.* Martin and Brocklesby, 1960, a piroplasn of Eland (*Taurotragus orxy pattersonianmus lydekker*, 1960). Research in Veterinary Science, 3, 334 - 344.

Brocklesby, D.W. and Barnett, S.F. (1966). The isolation of *Theileria lawrencei* (Kenya) from a wild buffalo (*Syncerus caffer*) and its serial passage through captive buffaloes, British Veterinary Journal, 122: 387-395.

Brown, C.G.D. (1987). Theileria. In *vitro* cultivation of protozoan parasites (Ed J.B. Jensen) pp 243-284 Boca Raton. Florida CRC press Inc.

Burridge, M.J. and Kimber, C.D. (1972). The indirect fluorescent antibody test for experimental East Coast Fever (*T. parva* infection of cattle): evaluation of a cell culture schizont antigen. Research in Veterinary Science, 13: 451-455.

Bwangomoi, D., Frank, H., Munyua, W.K. and Wandera, J.G. (1971). Gross lesions in natural bovine East coast fever at Kabete. Bulletin of Epizootic Diseases in Africa, 19: 271-277.

Chema S., Waghela S., James A.D., Dolan T.T., Young A.S., Masiga W.M., Irvin, A.D., Mulela G.H.M. and Wekesa L.S. (1986). Clinical trials of parvaquone for treatment of East Coast Fever in Kenya. Veterinary Record, 118: 588-589.

Conrad, P.A, Stagg, D.A, Grootenhuis, J.G, Irvin, A.D, Newson, J., Njamunggeh, R.E.G, Rossiter, P.B and Young A.S. (1987). Isolation of *Theileria* parasites from African buffalo (*Syncerus caffer*) and characterization with anti-schizont monoclonal antibodies. Parasitology, 94: 413-423.

Cowan M.K. (1981). Humoral responses in theileriosis In Advances in the control of theileriosis (Ed. A.D.Irvin, M.P. Cunningham and A.S., Young). Martin Nijhoff publishers. The Hague pp 368-382.

Creemers, P. (1983). Protein antigens of *T. parva* macroschizonts and immune precipitation studies. Journal of Parasitology, 69: 54-59.

Cunningham, M.P ,Brown, C.G.D, Burridge, M.J and Purnell, R.E. (1973). Cryo-preservation of infective particles of *T. parva*. International Journal for Parasitology, 3: 585-587.

Da Graca, H.M and Serrano, F.M.H. (1971). Contribuicao para o' estudo da theileriose cincerina maligna dos bovinos em Angola, Acta, Veterinaria, Nova Lisbona, 7:1-8.

De Kock, G. (1957). Studies on the lesions and Pathogenesis of East Coast Fever (*Theileria parva* infection) in cattle with special references to the lymphoid tissues. Onderspoort Journal of Veterinary Research, 27 :431-453.

DeMartini, J.C. and Moulton, J.E. (1973a). Responses of the bovine lymphatic system to infection by *Theileria parva*. 1. Histology and ultrastructure of lymph node in experimentally infected calves. Journal of Comparative Pathology, 83: 281-298.

DeMartini, J.C. and Moulton, J.E. (1973b). Responses of the bovine lymphatic system to infection by *Theileria parva*. 11. Changes in the central lymph in experimentally infected calves. Journal of Comparative Pathology, 38: 299-306.

Dobbelaere, D.A.E., Spooner, P.R., Barry, C.W. and Irvin, A.D. (1984). Monoclonal antibodies neutralise the sporozoite stage of different *Theileria parva* stocks. Parasite Immunology, 6: 361-370.

Dolan, T.T. (1989). Theileriasis: A comprehensive review. Revue scientifique et Technique de l'office International des Epizooties, 198 (1): 11-36.

Dolan, T.T., Linyonyi, A., McHardy, N., Bond, A.L. and Clampit, R.B. (1988). Chemotherapy of ECF: Parvaquone treatment of *Theileria parva* at intervals after infection. Research in Veterinary Science 44: 15-20.

Dolan, T.T., Young, A.S., Leitch, B.L. and Stagg, D.A. (1984). Chemotherapy of East Coast Fever: Parvaquone treatment of clinical disease induced by isolates of *Theileria parva*. Veterinary Parasitology, 15: 103-116.

Dolan, T.T., Young, A.S., Losos, G.J., McMillan, I. Minder, C.E. and Soulsby, K. (1984). Dose dependent responses of cattle to *Theileria parva* stabilate infection. International Journal for Parasitology, 14: 89 - 95.

