IMMUNOHISTOCHEMICAL CHARACTERIZATION AND QUANTIFICATION OF LYMPHOCYTES INFILTRATING LUNGS DURING EAST COAST FEVER.

VALLERY MSAFIRI JOHN KESSY

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ABSTRACT

A study was conducted to investigate the hypothesis that the lungs in East Coast fever are infiltrated by different subsets of lymphocytes that may be responsible in the pathogenesis of pulmonary edema. Four steers 7 – 10 months old were experimentally infected by subcutaneous injection with 0.5 ml of live *Theileria parva* sporozoites (Muguga stabilate 3087). Two others remained as uninfected controls. All six animals were clinically monitored daily before and after infection by screening peripheral blood smears, lymph node smears, rectal temperatures, lymph node enlargement, coughing and dyspnea. Also blood samples were taken at intervals of 3-4 days and analysed for total RBC and WBC counts, Hb, PCV and differential leukocyte count. Each of the infected animal was humanely exsanguinated to death under general anaesthesia at the onset of dyspnea, together with one of the controls for sampling of lung tissues. In addition, two lung tissue samples were collected from two ECF naturally infected cattle that were slaughtered during the advanced stage of the disease. The lung tissue samples from the experimentally and naturally infected animals were fixed in 4% neutral-buffered formaldehyde (pH 7.4) for 48 hours and subjected to routine tissue processing procedures to obtain 4 μm thin sections that were stained routinely by H & E for histopathological examination and immunohistochemically by monoclonal antibodies labelled by streptavidin-biotin peroxidase complex to visualize lymphocyte subsets $BoCD21^+$, $BoCD4^+$, $BoCD8^+$ and $BoWC1^+$. Rectal temperatures

started to increase above normal by day 8 post infection while ECF was confirmed by lymph node smear examination by day 7 and dyspnea by day 15 post infection. It was demonstrated that the majority of lymphocyte subsets infiltrating the lungs of cattle infected with *Theileria parva* are BoCD4$^+$ and BoCD8$^+$ in the acute and advanced stages of ECF, respectively. The BoCD8$^+$ T cells were about eleven times more than the rest of the other subsets in the naturally infected animals slaughtered in the advanced stages of ECF. The apparent higher mean number of both BoCD21$^+$ and BoWC1$^+$ during midway of the course of infection suggests that these may have more roles to play in this stage than in the advanced stages of *Theileria parva* infection. Notwithstanding the mechanisms that attract these lymphocytes into the lungs, their presence indicates that they likely bring about local release of cytokines that contribute towards development of inflammation and pulmonary edema. It was concluded that any endeavour to develop ways to treat *Theileria parva* infection should study the specific cytokines released during ECF.

DECLARATION

I, Vallery Msafiri John Kessy do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and has not been submitted for a degree award in any other University.

Signature .....

Date 18/11/2002

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DEDICATION

This work is dedicated to my beloved wife Loleni and our children Clement, Clementine and Christine.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	Percentage
γ/δ	Gamma-delta
^{0} C	Degree Celsius
ALT	Alanine aminotransferase
AP–1	Activator Protein–1
APC	Antigen presenting cells
AST	Aspartate aminotransferase
BCR	B cell antigen receptor complex molecule
BoCD	Bovine cluster of differentiation molecule
BoT4	Bovine T_H differentiation molecule
BoT8	Bovine T_C differentiation molecule
BoWC	Bovine Workshop Cluster antigen molecule
BSA	Bovine serum albumen
C3a	Complement component 3a
C5a	Complement component 5a
CD	Cluster of Differentiation molecule
CK	Casein Kinase
CMI	Cell mediated immunity
DC	Dendritic cells
DNA	Deoxyribose Nucleic Acid
ECF	East Coast fever
EDET	Early Diagnosis and Early Treatment
EDTA	Ethylene diamine tetra acetate
ELISA	Enzyme linked immunosorbent assay
Fc	Crystallisable fragment
FDP	Fibrinogen degradation products
H_1	Histamine receptor type-1

Hb	Hemoglobin
IFAT	Indirect fluorescent antibody test
IFN-γ	Gamma interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
ILRI	International Livestock Research Institute
ITM	Infection and Treatment Method
JAK-STAT	Janus Kinases – Signal Transducers and Activators
JKN	Jun NH2 terminal Kinase
KBB	Koch's blue bodies
mAb(s)	Monoclonal antibody(s)
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MHC	Major Histocompatibilty Complex
MNC	Mononuclear cells
NBF	Neutral buffered formaldehyde
NF-κ/β	Nuclear Factor-kappa beta
NK	Natural Killer cells
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
pi	Post infection
RBC	Red blood cell count
SABC	StreptAvidinBiotin Complex
TARP II	Tanzania Agricultural Research Project Phase II
TBS	Tris-buffered saline
TBS-AR	Tris-buffered saline – antigen retrieval
TBS-T	Tris-buffered saline – containing 0.25% Triton X-100

T_C	Cytotoxic T cell
TCR	T cell receptor complex
TGF-β	Transforming Growth Factor-beta
T_H	Helper T cell
TNF-α	Tumor Necrosis Factor-alpha
TNF-β	Tumor Necrosis Factor-beta
WBC	White blood cells count

CHAPTER ONE

1.0 INTRODUCTION

East Coast fever (ECF) is a tick-borne lymphoproliferative disease caused by *Theileria parva* that occasions high rates of morbidity and mortality in susceptible cattle.

It is a constraint in livestock keeping and production in eastern, central and southern Africa where it causes significant morbidity and mortalities (Norval *et al.*, 1992). In addition, ECF is one of the most important factors in the reduction of growth rate and productivity of infected animals (Moll *et al.,* 1986). The decreased production and productivity plus heavy costs incurred in implementing tick control in these countries cause losses estimated to be at least $168 million each year (Mukhebi *et al.*, 1992). This substantial loss entrenches poverty among the livestock keepers and livestock producers.

When inoculated into an animal by the tick, the sporozoite stage of *T. parva* rapidly enters and establishes infection within lymphocytes in circulation which later reach the regional lymph nodes. Differentiation of the sporozoite to a schizont form is associated with induction of proliferation of the host cell. The schizont resides free in the host cell cytoplasm and constitutes the pathogenic stage of ECF. The host cell is transformed clonally into a proliferating lymphoblast.

The dividing parasitized lymphoblasts enter the recirculating lymphocyte pool and spread throughout the lymphoid system and nonlymphoid tissues thus causing generalized parasitosis (Lawrence *et al.,* 1994).

The invasion of lymphoid and nonlymphoid tissues by infected cells results in organ dysfunction and severe immunopathological changes characterized by lymphoproliferation that is followed by lymphodestruction and destruction of tissues (Irvin and Morrison, 1987).

Understanding of pathogenetic mechanisms of tissue injury that accompanies ECF is crucial in the elucidation of the role of rapidly dividing lymphocyte infiltrations in causing death within 3 - 4 weeks after infection.

The immediate cause of death in ECF is considered to be pulmonary oedema (Soulsby, 1982; Jubb *et al.,* 1992). The pulmonary oedema has been attributed to a number of factors including the action of vasoactive molecules liberated from disintegrating lymphocytes, complement fragments C3a and C5a and fibrin degradation products (Shitakha *et al.,* 1983; Lawrence *et al.,* 1994).

Our understanding of immunophenotypes of T- lymphocytes and B-lymphocytes infiltrating nonlymphoid tissues in bovine infected with ECF and vasoactive molecules released is limited. Therefore, elucidation of types of lymphocytes

infiltrating nonlymphoid tissues particularly the lungs during *Theileria parva* infection is considered to be a useful clue to the understanding of the local pathogenetic mechanisms of tissue injury in this organ.

Due to technical difficulties related to detecting and assaying released vasoactive molecules *in situ*, the study seeks to approach the matter by identifying and characterising the lymphocytes infiltrating the tissues and there from deduce the vasoactive molecules most likely released in the course of ECF.

This study envisages using immunohistochemical technique to visualise, characterize and quantify lymphocytes infiltrating lung tissues and on the basis of existing biological knowledge deduce their role in the development of pulmonary oedema.

The knowledge acquired will enhance our resourcefulness in the development and optimisation of intervention regimes for the management of ECF.

1.1 Main objective

The main objective of the study is to attempt to unfold the pathological mechanisms behind the development of pulmonary oedema, with particular interest on the role of lymphocytes infiltrating the lungs during ECF.

1.2 Specific objectives

- To characterize the immunophenotypes of lymphocytes that infiltrate lungs of cattle infected with *Theileria parva* by using immunohistochemical technique.
- To evaluate the relative proportion of $BoCD21^+$, $BoCD4^+$, $BoCD8^+$ and $BoWC1^+$ in lungs of cattle infected by *T. parva*.
- To study the pathomorphological changes in the lungs of cattle with East Coast fever.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Introduction

East Coast fever occurs endemically in 11 countries of Eastern, Central and Southern Africa where it kills over one million cattle each year (Norval *et al.* 1992). Non-indigenous breeds and their crosses introduced to the endemic areas are fully susceptible where the morbidity and mortality rates are around 87% and 95% respectively (Brocklebys, *et al.*, 1961). However, mortality may not exceed 50% in previously exposed herds (Mukhebi *et al.*, 1992).