Duffus, W.P.H. and Wagner, G.G. (1974). Immunochemical studies on East Coast fever 3: Development of an indirect haemagglutination assay using *Theileria parva* piroplasms antigen. Journal of Parasitology, 60: 860.

Fawcett, D.W., Young, A.S. and Leitch, B.L. (1985). Sporogony in *Theileria* (Apicomplexa:Piroplasmida). A comparative Ultrastructural study. Journal of Submicroscopic Cytology, 17: 299-314.

Fivaz, B.H. and Norval, R.A.I. (1990). Immunity of the ox to the brown ear tick *Rhipicephalus appendiculatus* (Neumann). Experimental and Applied Acarology, 8: 51-63.

Goddeeris, B.M., Katende, J.M., Irvin, A.D. and Chumo, R.S.C. (1982). Indirect fluorescent antibody test for experimental and epizootiological studies on East Coast Fever (*T. parva* infection in cattle): Evaluation of a cell culture schizont antigen fixed and stored in suspension. Research in Veterinary Science, 33:360-365.

Hensen, J.B. and Campbell, M. (1977). Theileriosis report of a workshop held in Nairobi, Kenya, 7-9 December 1976. IDRC Ottawa Canada 112 pp.

Henning, M.W. (1956). Animal diseases in South Africa. 3rd Ed. Central News Agency Ltd. Capetown. 1239pp

Hill, R.R.H. and Matson, B.A. (1970). The haematology of experimental *Theileria lawrencei* infection. Journal of South African Medical Association, 41: 275-284.

Howell, C.J, De vos, A.J., Bezuidenhout, J.D., Potgieter, F.T. and Barrowman, P.R. (1981). The role of chemical tick eradication in the control or prevention of tick transmitted diseases in cattle: In Tick biology and control: Proceedings of an International Conference Held in Grahamstown, 27-29 January 1981. Editors G.B. Whitehead and J.D. Gibson, Tick research unit, Rhodes University, Grahamstown pp 61-66.

Hullinger, L., Wilder, J.K.H., Brown, C.G.D and Turner, L. (1964). Mode of multiplication of *Theileria* in cultures of bovine lymphocytes cells. Nature, 203: 728-730.

Hunsaker, W.R., Badri, H., Lombardo, L. and Collins, M.L., (1989). Nucleic acid hybridization assays. Detailed capture probes. II. Advanced Multiple capture. Methods in Analytical Biochemistry,181, 360 - 370.

Irvin, A.D. (1987). Characterization of species and strains of *Theileria*. Advances in parasitology, 26: 145-197.

Irvin, A.D., Cunningham, M.D. and Young, A.S.(1981). Advances in the control of theileriosis. Martinus Nijhoff Publishers. The Hague.

Jura, W.G.Z. and Losos, E.M. (1980). A comparative study of the disease in cattle caused by *Theileria lawrencei* and *Theileria parva* : Clinical signs and Parasitological observations. Veterinary Parasitology, 7 : 275-286.

Kariuki, D.P. (1988). Theileriosis in Kenya: In Theileriosis in Eastern, Central and Southern Africa.(Ed) T.T. Dolan ILRAD Nairobi pp 5-11.

Kariuki, D.P. (1990). Current status of Theileriosis in Kenya 1989: In progress towards the control of East coast fever (Theileriosis) in Kenya. Ed A.d Young, J.J Mutungi and A.C Maritim, KARI pp 17-26.

Katende, J.M., Goddeeris, B.M., Morzaria, S.P., Nkonge, C.G. and Musoke, A.J. (1990). Identification of a *Theileria mutants* - specific antigen for use in an antibody and antigen detection ELISA. Parasite Immunology, 12: 419-433.

Kimeto, B.A. (1978). Histopathologic and Electron microscopic studies of cutaneous lesions in calves with experimentally induced East Coast Fever (Theileriosis) American Journal of Veterinary Research, 39: 1117 - 1122.

Koch, R. (1903) Interim Report on Rhodesian Red water or African Coast Fever, Argus Printing and Publishing Co. Ltd Salisbury, 8pp (abstracted in Journal of comparative pathology and therapeutics, 16: 273-280.

Lawrence, J.A. (1981). Bovine theileriosis. In : Advances in the control of theileriosis. Proceedings of an International Conference held in ILRAD, Nairobi, 9-13 February 1981. (Ed. A.D. Irvin, M.P. Cunningham and A.S. Young) Martinus Nijhoff publishers the Hague pp 74-75.

Lawrence, J.A. and Mackenzie, P.K.I. (1980). Isolation of a non-pathogenic *Theileria* of cattle transmitted by R. appendiculatus. Zimbabwe Veterinary Journal 11: 27-36.

Lawrence, J.A., Norval, R.A.I. and Uilenberg, G. (1983). *Rhipicephalus zambenziensis* as a vector of bovine Theileridae. Tropical Animal Health and Production, 15: 39-42.

Lessard, P., L'Eplattenier, R., Norval, R.A.I., Kundert, K., Dolan, T.T., Croze, H., Walker, J.B., Irvin, A.D. and Perry, B.D. (1990). Geographical information systems for studying the epidemiology of cattle diseases caused by *T. parva*. Veterinary Record., 126: 255-262.

Lesan, A.C., Awich, J.R, Williamson, S.M., Linyonyi, A., Ondwasy, H. and Stagg, D.A. (1989). Characterization of Theilerial stocks using monoclonal antibodies. In the Twelfth Quarterly Report July-September 1989. pp 11-12.

McHardy, N. (1989). Buparvaquone. The new antitheilerial: Review of its efficacy and safety. In Theileriosis in Eastern, Central and Southern Africa. Proceedings of a workshop on East Coast Fever Immunization held in Lilongwe, Malawi, 20 - 22 September, 1988. Editor, T.T. Dolan. International Laboratory for Research in Animal Diseases, Nairobi. pp 157 - 165.

Malmquist, W.A., Nyindo, M.B.A. and Brown, C.G.D. (1970). East Coast Fever: Cultivation *in vitro* of bovine spleen cell lines infected and transformed by *Theileria parva*. Tropical Animal Health and Production, 2: 139-145.

Maribei, J.M. (1974). Experimental East Coast Fever: A study of clinical signs, haematology and biochemistry. Msc Thesis. University of Nairobi.

Matson, B.A. (1967). Theileriosis in Rhodesia: 1. A study of diagnostic specimens over two seasons. Journal of South African Medical Association, 38: 93-102.

Maxie, M.G., Dolan, T.T., Flowers, M.J., Jura, W.G.Z and Tabel, H. (1982). A comparative study of the diseases in cattle caused by *Theileria parva* or *Theileria lawrencei*: II: Haematology, clinical chemistry, coagulation studies and compliment. Veterinary Parasitology. 10: 1-19.

Melhorn, H. and Schein, E. (1984). The piroplasms: Lifecycle and sexual stages. Advances in Parasitology, 23, 37-103.

Minami, T., Spooner, P.R., Irvin, A.D., Ochama, J.G.R., Dobbelaere, D.A.E and Fujinaga, T. (1983). Characterization of stocks of *T. parva* by monoclonal antibody profiles. Research in Veterinary Science, 35, 334 - 340.

Morrison, W.T., Buscher, G., Emery, D.L. Nelson, R.T. and Murray, M. (1981). The kinetics of infection with *Theileria parva* in cattle and relevance of the development of immunity. In advances in the control of Theileriosis (Eds A.D. Irvin, M.P., Cunningham and A.S.Young), Martiunus Nijhoff Publishers. The Hague pp. 311 - 326.

Morrison, W.T., Lalor, P.A., Goddeeris, B.M. and Teale, A.J., (1986). Theileriosis: Antigens and host parasites interactions. In parasite antigens (Editor T.W. Pearson). Marcel Deaker Inc New York pp 167- 213.

Morzaria, S.P (1989). Identification of Theileria species and characterization of *Theileria parva* stocks: In Theileriosis in Eastern, Central and Southern Africa: Proceedings of a workshop on East coast fever immunization held in Lilongwe, Malawi 20 - 22 September, 1988. Editor T.T. Dolan, International Laboratory for Research in Animal Diseases. Nairobi pp 102 - 110.

Muhammed, S.I. (1975). The effect of leucocytes from cattle immunised against East Coast Fever on the migrations of bovine lymphoblasts infected with *Theileria parva*. Zeuteublad. Veterinary Medicine, 22: 455-60

Muhammed S.I., Johnson L.W. and Lauerman, L.H. (1975). Effect of humoral antibodies on the course of *Theileria parva* infection (East Coast fever) of cattle. American Journal of Veterinary Research., 36 : 399-402.

Munyua, W.K. (1971). Pathogenesis and Pathology of East Coast Fever in cattle. Msc Thesis. University of Nairobi, Kenya.

Musoke, D.J. Nantulya, V.M., Rurangirwa, F.R. and Buscher, G. (1984). Evidence of a common protective antigenic determinant on sporozoites of several different *Theileria parva* strains. Immunology, 52: 231-238.