East Coast fever is maintained by a continuous cycle of transmission between cattle and ticks. Within the endemic areas the prevalence of the disease is determined by the availability of the vector and infected animals that serve as a source of infection to susceptible animals. Ticks may live for 1-2 years but they lose their infection within 11 months (Radostitis *et al.*, 1994). In addition, the parasites ordinarily cannot survive through more than one molt in the tick. (Levine, 1977)

East Coast fever occurs in the field in a number of epidemiological states that depend on the intensity of parasite challenge and existing management practices (McKeever, and Morrison 1998). Usually, epidemic and three endemic states of ECF are recognized.

Epidemic state of ECF occurs when infection is introduced into a disease-free area with a full susceptible cattle population. In this state mortality in the absence of control measures may exceed 90% (Lawrence *et al.*, 1994). Endemic stability state is characterised by high levels of challenge and prevalence rates as well as low incidence of clinical disease together with low case fatality rates. Two types of endemic instability exist. One type of endemic instability is characterised by infrequent challenge, low prevalence of infection, low herd immunity, low incidence rates and high mortality rates. The second type of endemic instability is characterised by intermediate challenge, high incidence rate but variable prevalence and mortality rates (McKeever and Morrison 1998).

2.2 The etiological agent of ECF

Theileria parva, is a tick-borne hemoprotozoan parasite that infects T- and B-lymphocytes of cattle and buffaloes causing ECF (McKeever and Morrison, 1990; Dobbelaere and Heussler, 1999).

These parasites had existed in the African buffalo (*Syncerus caffer*) in eastern Africa for thousands of years (Uilenberg, 1981; Young, 1981) and the disease they cause was well known to the traditional cattle keepers. The earliest record of the disease dates back to the missionary chronicles of Father Monclaro from 1569 (Theal 1909; cited by Henning, 1932).

Robert Koch was the first scientist to detect and describe *Theileria* piroplasms in erythrocytes of cattle in 1898 in Dar es Salaam, Tanzania. Later, he recognised the presence of schizonts in lymphoid cells and subsequently described them in more detail (Koch, 1905, 1906 cited by Norval *et al.*, 1992).

Theileria parva is transmitted by the three-host tick *Rhipicephalus appendiculatus*. There are other *Rhipicephalus* species of lesser significance that may be involved in the transmission of the disease such as *R. zambeziensis* found in drier areas of southern Africa and *R. duttoni* found in Angola. East Coast fever cannot be maintained in the absence of these field vectors that are responsible for propagation of the parasite to non-infected animals.

Publications regarding the causative agent of ECF have caused considerable disparity of opinion among scientists, regarding their pathogenicity because different strains of *T. parva* in cattle are presented with different clinical and parasitological manifestations (Perry and Young, 1993). Strain differences of *Theileria parva* have been identified on the basis of differences in their pathogenicity (Barnett and Brocklesby, 1961), cross immunity and antigenic characteristics of the schizont (Radley *et al.* 1975a; Irvin, 1987; Anon 1989a), and by molecular biological techniques (Allsopp *et al.*, 1993; Bishop *et al.*, 1995; D'Oliveira *et al.*, 1995). This was the basis for the division of *T. parva* into three separate species: *T. parva, T. lawrencei* and *T. bovis* (Barnett, 1968; Norval *et al.*,

1992). Later, Uilenberg (1976) and Lawrence (1979) proposed a trinomial system for classifying the three forms of *T. parva* into subspecies namely: *Theileria parva parva* was designated for parasites causing classical ECF, *Theileria parva lawrencei* for parasites causing Corridor disease and *Theileria parva bovis* for parasites causing January disease in Zimbabwe (Uilenberg, 1981).

However, the three subspecies have been confirmed to be a single species through studies on cross immunity (Irvin *et al.,* 1989) carrier state (Young and Leitch, 1981, Young *et al.,* 1986; Maritim *et al.,* 1989), monoclonal antibodies profile, protein analysis, DNA and RNA nucleotide sequences (Conrad *et al.,* 1987; Conrad *et al.,* 1989; Allssop *et al.,* 1989; Norval *et al.,* 1992; Allsopp *et al.,* 1993).

Therefore at present a single species of *T. parva* is recognized, causing either the lawrencei type of infections (buffalo-derived *T. parva* infections) of low schizont and piroplasm numbers, accompanied by hemorrhagic anaemia or the parva type (cattle-derived *T. parva* infection) with high schizont numbers and high parasitemia, and intermediate syndromes such as the January disease with few schizonts, and no or very few piroplasms (Dolan, 1999).

2.1.1 Life cycle of *Theileria parva*

The *Theileria parva* parasite has a characteristic multistage life cycle. It assumes its sporozoite form in the salivary glands of the tick and enters the cattle when the tick

is feeding on blood (Fawcett *et al.* 1982). The sprorozoites rapidly enter lymphocytes and develop within them, becoming multinucleated schizonts after a period of about three days (Stagg *et al.*, 1981).

Although the schizont and the host cell divide synchronously, synthesis of schizont DNA occurs when the host cell enters mitosis and is immediately followed by division when the host cell is in metaphase. Thus there is a clonal expansion of the infected cells (Malmquist *et al.,* 1970; Brown, 1979) and, therefore, expansion of the parasitized cell population can occur without infection of other lymphocytes. Continuous lymphocyte proliferation depends on the presence of *T. parva* schizont in the host cell cytoplasm and the infected cells cease to require antigenic stimulation and exogenous growth factors to proliferate (Ahmed and Mehlhorn, 1999; Dobbelaere *et al.*, 1999; Dobbelaere *et al.*, 2000).

The intracytoplasmic schizont stage of *T. parva* is the only proliferative stage in the parasite life cycle. However, the transformed lymphocytes can be reversed to quiescent lymphocytes by treatment with anti-theileria agents, indicating therefore, that the alterations to the host cell do not involve permanent changes to the host cell genome such as mutations or chromosomal translocations (Pinder, *et al.*, 1981; Ole-Moiyoi, 1989). When the parasite is eliminated by treatment with the specific theilericidal drug BW720c, cells revert to a resting phenotype over a period of 3 – 4

days (Hudson *et al.* 1985, Dobbelaere *et al.* 1988) and eventually undergo apoptosis (Fich *et al.* 1998).

This quality of reversibility is a unique aspect of *T. parva* to transform lymphocytes (Dobbelaere *et al.* 1988, Hudson *et al.* 1985) and also is the only eukaryote known to transform another eukaryote (Heussler *et al.* 1999).

In a proportion of infected cells, merogony of the schizonts occurs resulting in disruption of the lymphocyte thereby releasing merozoites into blood that invade erythrocytes. The merozoites differentiate to intra-erythrocytic forms called piroplasms, which may subsequently undergo limited division by merogony in the erythrocytes (Conrad, *et al.,* 1986) but reinitiate the infective cycle upon being ingested by ticks (Schein, *et al.,* 1978). Piroplasms first appear on day 12 after infection and increase rapidly in number until death (Jubb *et al.* 1992).

Piroplasm-infected erythrocytes are ingested by the larval or nymphal stages of ticks and undergo a sexual cycle in the gut of the replete tick to produce zygotes, which in turn develop into motile kinete stages that infect the salivary glands of the next instar, the nymph or adult respectively (Mehlhorn and Schein, 1984; Fawcet, *et al.,* 1985).

All parasite stages are haploid except for a short diploid zygote stage in the tick intestinal cells (Gauer *et al.* 1995).

2.2 Pathology of ECF

2.2.1 Pathogenesis of ECF

The course of ECF has been well characterised in experimentally infected animals. Dendritic cells (DC) in the skin (Shaw *et al.,* 1993) and both T- and B-lymphocytes in circulation (Baldwin *et al.*, 1988) take up the parasites after inoculation and the parasite within the cells develop into intracytoplasmic multinucleated schizonts that induce proliferation of the host cell.

Dendritic cells are a feature of bovine dermis and are present in large numbers at the site of tick attachment in cattle (Matsubara *et al.*, 1995). However, *in vitro* studies have shown that *Theileria parva* sporozoites invade bovine DC and differentiate into schizonts and that the sporozoites within DC become associated with a comprehensive microtubular network near the centrioles but do not transform and there is no evidence of the occurrence of merogony (Wells *et al.*, 1999).

Entry of Theileria sporozoites into the cytosol of target cell is completed within a short period (seconds to few minutes) at 37°C (Fawcet *et al.,* 1982; Jura *et al.,* 1982; Shaw *et al.,* 1991). Shaw (1999) and Shaw *et al.* (1991) established that, the entry of sporozoites involved some cytochalasin-inhibitable rearrangement of the host cell membrane or cytoskeleton.