Musoke, A.J., Nene, V and Morzaria S.P. (1993). A sporozoite based vaccine of Theileria parva. Parasitology Today, 9: 385-388.

Neitz, W.O. (1957). Febrile reactions in East Coast Fever. A review of 150 cases. Bulletin of Epizootiological diseases of Africa, 10: 44-54.

Norval, R.A.I. and Lightfoot, C.J. (1982). Tick problem in wildlife in Zimbabwe: Factors influencing the occurrence and abundance of *Rhipicephalus appendiculatus*. Zimbabwe Veterinary Journal, 13: 11-20.

Norval, R.A.I., Perry, B.D. and Young, A.S. (1992). The epidemiology of Theileriosis in Africa. Academic Press, Havecourt, Brace Jovanovich Publisers. 481pp.

Pearson, T.W., Pinder, M., Roelants, G.E., Karsklundin, L.B., Mayor-Withey, K.S and Hewett, R.S. (1980). Methods of derivation and detection of antiparasite monoclonal antibodies. Journal of Immunological Methods, 34: 141 - 154.

Pinder, M. and Howett, R.S. (1980). Monoclonal antibodies detect antigenic diversity in *Theileria parva* parasites. Journal of Immunology, 124: 1000-1001.

Purnell, R.E. (1977). East Coast fever of cattle : Some recent research in East Africa. *Theileria*. Advances in Parasitology, 15: 83-132.

Purnell, R.E., Brown, C.G.D., Cunningham, M.P., Burridge, M.J., Kirimi, I.M. and Ledger, M.A. (1973). East coast fever: Correlation between the morphology and infectivity of *T. parva* developing in its vector. Parasitology, 66: 539-544.

Radley, D.E., Brown, C.G.D., Burridge, M.T., Cunningham, M.P., Pierce, M.A. and Purnell, R.E. (1974). East Coast fever : Quantitative studies of *Theileria parva* in cattle. Experimental Parasitology, 36:278-287.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Sejart, S., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlichs, H.A. (1988). Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science, 237: 487-491.

Schein, E. and Viogt, W.P., (1981). Chemotherapy of theileriosis in cattle. In advances in the control of theileriosis. Proceedings of an International Conference held at ILRAD, Nairobi, 9-14 February 1981. (Ed A.D. Irvin, M.P.Cunningham and A.S. Young). Martiunus Nijhoff publishers the Hague. pp 212-214.

Schalm, W.O. (1986). Haematology :Cattle. In, Veterinary Haematology, Lea and Ferbiger, Philadelphia.

Shapiro, S.Z, Fujisaki, K., Morzaria, S.P., Webster, P., Fujinaga, T., Spooner, P.R. and Irvin, A.D. (1987). A live cycle stage specific antigen of *T. parva* recognised by antimacroschizont monoclonal antibodies. Parasitology, 94: 29-37.

Shaw, M.K., Tilney, L.G. and Musoke, A.J. (1991). The entry of *Theileria parva* sporozoites in bovine lymphocytes: Evidence of MHC class I involvement. Journal for Cellular Biology, 133: 87-101.

Shields B., Mc Dougall, C., Tait, A. and Brown C.G.D. (1986). Antigenic diversity of *Theileria annulata* macroschizonts. Veterinary Parasitology, 5: 117-128.

Stagg, D.A., Dolan, T.T., Leitch, B.L. and Young, A.S. (1981). The initial stages of infection of cattle cells with *Theileria parva* sporozoites *in vitro*. Parasitology, 83: 191-197.

Stagg, D.A., Young, A.S., Leitch, B.L., Grootenhuis, J.G. and Dolan, T.T. (1983). Infection of mammalian cells with *Theileria* species. Parasitology, 86: 243-254.

Steck, W. (1928). Histological studies on ECF. 13th and 14th report to the Director of Veterinary education and research, Union of S. Africa. Government printing and stationary officer Pretoria pp 243-280.

Sugimoto, C., Mutharia, L.M., Conrad, P., Dolan, T.T., Brown, W.C., Goddeeris, B.M. and Pearson, T.W. (1989). Protein changes in bovine lymphoblastoid cells induced by infection with the intracellular parasite *T. parva*. Molecular Biochemistry. Parasitology,37: 159-169.

Toye, P.G., Goddeeris, B.M., Iams, K., Musoke, A.J., Morrison, W.I. (1991). Characterization of a polymorphic immunodominant molecule in sporozoites and schizonts of *Theileria parva*. Parasite Immunology, 13 (1): 49-62.