Inside the lymphocytes, the sporozoite makes a beeline for the microtubules that form the centrioles and sets them to divide uncontrollably (Brown *et al.* 1973, Dobbelaere *et al.* 1988). Association of the schizont with the host nuclear spindle ensures that daughter host cells remain infected during cytokinesis (Hulliger *et al.,* 1964; Vickerman and Irvin, 1981; Carrington *et al.,* 1995).

Thus, the parasite ensures its survival by taking up residence inside those cells that have evolved to destroy invading organisms while inducing uncontrollable proliferation of the host cell. Therefore the parasite becomes inaccessible to serum humoral factors during much of the infection period.

As the parasite grows into the schizont, the host cell becomes transformed into a lymphoblast. At present, the mechanism of transformation process is not clearly understood (Ahmed *et al.,* 1999; Shayan *et al.,* 1999). Ole-Moiyoi (1989) suggested that reversible lymphocyte transformation process could be induced by *T. parva* through constitutive production of Casein kinase II (CK II) or other molecule with G–protein like activity that may precede CK II in a mitogenic enzyme cascade. Dobbelaere and Heussler (1999); Galley *et al.,* (1997) lately have observed that infected cells show increased activity of CK II and Jun NH2 terminal kinase (JKN) while the extracellular signal – related pathways are not activated. More importantly, the presence of the parasite in the host cell cytoplasm is known to

activate several transcription factors such as Nuclear Factor-kappa beta (NF-κ/β) and AP–1. It has also been shown that the parasite induced NF-κ/β activation plays a crucial role in the transformation and survival of *T. parva* transformed T cells by conveying protection against an apoptotic signal that is required to maintain the transformed phenotype that underlies the pathogenesis of ECF. (Heussler *et al.,* 1999; Dobbelaere *et al.*, 1999).

It has also been reported that continuous proliferation of infected lymphocytes requires surface stimulation through cell-to-cell contact (Dobbelaere *et al.,* 1991). However, not all infected cell types become transformed and the transformed cells may not necessarily grow more rapidly than other cells (Goddeeris *et al.,* 1986; Baldwin *et al.,* 1988).

The transformed cell divide rapidly at regular intervals in such way that it often gives up to 10 fold increase in the number of infected cells in about every three days (Jarrett *et al.,* 1969; Radley *et al.,* 1974). Proliferation of infected lymphoblasts is first observed in the lymph node that drains the site of inoculation. Then the rapidly dividing parasitized lymphoblasts enter the recirculating lymphocyte pool and spread throughout the lymphoid system causing generalized parasitosis.

Parasitized lymphoid cells can be detected in the lymphoid tissue throughout the body usually within 14 days of infection (Urquhart *et al.,* 1996). In addition to the spread throughout the lymphoid system, the hyperplastic parasitized and non-parasitized lymphoblasts establish in many parenchymatous organs, (notably the liver, kidneys, lungs, myocardium and adrenals), bone marrow, gastrointestinal tract mucosa and sometimes the brain (Lawrence, *et al.,* 1994). The generalized parasitosis is associated with initial lymphocyte proliferation in lymphoid organs and later ensuing lymphocytolysis and cellular depletion that may lead to rapid death of the host (Theiler, 1904; Morrison, *et al.*, 1981). Also, there is evidence of necrosis and depletion of lymphocytes in lymphoid foci in parenchymatous organs (Lawrence, *et al.,* 1994). Both parasitized and non-parasitized lymphocytes are destroyed as a result of activation of specific protective cytotoxic T-lymphocytes and non-specific cytotoxic T-lymphocytes ('natural killer' cells) (Emery *et al.,* 1981). The rapid proliferation of infected lymphoid cells and the accompanying destruction of the invaded tissues are believed to produce the main pathogenic effect of the disease (Jarrett *et al.,* 1969; Radley *et al.,* 1974; Maxie *et al.,* 1982; Lawrence *et al.,* 1994).

Conceivably, of more relevance to the cause of death is the infiltration of other tissues, particularly the lungs and gastro-intestinal tract, with parasitized lymphoblasts, and severe pulmonary edema and associated dyspnea that are commonly observed late in the course of the infection (Irvin and Morrison, 1987).

2.2.2 Pathogenesis of pulmonary edema

There are inadequate publications that explain in detail the pathogenesis of pulmonary edema observed during ECF notwithstanding its clinical importance in the disease.

However, the few publocations available (Shitakha *et al.* 1983; Lawrence *et al.* 1994) indicates that pulmonary oedema occurs due to local increase of vascular permeability mediated by vasoactive molecules released by infiltrating and disintegrating lymphocytes in the lungs. The vasoactive molecules activate the complement cascade and subsequent formation of anaphylatoxins (C3a and C5a) in the lungs responsible for the development of pulmonary oedema. Both C3a and C5a cause increased vascular permeability and smooth muscle contraction (Cotran *et al.,* 1994). Although the mechanism involved in increased vascular permeability is multifactorial, it is generally accepted that anaphylatoxic effect is largely through degranulation of mast cells and formation of leukocyte intermediaries (Slauson and Cooper, 1990). Mast cells are within the lamina propria of the trachea, bronchi and bronchioles and participate in normal baseline immune and inflammatory processes (Jubb *et al.*, 1992). The consequence mast cell degranulation is the release of histamine that in turn mediates vascular permeability or causes the release of prostaglandin and leukotrienes from leukocyte following activation of arachdonic acid (Fantone and Wark, 1990; Slauson and Cooper, 1990).

A study by Gwamaka (2001) showed that use of antihistaminic promethazine, a drug that specifically binds to H_1-receptor in tissue cells significantly suppressed the development of pulmonary edema in ECF. This result confirms other findings that histamine has a role in the pathogenesis of pulmonary edema during ECF.

It is known that living parasites, parasite antigens or parasitized cells can activate the complement system (Kassis and Tanner, 1976; Musoke and Barbet, 1977; Hammerberg *et al.,* 1977). Furthermore, Slauson and Cooper (1990) pointed out that a variety of enzymes from sources not intrinsic to the classic complement pathways such as plasmin, kallikrein and lysosomal enzymes of leukocytes can activate some of the complement through the alternative pathway. Also the complement system is believed to be activated in any situation associated with leukocyte accumulation since lysosomal enzymes can be released from leukocyte during the process of phagocytosis or following their disintegration. It is possible that the same happens in ECF, as the disease is characterised by leukocyte infiltration and destruction in tissues (Jubb *et al.,* 1992; Norval *et al.,* 1992; Mbassa *et al.,* 1995). The activation of complement via either the classical or alternate pathway in ECF is assumed to trigger the formation of anaphylatoxins C3a and C5a (Shitakha *et al.,* 1983).

The attribution of pulmonary edema to increased vascular permeability was shown by Matovelo *et al.*, (2000) in an experimental study with ECF that traced the

diffusion of carbon particles sized between 0.55 μm and 1.37 μm from circulation into the alveolar and extracellular spaces. In this study it was demonstrated that increased vascular permeability is the mechanism by which vascular fluid and proteins escape into the interalveolar and alveolar spaces.

2.2.3 Immunopathology of ECF

Since ECF is a disease of the lymphoid system, the outcome of challenge with the parasite is reflected by events in the lymph node that drains the site of tick attachment. It is here that the initial amplification of the infection occurs and, in immune cattle, it is in the lymph node where the parasite is eliminated. It has been shown that killing of the parasite occurs in the lymph node of immune cattle from day seven after challenge, about a day before the parasite is detected in the blood (McKeever *et al.* 1994).

Cattle that recover from the disease develop a strong parasite-specific MHC-class I-restricted cytotoxic T-lymphocyte response (McKeever, 2001). The response is remarkably powerful and effective in elimination of homologous challenge in a short period of time.

The response coincides with the clearance of parasitized cells from lymphoid tissue (Eugui and Emery, 1981; Morrison *et al.*, 1987; McKeever, 2001). The parasite-specific MHC-class I-restricted cytotoxic T-lymphocytes are within the BoCD8$^+$

subpopulation of T cells. However, there is evidence that this response is also dependent on the input of activated BoCD4$^+$ T cells (Taracha, 1998; McKeever, 2001) and in immune cattle some parasite-specific BoCD4$^+$ T cells have been shown to be parasite strain-specific (Baldwin *et al.* 1987; Brown *et al.*, 1989; Morrison *et al.*, 1987; Morrison and Goddeeris, 1990). Although the response is parasite strain-specific, also there is evidence that the parasite can evade the response through antigenic diversity, which is maintained through sexual recombination in the tick vector (McKeever, 2001).

T. parva infects and transform bovine B cells and α/β and γ/δ T cells and transforms them into continually proliferating cells (DeMartin and Baldwin, 1991). Not all transformed cells retain surface differentiating antigens characteristic of a subpopulation of proliferating T cells (Naessens *et al.,* 1985; Pinder *et al.,* 1981). However, most or all of the T-cell differentiating antigens acquire a low level of expression of BoT4, BoT8, or the null cell marker recognized by monoclonal antibody IL-A29.

Phenotypic studies of parasitized cells isolated from lymphoid tissues of infected cattle suggest that very few arise from B cells, and while substantial numbers of infected BoCD8+ cells are observed, the majority of parasitized cells appear to be derived from the BoCD4+ subpopulation of T lymphocytes (Emery, *et al.,* 1988).

The induced lymphocyte transformation in ECF is accompanied by the expression of a wide range of different lymphokines and cytokines, some of which may contribute to proliferation or may enhance spread and survival of the parasitized cell in the host (Dobbelaere and Heussler, 1999).

2.2.4 Cytokines in disease

Cytokines are important and specialized messengers of the immune system produced by white blood cells and mostly by T helper cells or macrophages (Kuby, 1997).

In many instances, individual cytokines have multiple biological activities. Different cytokines can also have the same activity, which provides for functional redundancy within the inflammatory and immune systems and is not known precisely how the cytokine networking operates (Goldsby et al., 2002).

The general mechanism of action of cytokines seems to be signal transduction via cytokine receptors. Cytokine receptors are found on more than one cell type and engagement of the receptor may lead to different reactions in different cell types. When a cytokine binds to its receptor, the signal is transduced and the JAK-STAT pathway is activated. The end result of this pathway is to activate the transcription of certain genes, usually genes involved in cell activation or in cell growth and differentiation (Roitt *et al.*, 1998).

Antigen exposure and processing drive the differentiation of naïve T helper cells (Th0 cells) into two classes of cells termed Th1 and Th2, which differ in terms of their patterns of cytokine production. Substances that stimulate production of cytokines include components of microbes, parasites, materials released as the result of tissue damage and cytokines themselves (cytokine cascade). The biological activity of one class regulates the activities of the other class (Kuby, 1997; Roitt *et al.*, 1998). Their ratios and the predominance of certain cytokines can vary, and this may mediate the pathology and outcome of certain microbial infections. More importantly, cytokines usually do not act alone, but act as part of a huge, complicated network as messengers of the immune system.

Th1 cells are able to recognize ("see") foreign antigen on the surface of antigen presenting cells in the context of MHC II. Th1 cells produce key cytokines interleukin-2 (IL-2), interferon-gamma (IFN-γ) and tumor necrosis factor-beta (TNF-β) after being stimulated by IL-12 produced by macrophages, dendritic cells and B cells (Tizard, 1996; Roitt *et al.*, 1998). Only Th1 cells produce IL-2, and its targets include T cells, B cells, NK cells and macrophages. IL-2 activates helper (T_H) and cytotoxic (T_C) T cells, B cells, and NK cells. IL-2 is important for the initiation of both Th1 and Th2 responses. IL-2 is very similar in its actions to Tumor Necrosis Factor (TNF) and TNF acts in synergy with IL-2. IFN-γ activates CD8 T cells and macrophages; increases Th1 response and decreases Th2 responses. TNF-β activates macrophages and neutrophils. All interferons act largely in synergy with

IL-1 and TNF to promote resistance to pathogenic attack. The $CD8^+$ cytotoxic T cells also come in two subsets, Tc1 and Tc2. Tc1 like Th1 secretes IFNγ while Tc2 like Th2 secretes IL-4 (Tizard, 1996; Ibelgauft, 2002).

Th1 cytokines usually correlate with resistance to microbial diseases and are known to induce cell-mediated immunity and are most effective against intracellular organisms and tumor cells, and also promote delayed hypersensitivity responses which is usually evident in chronic cell-mediated immune infections e.g. tuberculosis or persistent bacterial, fungal and protozoal infections (Goldsby *et al.*, 2002).

Th1 response during inflammation and helping Tc cells destroy a pathogen it involves producing one or more cytokines such as IL-1, IL-2, IL-6, IL-12, TNF-α, TNF-β and IFN-γ (Roitt *et al.*, 1998). These cytokines promote even more inflammation and encourage an ever-increasing potent reaction against a pathogen. Systemic effects of the main pro-inflammatory cytokines IL-1, IL-6, and TNF-α include: a) acting as endogenous pyrogens on hypothalamus to induce fever; b) acting on the liver to produce acute-phase proteins that help the body control infection, and c) wasting (cachexia) and shock due to TNF-α. The local effect of tumor necrosis factor is to increase vascular permeability (Tizard, 1996; Ibelgauft, 2002).

Once a threat diminishes and a pathogen has been destroyed the Th1 cells gradually switches to a Th2 type response that involves production of cytokines like IL-4, IL-10 and TGF-β. These cytokines deactivate Tc and Th1 cells and so down regulate an inflammatory response and the T cells to a resting state. Cysteine rich TGF-β is arguably the most important suppressor cytokine identified so far. It inhibits production of inflammatory cytokines like IFN-γ and IL-2. It is mainly produced by the T cells themselves, but can be found in a range of cell types (Kuby, 1997).

Th2 cells also "see" foreign antigen on the surface of antigen presenting cells in the context of MHC II. Th2 secrete a mixture of cytokines, including IL-4, IL-6, IL-10 and IL-13 and generally provide helper activity for B cell immunoglobulin production. They are co-stimulated to produce these cytokines by IL-1 (Tizard, 1996; Roitt *et al.*, 1998). IL-1 is a key mediator in amplifying many immunological actions. There is always a slow release of IL-1 into the body and the key producers are macrophages. IL-1 concentration increases rapidly when tissue is damaged or threatened by pathogens. IL-1 acts directly on target cells and also promotes the production of other cytokines that further stimulate.the immune system (Tizard, 1996; Ibelgauft, 2002).

Th2 cytokines induce humoral immunity and most effective against extracellular organisms (including parasitic worms). They tend to activate B cells to proliferate,

differentiate into plasma cells, increase immunoglobulin production and lead to Ig class switching. They also affect basophils and eosinophils. IL-4 is responsible for B cell growth, class switching to IgE, and increases Th2 responses. IL-6 increases Ig production. IL-10 decreases production of IL-2 and IFN- by Th1 cells, and also decreases production of IL-12 by macrophages. IL-13 decreases macrophage activation (Ibelgauft, 2002).

Usually, Th2 cytokines correlate with susceptibility to microbial diseases, and may also favour the development of allergic inflammation by stimulating the production of IgE (Goldsby *et al.*, 2002).

There is also evidence that late in the response, negative feedback mechanisms come into play to dampen the response in that IL-4 kills the precursors of the dendritic cells-2 (DC2) by apoptosis, thus inhibiting the Th2 path and further production of IL-4 and that IFNγ may eventually turn off the Th1 response that produced it (Hemond, 1999).

2.2.5 Cytokines in East Coast fever

Dobbelaere and Heussler (1999) showed that, presence of the parasite in the host-cell cytoplasm modulates the state of activation of a number of signal transduction pathways. This in turn leads to the activation of transcription factors, including

nuclear factor-κβ, which appears essential for the survival of *Theileria*-transformed T cells.

This is consistent with earlier studies by Preston *et al.,* (1992) which showed that using recombinant (r) bovine (Bo) and human (Hu) cytokines — Bo rTNF-α, Bo rIFN-γ, Hu rIFN-α, Hu rIL-1 and Hu rIL-2 significantly inhibited the *in vivo* development of trophozoite-infected cells of three stocks of *T. parva* (Muguga) and of *T. annulata* but did not inhibit the proliferation of established *T. parva* and of *T. annulata* macroschizont-infected cell lines. However, they suggested that the inhibition of *in vivo* development of trophozoite-infected cells perhaps could help in resistance to challenge infections by preventing further development of the trophozoite-infected cells. Moreover, both TNF-α and IL-2 could play a role in the pathogenesis of Theileria infections by promoting the proliferation of macroschizont-infected cells and the associated hyperplasia.

Shayan *et al.* (1999) reported that *Theileria*-infected cells do not secrete IL-2 and that IL-2 does not play an important role in the autocrine proliferation of the parasitized host cell. This is contrary to what have been shown by others that *T. parva*-infected lymphocytes produce and consume T-cell growth factors (TGF) and interleukin-2 (IL-2) (Brown and Logan, 1986; DeMartin and Baldwin, 1991; Preston *et al.,* 1992; Dobbelaere and Heussler, 1999).

Results of DeMartin and Baldwin (1991) indicated that gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α) and IL-2 did not have profound inhibitory effect *in vivo* on *T. parva* infections. Instead, IFN-γ and IL-2 perhaps facilitated the establishment of infection by *T. parva.* However, *in vitro* studies they had conducted revealed that rIFN-γ has neither enhancement nor inhibitory effects on the growth of the established *T. parva*-infected T cell clones.

Also, the expression of IL-2 has shown to be dependent on the continuous presence of the parasite in the host cell cytoplasm (Dobbelaere, *et al.,* 1988) although not all lymphoblastoid cell lines of T or B cell origin were dependent on IL-2 for their proliferation (Heussler *et al.*, 1992). In studies conducted by Ahmed *et al.,* (1999) on *Theileria annulata* and *Theileria parva*-infected lymphoblastoid cell lines revealed that only *Theileria parva*-infected cells of T cell origin were capable of producing IFN-γ.