Wagner, G.G., Brown C.G.D., Duffus, W.P.H., Kimber, C.D, Graw, J.E. and Lule, M. (1974). Immunochemical studies on East Coast Fever, Partial segregation and characterisation of the *Theileria parva* schizont antigen. Journal of Parasitology, 60: 848-853.

Walker, J.B. (1991). A review of the Ixodid ticks (Acari.Ixodidae) occurring in Southern Africa. Onderstepoort Journal of Veterinary Research, 58: 81-105.

Wilde, J.K.H. (1967) East coast fever. Advances in Veterinary Sciences, 11: 207-259.

Willadesen, P. (1990). Perspectives for subunit vaccines for the control of ticks. Parasitolgia 32: 195-200.

Willadsen, P. and Kemp, D.H. (1988). Vaccination with concealed antigens for tick control. Parasitology Today, 4 : 196-198.

Williamson, S.M., Lesan, A.C. and Awich, J.R. (1990). Immunological and molecular tools for use in ECF immunisation and for assessment of other tick born diseases. In Progress towards the control of East Coast fever (Theileriosis) in Kenya. Ed A.S.Young, J.J.Mutugi and A.C.Maritim, Kenya Agricultural Research Institute Nairobi, pp. 66-71.

Young, A.S. (1981). The epidemiology of Theileriosis in East Africa. In Advances in the control of theileriosis. Proceedings of an international conference held at ILRAD, Nairobi 9-13 February 1981. Editors A.D Irvin, Cunningham and A.S Young. Martinus Nijhoff publishers The Hague pp 38-55.

Young, A.S., Brown, C.G.D., Burridge, M.J. Grootenhuis, J.G., Kariuki D.P, Purnell, R.E. and Stagg D.A. (1978). The incidence of *Theileria* parasites in East Africa buffalo (Syncerus Caffer). Tropical Medicine Parasitology 29: 281-288.

Young, A.S., Groocock, C.M. and Kariuki, D.P (1988). Integrated control of ticks and tick borne diseases of cattle in Africa. Parasitology, 96: 403-432.

Young, A.S., Leitch, B.L. and Newson, R.M. (1981). The occurrence of *Theileria parva* carrier status in cattle from an East Coast fever endemic area in Kenya. In advances in the control of theileriosis (Ed A.D. Irvin, M.P. Cunningham and A.S. Young) Martinus Nijhoff Publishers. The Hague, pp. 60-62.

APPENDIX I.

Preparation of Media, Buffers and Solutions:

(MEM) Minimum Essential Media & RPMI 1640

a) MEM (5L)

i) Dissolve 47.633g (but this may vary as labels of the Manufacture varies) powder in 1L of purified water. Add another 1L of water for completing the dissolving process.

ii) Dissolve 11g of $NaHCO_3$, add to the above solution.

iii) Add 0.5g of Asparagine. Bring volume to 5L by adding purified water.

iv) Dissolve antibiotics in 5ml of water. Use 2.5ml of Benzylpenicillin and streptomycin for 3ml kanamycin use 1.5ml of 100mg/ml

v) Adjust PH to 7.2 by adding 1ml of conc. HCL (1M)

vi) Sterilize medium using 0.22m membrane, use positive pressure pump (to minimize loss of carbon dioxide). Collect medium in sterile bottles and store at 4+° celsius.

Sterility test.

Add approximately 10ml medium into nutrient broth at the beginning and end of each collection and incubate at 37°C for at least 3 days.

Preparation of working media

Preparation of other associated reagents :

a) Phosphate Buffered solution (PBS) PH 6.8 0.0132M

i) 1.874g $NaHPO_4$ in 1L distilled water

ii) 1.796g KH_2PO_4 in 1L distilled water

iii) Prepare stock phosphate solution PH 6.8 0.0132M

iv) Mix equal parts (i) and (ii) to the 2l after mixing add 16 gms of NaCL sterilize by autoclaving. Dispense into 100mls bottle, store at 4°C during use otherwise leave in a cool place, (Room temp).

b) Lymphoprep : to make 100ml

i) Take 70ml above 8% ficoll (stock) and sterilize by autoclaving 10 minutes at 15 psi.

ii) Add 1 vial(30ml) sodium metrizoate solution 32.8% (i.e. Metrozoic acid) (Nyegaard) also to 70ml 8% ficoll and pipette thoroughly for 2 minutes.

Attention : to dissolve powder, add to water, put in water bath 37°C and shake at intervals. This is "Lymphoprep" S.G. approx. 1.077 kept sterile at 4°C.

Preparation of Glutamine

Glutamine - 29.23gm

D.D H_2O (deionized distilled water is used)

a) Dissolve glutamine in the amount of A.D. required (A.D. aquadest) distilled water in water bath (37^0C) to speed up the process.

b) Sterilize by filtration thro' 0.22m millipore /-ve pressure 15 psi

c) Bottle into 20ml amounts & freeze at -20°C

d) ' Use 1ml in 100ml (G.M.) growth media

APPENDIX 2

Collection of blood for Haematology & serology

Haematological slides

- Collect blood in EDTA tubes about 10ml
- Mix well in the tube to avoid clotting lab.
- Determine Hb, Rbc count, WBC count and PCV.
- Make a smear.

Isolation of Bovine peripheral blood lymphocytes

Procedure

1. Collect venus blood (jugular vein) using a 10ml EDTA bottle
2. In the laboratory spin the blood at 4°C centrifuge at 2500 rpm (800-1000 x g) for 20 minutes to collect the buffy coat, carrying the lymphocyte cells.
3. Remove most of the supernatant plasma with a 10ml pipette and discard. Using a pasteur pipette remove the remaining plasma within 1-2mm of the buffy coat, and the buffy coat layer (Grey layer above RBC).
4. Re-suspend the cells in Buffy coat in an equal volume of PBS and layer onto 3ml lymphoprep for washing and separating. (max 6ml of cell suspension to 3ml lymphoprep)
5. Spin at (800- 1000 x g) 2500 rpm for 20 minutes.

6. Recover the mononuclear cells at and below the interface using a 5ml pipette.
7. Re-suspend the cells into 9ml PBS and spin at (180 x g) 1000 rpm to 10 min x 2
8. Suspend the pellet (cells) in 5mls of MEM (working media). Transfer into monolayer into a 25 cm^2 flask and incubate at 37°C. (for straight cultures, gas using 5% carbon dioxide).
9. Make a (smear) cytospin.
10. Stain and observe for schizont in the lymphocyte cells.

Lymph node biopsy culture Preparation

Procedure

1. Place 5-10ml heparinized media into a universal bottle.
2. Shave and surface sterilize the skin over the lymph node.
3. Draw 5ml heparinized medium into 10ml syringe via a 4 cm 14 gauge biopsy needle
4. Immobilize the lymph node with one hand.
5. Biopsy the lymph node, squeeze and withdraw the needle and put the biopsy into the universal bottle.

In the lab

1. Pipette the tissue material to break up the clumps
2. Centrifuge at 1000rpm for 5 min (180xg)
3. Collect supernatant and spin at 1000rpm for 5 min (use 9ml of PBS)
4. Collect the cells and suspend in a little growth medium and gas using 5% carbon dioxide.
5. Make smears using cytospin, stain observe for the presence of schizont.

To passage (subculture) bovine embryo spleen cells (BESP).

1. Check the BESP for viability and for contamination i.e bacteria, fungi,etc, both visually and microscopically.
2. Pour off the media and add 2ml ATV (antibiotic trypsin and versene). Shake and pour off the ATV.
3. Using a pipette add 4ml of fresh ATV into the flask, incubate at 37°C for 10 min or until the monolayer detaches.
4. Pipette the detached monolayer cell suspension and dispense it into a centrifuge tube containing 10mls of culture medium. Centrifuge the suspension at 180xg for 10 minutes.

5. Pipette off the supernatant and re-suspend the pipette in growth media. Distribute the suspension into 25cm^2 culture flasks in 5ml aliquots.

6. Incubate in 37°C after gassing with 5% carbon dioxide.

Maintenance of Established cell lines

Static cultures

1. Cultures are routinely incubated at 37°c in 25 cm plastic disposable flasks (Nunc) held horizontally and containing 5-10 ml cell suspension in growth medium.

2. Though sealed, if using a medium with high bicarbonate levels, it is advisable to gas the culture with 5% carbon dioxide in air when sub-culturing. (This is unnecessary when flasks are kept, with tops loose, in the gassed incubator).

3. Healthy culture will grow rapidly healthy, increasing Log10 ever 48-72 hours. They thus require subculture every 2-3 days if in complete growth medium. The seeding rate at subculture is usually 1-2 x 15/ml. This is usually simply affected by seeding at 1:10 i.e. 1ml into 9ml fresh medium or 0.5ml to 4.5ml. This "subculture" need not necessarily be to a new flask provided the cells are fully in suspension. Where adherent they can usually be removed by

vigorous pipetting or the use of a silicone rubber policeman on a glass rod, re-suspended and seeded as for cells in suspension.