In another in vitro study, Campbell et al., (1998) showed that IFN-γ could block *Theileria parva* development in newly infected cells and inhibits the growth of fully differentiated macroschizont stage infected cells. In addition, they found that *Theileria parva* specifically induce IFN-γ production by T cells and also appears to flourish in the face of this T cell derived response in vivo.

It is also known that inflammatory cytokines that includes IL-1, IL-6 and Tumor Necrosis Factor-alpha produced during *Theileria parva* infection can cause widespread metabolic effects including induction of acute-phase response, fever, hormone and corticosteroids synthesis, decreased fat storage, loss of appetite, and loss of weight. In addition, these cytokines are known to directly or indirectly increase vascular permeability that leads to loss of fluid to the tissues (Tizard, 1996).

2.2.6 Clinical pathology of ECF

The acute disease is characterized by massive lympholysis, severe panleukopenia and bone marrow hypoplasia (Jubb *et al,* 1992; Lawrence *et al.*, 1994) with little or no anaemia (Radostitis *et al.*, 1994). It has been found that the severe leukopenia coincided with the onset of the febrile reaction (Steck, 1928 and Wilde, 1966 cited by Lawrence *et al.,* 1994). The panleukopenia is characterised by neutropenia, lymphopenia and eosinopenia (Mbassa *et al.,* 1994; Mbassa *et al.,* 1995).

When anaemia occurs it is usually mild normocytic, normochromic and non-responsive (Maxie *et al.,* 1982). Mbassa *et al.,* (1994) suggested that this type of anaemia is perhaps due to precursor cell destruction..

The erythrocyte and leukocyte counts, haematocrit and haemoglobin concentration may be greatly decreased (Mbassa *et al.,* 1994) however, the red cell indices (MCV, MCH and MCHC) are not affected.

The decline of total plasma protein in ECF has been reported to be the result of reduced feed intake, malabsorption, increased loss of metabolites and protein in diarrhoea during the course of the disease, or increased protein catabolism due to fever (Maxie *et al.,* 1982).

Four to six days after the onset of clinical disease there is increased activity of serum aspartite aminotransferase (AST) but no changes are observed in the activity of serum alanine aminotransferase (ALT) (Munyua and Wamakina, 1979). The increased activity of AST indicates liver damage. Furthermore, Maxie *et al.* (1982) reported a marked increase in serum creatinine and serum lactate dehydrogenase concentration. These findings implied renal dysfunction and liver damage respectively.

2.2.7 Gross pathological changes associated with ECF

Signs of diarrhoea, emaciation, and dehydration may be seen on the carcass that has died from ECF. Lymph nodes including Peyer's patches are progressively enlarged but terminally shrinks back to normal size (Jubb *et al.* 1992). The enlargement is attributed to lymphocyte proliferation. In acute cases, lymph nodes are enlarged, oedematous, hyperaemic or congested and hemorrhagic but often become necrotic and shrunken in advanced chronic disease. The spleen is enlarged in acute disease but becomes shrunken and strap-like in prolonged course of the disease. In some cases, subcutaneous edema may be seen. Connective tissue

shows serous effusions and sometimes may appear gelatinous. Muscles and fat appear normal in most cases but, in acute stages of the infection, fat may become greatly depleted showing serous atrophy. Occasionally muscles show small hemorrhages (Jubb *et al,* 1992).

Serosal surfaces have petechial and ecchymotic hemorrhages. Hemorrhages and ulceration may be seen on mucous membranes throughout the gastrointestinal tract particularly in the abomasum and small intestine, where necrosis of Peyer's patches can be observed. The mucosa of the abomasum is thickened and congested (Lawrence *et al.*, 1994).

Small nodular grayish-white foci referred to as pseudoinfarcts appear in the liver and kidney in longer standing cases. These pseudoinfarcts project slightly above the general surface of the organ (Jubb *et al,* 1992). The liver is pale as a result of mild degeneration.

The most remarkable changes are seen in the lungs. In most cases of ECF, interlobular emphysema and severe pulmonary edema appear. The lungs are reddened and filled with fluid, and the nasal passages, trachea and bronchi are filled with copious frothy exudate (Gray and Robertson, 1902; Lawrence *et al.*, 1994). A moderate hydrothorax and hydropericardium may also be present

whereby the fluid is straw coloured. Also there is increased texture on palpation and increased weight (Jubb *et al,* 1992).

2.2.8 Histopathological changes associated with ECF

During the stage of lymphoid proliferation, lymphoblasts establish in many parenchymatous organs as lymphoid foci especially in the liver, kidneys, lungs, myocardium, adrenals and glandular mucosa of the abomasum (Steck, 1928; Lawrence *et al.,* 1994).

In the lymph nodes there is diffuse lymphoid hyperplasia in the early stages of infection and later on wide spread lympholysis with hemorrhage and fibrinous or hyaline exudate occur throughout the cortical areas (Jubb, *et al.* 1992). Lympholysis of small lymphocytes is prominent in germinal centers while the remaining lymphocytes appear large and blastic. At the time of death there is a marked depletion of lymphocytes in lymphoid tissues (Jarrett *et al,* 1969; Morrison *et al.* 1981)

In the liver there is periacinar and slight centriacinar lymphocytic infiltration and focal lymphocytic infiltration of the hepatic capsule that is seen grossly as grayish-white "infarcts". In addition, periacinar necrosis and irregular canalicular cholestasis with foci of inspissated bile are encountered.

In the spleen there is early lymphoid hypertrophy and late lympholysis where germinal centers remain prominently surrounded by areas of hemorrhage. The hypocellular follicular centers are occupied by fibrinous or hyaline exudate.

The kidneys are congested and have focal hemorrhages. There is interstitial infiltration with lymphocytes, which is prominent around vessels and often around the parietal layer of Bowman's capsule (Munyua *et al.* 1973). These are what are seen grossly as pseudoinfarcts. Some parenchymal necrosis with formation of hyaline casts and brown pigmentation of the remaining epithelium is observed.

Lungs show lymphocytic infiltration of the septa and interstitial tissues characterizing severe interstitial alveolitis (interstitial pneumonia) but mostly around bronchioles (Munyua *et al.* 1973). The commonly observed infiltration of parasitized lymphoblasts particularly in the lungs and gastrointestinal tract together with the associated severe pulmonary edema late in infection has more bearing to the cause of death (Irvin and Morrison, 1987).

The bone marrow is hypocellular, showing asynchrony of granulocytic system with less severely affected erythroid while there is proliferation of large lymphocytes.

In animals that recover, occasional relapses occur and a nervous syndrome called 'turning sickness' is sometimes seen. In this syndrome (cerebral theileriosis),

intravascular and extravascular aggregations of schizont-infected lymphocytes may be detected, accompanied by meningeal haemorrhages, cerebral perivascular haemorrhages, choroiditis, thrombosis and ischemic necrosis (malacia) in the brain (Mwamengele, 1989).

2.3 Clinical signs of ECF

Clinical signs of ECF range from inapparent, moderate to severe and fatal according to the level of challenge and strain of *T. parva* involved (Anon, 1989b).

The incubation period of the disease varies between one and three weeks depending on the virulence of the strain and the infecting dose, (Irvin *et* al., 1987; Radostitis *et al.*, 1994). In closely monitored animals, the first clinical sign to be detected is the enlargement of lymph nodes draining the area of tick attachment or the site of sporozoite inoculation (Sewell and Brocklesby, 1990). Lymph node enlargement is seen 8-16 days after tick attachment (Radostitis *et al.*, 1994). However, KBB first appear in draining lymph nodes five to six days post infection coinciding with the onset of fever (Lawrence, *et al.,* 1994).

Five to six days post-infection, the animal becomes febrile with temperatures rising up to between 41^0 - 42°C. Thereafter, the animal shows non-specific clinical manifestations including depression, anorexia and drop in milk production in lactating animals (Irvin and Mwamachi, 1983).

Sometimes there may be diarrhoea or dysentery and central nervous involvement characterised by circling, head pressing and tremors (Norval *et al.*, 1992; Mbassa *et al.*, 1994; Radostitis *et al.*, 1994). Petechiation of the visible mucous membranes is almost a common finding. In older animals these petechiations tend to be pinpoint and numerous, whereas in calves they tend to be fewer and ecchymotic in nature (Kiptoon *et al.*, 1983).

In terminal stages, respiratory distress as a consequence of pulmonary oedema predominates (Jubb *et al.*, 1992). Also, there may be ocular and frothy nasal discharges, emaciation, weakness, and recumbence. Invariably death occurs within three weeks in severe untreated cases.

2.4 Diagnosis of ECF

2.4.1 Overview

Presumptive diagnosis of *T. parva* infection in live animal is based on enlargement of lymph nodes, fever, a gradually increasing respiratory rate, dyspnea and diarrhea. The fever, depression and lymphadenopathy of ECF can be confused with theileriosis of *Theileria annulata*, trypanosomiasis and malignant catarrhal fever. However, ECF is only found in association with its known tick vectors, *Rhipicephalus appendiculatus, R. zambeziensis* and possibly *R. duttoni* and *R. nitens* (Norval *et al.* 1992). Furthermore, identification of the agent, serological tests and molecular techniques assist in reaching the definitive diagnosis.