Assessment of cultures.

Cell growth and behaviour of *Theileria*-infected cells need to be assessed routinely to ensure that cultures are behaving normally. Cultures may be monitored intermittently, at each subculture, or, as indicated for isolates or more subcultures are indicated :-

a) Determination of cell number and viability. Cell concentration in a culture can be assessed by doing direct hemocytometer counts or using an electronic particle counter (Coulter Electronics).

Haemocytometer counts.

1. Prepare the sample of culture by (a) gently pipetting to mix the clumps of a *T.parva* culture, or (b) vigorously pipetting the surface of the flask to remove adherent cells in a *T.annulata* culture. Take an approximate 1ml sample into a bijou, which hereafter for counts and cytospins need not be handled aseptically.

2. Prepare a moist chamber (with a piece of damp filter paper) to contain the hemocytometer slides until they are counted.

3. "Fix" the coverslip to the hemocytometer by moistening the edges of both the coverslip and the hemocytometer by moistening the edges of both the coverslip and the hemocytometer which should be clean and grease free. Placing the hemocytometer on the flat bench top, the coverslip in position, press firmly but carefully with thumbs on the sides of the coverslip. If properly fixed a rainbow effect (Newton's rings) should be visible. Keep the slide in the moist chamber now.

4. Using a Pasteur pipette, mix the culture sample (vigorously to break up clumps especially in *T.parva* or *T.annulata* (Hissar cultures) and take a small amount, e.g. 5mm, in the tip of the Pasteur pipette, lightly touch the tip against the edge of the coverslip and the slide, and allow fluid to fill the square. It is important that the fluid should not overflow into the channels.

5. For the viable cell count, mix equal volumes of dye and culture sample and place into the other side of the hemocytometer slide.

6. Counting the cells - Visualizing the hemocytometer grid as a noughts and crosses grid, count all the cells in the four corner squares of the undiluted sample (which are subdivided lines of each square but disregard cells on the right and top lines. Clumps of cells

(from a poorly pipetted sample) should be counted as single cells. The dimensions of the 4 large corner squares are 1mm x 1mm x 0.1mm depth, giving a volume of 0-1mm (3) = 0.0001 cm (3) or ml. Each large square conveniently fits in the x 10 objective field under the microscope.

7. In the dyed sample grid, again count cells in all 4 large squares to find the percentage viable. Viable cells do not take up the dye. Making two separate counts for total cells and for percentage viability is preferable to simply doubling the viable count numbers. The latter introduces an extra error in dilution, also, the greater the cell density, the more accurate the count.

Cell morphology and characteristics

- Giemsa stained cytospin smears.

The character of cells and schizont within a culture is best assessed on Giemsa -stained smears of thin air-dried preparations. Such preparations can be made by spinning 50m aliquots of culture suspension onto clean glass microscope slides at 600-1000rpm for 5 minutes in a cytocentrifuge (Cytospin 2, Shandon). Note that for optimal preparations it may be desirable to pre-wet the slides by

doing a preliminary cytocentrifugation of tissue culture medium onto the slide. Cytocentrifuge smears made in this way are air-dried, fixed methanol and stained for 40 minutes with 5% Giemsa buffered at pH 7.2. For optimal visualization of schizont nuclei the smears can be hydrolysed after fixation but before staining by immersing in HCl for 6 minutes then washing in buffer solution.

Observations should include counts to give :

Percentage infected cells (PIC)

Mean schizont nuclear number (MSN)

Multinucleate cells (MNC)

Mitotic index (MI)

Resuscitation of cultures cryopreserved in DMSO

1. Prepare a 10ml centrifuge tube with 8ml of warm (37°C) complete medium
2. Thaw a vial rapidly in a 37°C water bath. Immediately add contents of vial into the tube.
3. Centrifuge at 1000rpm for 5 minutes.(180xg)
4. Re-suspend in 5ml medium

5. Add cell suspension to 25 ml flask and gas well with 5% carbon dioxide.

6. Depending on cell concentration add 5ml the same day or following day.

   24 hours later spin down cells gently (180xg) 1000rpm for 5 minutes. If the culture looks healthy, viable and high density treat it like any other normal culture.

8. Ensure that records in cryopreservation files are altered accordingly.

T.parva schizont antigen is prepared from a *T.parva* infected lymphoblastoid cells.

Preparation of antigen spot slides for Mabs profiles.

1. Centrifuge culture suspension at 1500rpm (200-400xg) for 10 minutes at +4°c (clean up on lymphoprep).

2. Wash the cells (pellets) twice in cold PBS and spin down at(180xg) 1000rpm for 5 minutes at 4°C.

3. Re-suspend the cells in cold PBS at 1:50th of the original volume 50ml : 1ml, 100ml : 2ml etc.

4. Determine cell viability. (the viability of cells is determined by trypan blue exclusion).

5. Adjust cell concentration to 1 x 10^7 cells/ml in PBS

6. Fix the cells with equal volume of 3.7% FA (formaldehyde 40% v/w) in PBS PH 7.2. in ice bath for 10 minutes and add cold PBS then spin down at 1000rpm for 5 minutes at 4°C.

7. Discard the supernate and wash x 3 with cold PBS then spin at 1000rpm for 5 minutes.

8. Re-suspend the cells in cold PBS at 1:2 of the step 5 volume and 'count the cells.

9. Adjust to 1 x 10^7 cells/ml PBS 3 x 10^7 cell suspension stirred.

10. Distribute auto multi spot slides.

11. Wrap the slides with aluminum foil and store at -20°C

ECF Cytospin smear using cytospin shandon machine.

1. Add 0.1ml or a drop of culture suspension into the cytocentrifuge bucket.

2. Spin at 1000rpm for 5 minutes (cyto-centrifuge)

3. Air dry and fix in methanol for 2-3 minutes

4. Stain in 10% (Giemsa Buffer) for 20 minutes

5. Rinse in tap water.

6. Examine for schizont.

APPENDIX 3:

Sodium Dodecyl Sulphate - polyacrylamide gel electrophoresis

A. Solutions required

Acrylamide : Bisacrylamide 30% : 0.8% make up to 333 ml

100g Acrylamide

2.66 g Bisacrylamidey at 4 °C in a dark bottle

B. Tris - HCL PH 8.8. 1.65 M 100l 500ml at 4°C

C. 'Tris - HCL PH 6.8 1.65 M 20g/100ml

D. EDTA 0.2 M PH 8.0. at 4°C 5.84g/100ml

E. Ammonium Persulphate 1g/10mls d.d H_2O 10% make 10ml and divided into 1.0ml and divided into 1.0ml aliquots and store at -20°C.

F. TEMED (N,N,N,N Tetramethethlene diamine). (BDH UK)

2. Sample buffer

Tris - HCL PH 6.8	1.0 ml
Glycerol	2.0 ml
SDS 10%	3.0 ml
2 - Mercaptoethanol	300.0 ml
Bromophenol blue	1.0 ml
d.d. H_2O	3.0 ml

3. Electrode buffer (10xTG)

Tris	30 gm/L
Glycine	144 gm/L
SDS	10 gm/L

4. Blotting buffer

Tris	12.11gm
Glycine	57.80gm
Methanol	1 L

Mix and dissolve in 5 litre of D.D H_2O PH 8.3

5. Blocking buffer

Fat free milk	10gm/100ml 1 x PBS

6. Washing buffer

10x PBS	100ml
10% Milk	10ml
Tween (20)	0.5ml
Make up	1L

7. 10 x PBS

$NaH_2 PO_4 HO_2$	3.6 gm
$Na_2 HPO_4$	24.74gm
NaCl	146.0gm

Make up to 1L

8. Antibody dilution buffer and substrate buffer

Milk 1%

PBS

Make up to required volume.

9. Substrate.

3-3 Diamino Benzidine Tetrahydrochloride (DAB) stored at -20

DAB - 50 mg.

Substrate buffer 50 ml

10. Hydrogen Peroxide 1/300 use 20ml

Gel solutions was made as follows

	Separating gel		Stacking gel
	7.5%	10%	5%
Acrylamide	17.5gm	23.3gm	5.0
Tris- HCL PH 8.8.	14.0g	14.0gm	3.8 PH 6.8
EDTA	0.70g	0.70g m	0.3
20% SDS	0.7gm	0.7gm	0.3
H_2O	37.1gm	31.3gm	20.6

Degas for 15 minutes using water pump

Ammonium persulphate	35ml	16 ml
TEMED	0.7gm	0.3gm

The glass plates were cleaned with alcohol and then assembled while placed o spirit level. The separating gel was then poured (31ml) and toped with wate polymerization was allowed to take place for 1 hour. The toping water was poured and stacking gel added. The comb size was placed and polymerization allowed to oc for 20 minutes. The comb was removed and 1 x electrode buffer added.

Sample preparation 0.5×10^7 cells was added to 1ml sample buffer then boiled for minutes in a pierced microfuge tube. It was cooled and loaded on to the gel.