2.4.2 Identification of the agent

The schizont stage of *T. parva* in lymphocytes is a characteristic diagnostic feature of the infection with ECF in Giemsa-stained fine-needle aspirates of superficial lymph nodes or lymph node and spleen impression smears. Small piroplasms in erythrocytes are suggestive of ECF, but diagnosis must be confirmed by the detection of schizonts in lymphocytes.

The schizonts in lymphocytes are usually detected in lymph node biopsy four to twelve days following inoculation (Sewell and Brocklesby, 1990). The macroschizonts in the cytoplasm of infected lymphocytes are usually identified in Giemsa-stained smears as blue bodies. Because of the colour imparted by Giemsa stain and the first author, Koch, of *T. parva*, the blue bodies are known as "Koch's blue bodies" (KBB). KBB are detected in more than 60% of lymphocytes during the acute phase of disease (Jubb *et al,* 1992). The schizonts frequently appear extracellularly in smears as a result of rupture of the host cell (Lawrence *et al.,* 1994).

During schizont replication, the schizonts are seen initially to have large chromatin particles and are called macroschizonts. Later on a generation of microschizonts with small chromatin particles develops (Lawrence *et al.*, 1994). The size of schizonts, number of chromatin bodies that they contain and the number of lymphocytes infected with schizonts increase as the disease progresses (Barnett and Brocklesby, 1961 cited by Lawrence *et al.*, 1994).

Confirmative diagnosis can also be made by demonstrating the schizont in routine histological preparations of lymph nodes, spleen and infected lymphocytes infiltrating parenchymatous organs such as kidneys, liver, lungs and brain (Lawrence *et al.*, 1994).

2.4.3 Serological tests

The most widely used serological diagnostic test for *Theileria* spp. is the Indirect Fluorescent Antibody Test (IFAT), which uses schizonts grown in culture as antigen, or, where these are not available, piroplasm-infected erythrocytes from cattle can be. The IFAT test is sensitive, fairly specific, and usually easy to perform. Because of the acute nature of the disease, serological tests are useful in detecting a changed immune status of recovered animals within an exposed herd.

For IFAT, both schizont and piroplasm antigens may be prepared on slides or in suspension which is stored at 4^0C. Test sera are diluted with bovine lymphocyte lysate and incubated with the antigen in suspension, and anti-bovine immunoglobulin conjugate is then added. However, because of the problems of cross-reactivity among some Theileria species, the test has limitations for large-scale surveys in areas where these species overlap.

Theileria-specific antibody has also been detected by the enzyme linked immunosorbent assay (ELISA) (Katende, 1998; Katende *et al.*, 1990) The ELISA provide higher sensitivity and specificity than the IFA tests (Musoke *et al.*, 1994). In

ELISA, serum samples from animals are assayed for antibodies to *Theileria parva* using recombinant polymorphic immunodominant molecule as the antigen (Katende, 1998). Optical density readings for test sera are then expressed as percentage positivity.

2.4.4 Molecular approaches

Since the morphological features of the schizonts or piroplasms do not represent a reliable indicator for differentiation among *Theileria* species, developments in molecular biology provide possibilities for new, more sensitive and specific tests for differentiating species of *Theileria*.

Nowadays deoxyribose nucleic acid (DNA) technologies including polymerase chain reaction (PCR) and oligonucleotide probes are used for direct detection and differentiation of piroplasms of diferent *Theileria* species (Conrad *et al.,* 1989; Norval *at al.,* 1992, Chen *et al.* 1991; Allsopp *et al.,* 1993; Bishop *et al.,* 1995; D'Oliveira *et al.,* 1995;).

Theileria parva DNA detection assays based on the PCR using primers derived from a conserved region of the *Theileria parva* antigen gene are useful in monitoring long-term *Theileria parva* infections in cattle and in the investigating the epidemiology of theileriosis (Skilton *et al.,* 2002). These techniques are much more

sensitive and specific than lymph node biopsies and blood smear examination in differentiation of *Theileria* species.

2.5 Control and treatment of ECF

The current primary methods of controlling ECF in cattle are application of chemical acaricides on cattle, treatment with anti-theilerial drugs and immunization (treatment and infection method) or a combination of these methods and other measures such as effective fencing, pasture management, rotational grazing, and selection of tick resistant cattle (Young *et al.,* 1988).

The costs for these control measures is becoming excessive and the farming economies of the Eastern, Central and Southern Africa countries are unable to sustain them thus allowing the disease to continue causing great losses and impoverishing the peasant farmer in a vicious cycle.

2.5.1 Vector control

Acaricides continue to be the principal means of controlling ECF often as part of a general tick-borne disease control strategy although this practice also has detrimental effects on the environment.

Tick control has become less reliable because acaricides are expensive, resistance has developed to many of them, regulations regarding livestock movement and

quarantine are not strictly enforced, and management and maintenance of dips and spray races are often poor (Musisi, 1990). Other effective methods for controlling ticks include acaricides impregnated ear tags and pour-on preparations.

Extensive use of acaricide(s) is associated with the development of tick resistance to the acaricide(s), creation of endemic instability for tick-borne diseases and also environment and food contamination by the acaricides (Norval *et al.,* 1992).

The rate of emergency of resistance to any one acaricide has varied among different countries and among tick species (Dolan, 1999). Previously resistance was thought to arise rapidly due to the use of acaricide at a lower concentration than the recommended strength. However, more recent thinking advocate that under-strength dips delay development of resistance because at concentrations below the recommended strength, more ticks with a greater variety of resistance states survive thus, total resistance develops more slowly and the acaricide has a potentially longer effective life. This is in contrast with the earlier concept that at the recommended dip strength only those ticks capable of surviving concentration at or exceeding the recommended strength will survive and therefore selection will be for the most resistant ticks from the population (Dolan, 1999).

Traditional livestock keepers in Tanzania have been using acaricidal herbs to ticks, lice and flies on animals for a long time now as an alternative to the commercial

chemical insecticides including acaricides. One of the acaricidal herbs widely used by traditional livestock keepers is the fish bean, *Tephrosia vogelii.*

Tephrosia vogelii (Utupa) is an environmental friendly acaricidal herb that is been currently studied in respect to tick control in the southern highlands of Tanzania (Matovelo *et al.*, personal communication). It is a potential source of rotenone largely from the leaves, that is an important nonresidual insecticide (Echo, 1999).

2.5.2 Chemotherapy

Currently, there are three effective drugs for the treatment of clinical ECF: halofuginone lactate (Terit®), parvaquone (Clexon®) and buparvaquone (Butalex®). The first two have been in use since the mid-1980s and buparvaquone since early 1990s (Dolan, 1999). However, these drugs do not always effect cure in the advanced stages of the disease when extensive destruction of lymphoid tissues has occurred. There are no reports of resistance to these drugs (Dolan, 1999).

These drugs are being used in the treatment of clinical ECF with varying degree of effectiveness (Dolan, 1981; McHardy and Wekesa, 1985; Mbwambo *et al.*, 1986; Dolan, 1986a, 1986b; Dolan *et* al., 1988; Dolan *et* al., 1992; Thaiyah *et al.*, 1993).

Halofuginone is a quinozoline compound (di-trans-7-bromo-6-chloro-3-[3-(hydroxyl-2-piperidinyl) -2-oxopropyl] -4 (BH)-quinazolinone). It has been effective against

theileriosis with marked effect on the schizont stages and no effect on piroplasms stage since it is found to be effective when used in early infections (Mehlhorn *et al.,* 1981). However, recrudescence is a common feature following halofuginone treatment (Dolan, 1986a; Mbwambo *et al.,* 1986).

Parvaquone is a napthoquinone compound (2-cyclohexyl-3-hydroxy-1, 4-napthoquinone) with a wide margin of safety. Its mode of action is thought to inhibit electron transport within cells (McHardy, 1984). Early treatment is recommended for satisfactory results (Mbwambo *et al.,* 1987; Dolan *et al.,* 1988). However, the parasitosis recrudescence has been noted in some of the treated animals (Mbwambo *et al.,* 1987). The continued low parasitemia for more than three months after treatment is suggestive of a carrier state in recovered animals (Dolan, 1986a).

Buparvaquone is also a derivative of napthoquinones called compound BW 720C; 2-hydroxy-3 (trans-4-t butylcyclohexylmethyl) 1,4-napthoquinone. Although previously thought to be eight times more effective than parvaquone against *T. parva* infections (McHardy and Wekesa, 1985), recent studies have shown that the two drugs are equally effective in curing ECF (Muraguri *et al.,* 1999).

There are two constraints to the widespread use of chemotherapy. Firstly, the drugs are too expensive for most farmers, and secondly, it is crucial that the diagnosis is made early for effective treatment (Norval *et al.* 1992). In addition,

these treatments do not sterilise the infections. Hence, the success of treatment is a combination of drug destruction of the parasite load, and the animal's immune response controlling the residual infection (Dolan, 1999).

Notwithstanding the clinical uses of the above drugs, none is effective once the severe pulmonary oedema has set in (Norval *et al.* 1992). It is therefore a common routine in the field to combine antitheilerial drugs with supportive drugs like oxytetracyclines, diuretics, anti-inflammatory, antihistaminic and hematinics (Thaiyah *et al.,* 1993; Matovelo *et al.,* 2000).

2.5.3 Immunization

Immunization is meant to safeguard valuable susceptible exotic or crossbreeds, or local stock in areas with an unstable endemic situation or where the disease is threatening naïve cattle. According to the cost/benefit principle, in a stable endemic area where all young stock come into contact, immunization of local cattle with a high degree of innate resistance and relatively low monetary value is unlikely to be economical (Uilenberg, 1999). In such circumstances, and in the absence of tick control, mortality is limited and mainly confined to calves (Barnett, 1957; Grindle, 1981; Tyler, 1981). Moreover, immunization also aims to reduce the use of acaricides although this often leaves the questions about other tick-borne diseases unanswered because relaxing tick control in protected herds is risky. Immunization against the disease is based on an infection and a simultaneous injection of a long-

acting oxytetracycline to control infection (Radley *et al.* 1975a; Radley, 1981). The method involves inoculation of an aliquot of ground-up ticks infected with the nonattenuated viable sporozoite stage subcutaneously and the animals are treated simultaneously with long-acting tetracyclines. Usually a mild or inapparent reaction occurs and the immune response controls the infection.

This infection and treatment method (ITM) possibly results in life-long immunity against the immunising parasite (Burridge *et al.* 1972; Uilenberg, 1999). Two different approaches are being followed to determine the strain composition of vaccine stocks. One is "local" strain approach that uses a broadly protective local stock of *T. parva*; and the other one a "cocktail" approach that uses a combination of three stocks (trivalent cocktail vaccine) to provide broad immunity over most of the ECF region. The ITM has not gained widespread use as a control option because of a number of reasons. These include dependence on reliable cold chain for maintenance of live parasites; immunised animals are often susceptible to heterologous challenge, and that it gives rise to a carrier state raising concern over the possibility of introducing vaccine strains to native tick population previously free of them (Young *et al.* 1981; Dolan, 1986a; Young *et al.* 1986; Kariuki *et al.* 1995; Geysen *et al.*, 1999). Under such a circumstances it complicates the epidemiological state of the disease in areas where mass vaccinations are attempted also taking into account that wild buffalo and waterbuck are important reservoirs of infection

(Grootenhuis and Young 1981, Conrad *et al.* 1987a, Norval *et al.* 1992, Stagg *et al.* 1994).

Also there is the risk of contamination with other pathogens in the tick-derived material. For instance, it must be ascertained whether cattle are a reservoir of Nairobi sheep disease virus, of which *R. appendiculatus* is the main vector. There is another risk of immunising calves that are already in the advanced stages of naturally contracted ECF, consequently aggravating the condition.

Studies are under way to explore the potential that early diagnosis and early treatment (EDET) of ECF could be a tool for Zebu cattle especially calves for acquiring immunity and consequently developing resistance against subsequent homologous challenge of local strain. This will be advantageous to the ITM since it will eliminate the need for cold chain, reduce immunization costs and do away with risk of introducing vaccine strains (Chilongola *et al.*, 2002).

2.5.4 Breeding for genetic resistance

Genes that are associated with resistance to infectious diseases have been described in laboratory animals, and there is now evidence for such genes in livestock species. It has been shown that subclinical progression of enzootic bovine leucosis segregates with MHC haplotypes in sire families (Lewin and Bernoco, 1986) and several other important conditions including tick resistance and susceptibility to

ocular squamous cell carcinoma are associated with MHC type (Stear, 1988). Studies of the immune response *in vitro* and *in vivo* to *T. parva*, by Bensaid *et al.* (1988) and Shaw *et al.*, (1995) indicate that genes within the bovine MHC modulate the response.

Shaw *et al.*, (1995) showed that MHC I molecules are an essential component of the host cell surface receptor involved in *Theileria parva* sporozoite invasion and that sporozoite binding and entry clearly correlated with the expression of the level of MHC I molecules. Goddeeris (2000) in his study to demonstrate the MHC restriction and antigen-specificity of the *Theileria*-specific cytotoxicity found that not only the immunizing parasite but also the bovine MHC haplotypes of the respective immunized animal influenced whether the *Theileria*-specific CTL were strain-specific or cross-reactive between strains and that these results have important implications for the identification of cross-reactive CTL epitopes of the parasite in the development of subunit vaccines for outbred animals.

2.6 Immunohistochemical staining

2.6.1 Overview of lymphocyte surface markers

Leukocytes express distinct assortments of molecules on their cell surfaces, many of which reflect either different stages of their lineage-specific differentiation or different states of activation or inactivation. Leukocyte cell surface molecules are routinely detected with anti-leukocyte monoclonal antibodies (mAbs). Using

different combinations of mAbs, it is possible to chart out the cell surface immunophenotypes of different leukocyte subpopulations, including the functionally distinct mature lymphocyte subpopulations of B-cells, helper T-cells (T_H), cytotoxic T-cells (T_C), and natural killer (NK) cells.

The most important surface molecules are those that form part of the antigen-receptor complex molecules, such as T cell receptor (TCR) or B cell receptor (BCR) molecules for the T cells and B cells, respectively (Tizard, 1996). Each BCR has unique antigen specificity derived from somatic rearrangements of immunoglobulin genes that are made before the cell ever encounters an antigen (Cotran *et al.*, 1994).

In addition to membrane IgM, the B-cell antigen receptor complex contains a heterodimer of nonpolymorphic transmembrane proteins: Igα and Igβ. Like the Cluster of Differentiation 3 (CD3) proteins of TCR, Igα and Igβ do not bind antigen but are essential for signal transduction through the receptor. The B-cells also express several other nonpolymorphic molecules that are essential for B cell function. These include cytokine receptors, complement receptors, Fc receptors, adherence molecules, MHC and related molecules (Tizard, 1996).

Each T cell is genetically programmed to recognize specific cell-bound antigens by means of antigen-specific TCR complex molecules exposed on their surface (Weiss,

1990). Each TCR complex is similar, but not identical to an antibody. TCR diversity is generated by somatic rearrangement of the genes that encode the α, β, γ and δ TCR polypeptide chains (Cotran *et al.*, 1994). Two types of T cells, distinguished by surface expression of either an α/β TCR complex or a γ/δ TCR complex, develop independently as separate lineages in vertebrates (Haas *et al.* 1993). These constitute the total pool of peripheral T cells and are effectors of both cell-mediated immunity (CMI) and T-cell help.

The majority of mature α/β T cells express either CD4 or CD8 accessory molecules and recognize peptide antigens (Ags) in association with class I or class II major histocompatibility complex (MHC) molecules respectively. The CD4 and CD8 accessory molecules are distinguishing surface markers that are expressed on two mutually exclusive subsets of T cells that serve as co receptors in T-cell activation (Springer, 1990). The importance of CD4 and CD8 molecules lies in signal transmission and that stimulation of a T cell by an antigen-presenting cell is potentiated about 100-fold when CD4 or CD8 is associated with the TCR (Tizard, 1996).

T cells bearing CD4 molecules always recognize antigens in association with class II MHC proteins on the surface of some other cells called antigen-presenting cells (APC). APC include dendritic cells and phagocytic cells like macrophages for the cell-mediated immunity and B cells for the antibody-mediated immunity. CD4 T

lymphocytes generally function as T helper cells. T helper cells require co stimulation by cytokines that include IL-1 and IL-12 secreted by APCs plus cell surface adhesion molecules generated as a result of binding between T cells and APCs.

T cells bearing CD8 molecules always recognize antigen in association with class I MHC proteins and typically function as cytotoxic T cells. However, the CD4 and CD8 T cells perform distinct but somewhat overlapping functions (Cotran *et al.*, 1994).

The effector function of γ/δ T cells in immune responses in general, and in infectious diseases in particular, is poorly understood, and no consensus has yet emerged about the overall role of these cells in the immune systems of different species (Daubenberger *et al.*, 1999).

Chien *et al.,* (1996) showed that γ/δ T cell recognition of three murine protein antigens and the Herpes virus glycoprotein gI does not require antigen processing and that the proteins are recognized directly. They suggested that pathogens, damaged tissues, or even B and T cells can be recognized directly, and cellular immune responses can be initiated without a requirement for antigen degradation or specialized APCs, therefore giving γ/δ T cell greater flexibility than the more classical type of α/β T cell-mediated immunity.

In ruminants, γ/δ T cells respond predominantly in the mixed lymphocyte reaction, suggesting that they can recognize foreign MHC antigens and may also be cytotoxic. They have been found infiltrating the lesions of contagious ecthyma (orf) and around *Taenia hydatigea* cysts in the liver (Tizard, 1996).

2.6.2 Immunohistochemical detection of T- and B-lymphocytes in lung tissues of bovine infected with *Theileria parva*

In veterinary pathology the identification of components of the immune system by immunohistochemical technique has proved to be useful for both diagnostic and research purposes and has made possible to visualize lymphocyte populations in tissue samples (Kelley *et al.,* 1997; Gutierrez, *et al.*, 1999).

Monoclonal antibodies to lymphocyte markers are widely used in flow cytometry and immunohistochemistry (IHC) to detect molecules (qualitative IHC) and attempt measurement of just how much protein or nucleic acid is present in relative or absolute units (quantitative IHC) (Taylor, 2001).

Antibodies with that can detect antigens in formalin-fixed paraffin-embedded tissues are valuable in immunohistochemical studies in animals notwithstanding the general perception that formalin-fixed paraffin-embedded tissues are not best suited when compared to frozen material.

Some lymphoid antigen markers that correspond to CD antigens known in humans have been identified in animal species (Baldwin *et al.,* 1986; Crump, *et al.,* 1988; Naessens *et al.,* 1990; Howard and Morrison, 1991; Naessen and Howard, 1993; Kydd *et al.,* 1994; Naessens *et al.,* 1996; Naessens *et al.,* 1997;). Furthermore, T-cell specific markers for animal species are also available (Howard and Morrison, 1991; Lunn *et al.,* 1991; Naessen and Howard, 1993; Kydd *et al.,* 1994), and so are specific B cell markers although to a lesser degree (Naessens *et al.,* 1988; Naessen and Howard, 1993; Kydd *et al.,* 1994; Zhang *et al.,* 1994; Naessens *et al.,* 1990; Naessen and Howard, 1991). Some of these antibodies can differentiate animal lymphocyte subclasses in formalin-fixed paraffin-embedded tissues.

Since mAbs to leukocyte differentiation antigens cross-react very poorly between distantly related species, it has become necessary to produce antibodies for ruminants (Howard *et al.,* 1991).

However, mAbs to ruminant leukocyte antigens frequently, but not invariably, exhibit cross-reactivity between different ruminant species, and mAbs to leukocyte antigens of cattle, sheep, goats and other ruminants have been characterised. Moreover, the nomenclature and characterization of leukocyte differentiation antigens in ruminants now follows the CD nomenclature of the widely accepted practical system of the human workshops for identification of differentiation antigens (Naessens *et al.,* 1997) that will also facilitate comparisons of

immunological observations between species. The prefix Bo is used to discriminate the bovine from the human antigen where necessary.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental animals

3.1.1 Handling of experimental animals

Six apparently healthy steers aged between 7 - 10 months were purchased from Sokoine University farm, which practices rigorous tick control regime. The animals were allowed to acclimatize to their new premises for 9 days during which they were screened for haemoparasites by examining Giemsa stained lymph node and blood smears. Also, fecal smears were examined for eggs of gastrointestinal worms. The animals were drenched once with a broad-spectrum antihelmintic Nilzan Plus™ that contains 3% Oxyclozanide, 1.5% Levamisole Hydrochloride and 0.382% Cobalt sulphate. The animals were maintained indoors in a tick-free and insect-proofed pen on a high nutritional plane of a ration composed of hay, green grass and molasses and water was provided *ad libitum*.

During the nine days of acclimatization, baseline data on rectal temperatures and haematological parameters were determined by taking rectal temperatures daily and blood samples via the jugular vein for haematological parameters [Total red blood cell (RBC), Total white blood cell (WBC) and differential leukocyte counts,

hemoglobin concentration (Hb) and packed cell volume (PCV)] determinations at days -9, -4 and 0 pre-infection.

3.1.2 Infection of experimental animals with *Theileria parva* sporozoites

Four out of the six steers (group I) were randomly picked and each infected by subcutaneous injection anterior to the left prescapular lymph node with 0.5 ml of inoculum of *Theileria parva* sporozoites (Muguga stabilate 3087) purchased from the International Livestock Research Institute (ILRI) Nairobi, Kenya. The remaining two steers (group II) were kept as non-infected control animals.

3.1.3 Clinical examinations and hematological analyses

After infection, rectal temperatures of all animals were taken daily and the size of palpable lymph nodes and respiration dysfunction indicators (coughing, lung sounds and dyspnea) were assessed daily.

Lymph node biopsies from left prescapular lymph nodes of all animals were taken at days 4, 6 and 8 post-infection, stained with Giemsa and examined under the light microscope for presence of schizonts.

Five millilitres of blood were collected from each animal in ethylene diamine tetra acetate (EDTA) coated tubes for total RBC, WBC counts and differential counts, Hb and PCV at days 4, 8, 11 and 15 post infection.

Hb and PCV were measured by the cyanmethaemoglobin and standard microhematocrit centrifugation methods respectively (Coles, 1986) while total RBC and WBC counts were determined by the haemocytometer method. Differential leukocyte count was done by the microscope slide method (Benjamin, 1978).

3.1.4 Slaughter and post-mortem examination of experimental animals

Each of the four infected experimental animals (in group I) was exsanguinated by severing the carotid artery under general anaesthesia given intravenously using Pentobarbitone sodium (Pentobarbital – 6; VMD –CHEMIE N.V/ S.A B2370 ARENDONK – BELGIUM) at a dose rate of 15-mg/kg body weight at the onset of respiratory distress. The two non-infected control (group II) animals were also slaughtered by the same method at the same time when the infected animals were slaughtered. After slaughter, a post-mortem examination was conducted paying particular attention to the appearance, size, shape, colour, and consistency and cut surface of the lungs, and presence of fluid in the airways.

3.1.5 Tissue sampling, preparation and staining

Lung tissue samples from middle portions of cranial and caudal lobes of both right and left lungs were taken within 10 minutes of slaughter and immediately put in 4% neutral-buffered formaldehyde (NBF), pH 7.4 for 48 hours. There after, processing of the tissues was done in an automatic tissue processor (SHANDON, UK) where the tissues were dehydrated in alcohol, cleared in chloroform and embedded in melting paraffin wax at 56^0 C. The tissues embedded in paraffin blocks were sectioned at 4 μm thickness, flattened on mounting warm water bath (40^0 C) containing gelatin and then mounted on microscope slides coated with chrome-gelatin to avoid detachment of sections during subsequent stages of staining. The slides were dried for 24 hours in an oven at 40^0 C. Then the slides were removed from the oven and kept at room temperature, ready for routine and immunohistochemical staining.

Half of the slides were stained with haematoxylin and eosin (H & E) (Bancroft and Stevens, 1990) and examined under light microscope while the other half were subjected to immunohistochemical staining using streptavidin-biotin immunoperoxidase staining technique as described by Gutierrez, *et al.,* (1999) with modifications as shown in the immunohistochemical protocol (APPENDIX I).

Briefly, for the immunohistochemical staining, serial 4 μm thick tissue sections mounted on microscope slides were warmed to 56 - 60^0 C in an oven, then

immediately deparaffinized in three sequential xylene washes, then rehydrated in ethanol baths, and then brought to water. The tissue sctions were then incubated in an antigen retrieval solution, in either 10mM citrate buffer, pH 6.0 (APPENDIX IV) (Norton *et al.*, 1994) or Tris-buffered saline solution as antigen retrieval solution (TBS-ARS), pH 8.2 (APPENDIX V) depending on the mAb that attained optimum immunoreactivity (as indicated in Table 1) at temperature of $90^0C - 95^0C$ for 20 minutes.

The sections were cooled for 15 minutes in the same retrieval solution then washed in 50mM Tris-buffered saline solution, pH 7.6 (TBS) (APPENDIX VI). The sections were then treated with 0.3% H_2O_2 in TBS for 30 minutes at room temperature to quench endogenous peroxidase activity (Hittmair and Schmid, 1989) and then washed in Tris-buffered saline solution containing 0.25% Triton X-100 (TBS-T) (APPENDIX VI).

The slides were dried around the tissue section with an absorbent wipe, and using a colourless fingernail polish, a circle was drawn on the microscope slide around the section to help retain solution on the section during subsequent incubations with reagents.

The sections were preincubated in a humidified chamber for 60 minutes with 10% normal rabbit serum (X0902, DAKO) in TBS-T in which 1% Bovine Serum Albumen

(Sigma A-4503) (BSA) and 0.5% full cream milk Nestle® was added in order to block non-specific binding sites and to reduce background staining.

In this study monoclonal antibodies directed against lymphocyte subpopulations of B cells, helper T cells (T_H), cytotoxic T cells (T_C) and gamma-delta (γ/δ) T cells were employed to differentiate lymphocyte subclasses in formalin-fixed paraffin-embedded lung tissues of cattle infected with *T. parva*. These monoclonal antibodies summarised in Table 1 below are: -

1) Mouse anti-bovine monoclonal antibody IL-A65, isotype IgG2a of specificity BoCD21 (Naessens *et al.,* 1990; Naessens *et al.,* 1997) was produced as mouse ascitic fluid, clarified by centrifugation and filtered through a 0.2 μm filter.
2) Mouse anti-bovine monoclonal antibody IL-A11, isotype IgG2a of specificity BoCD4 (Baldwin *et al.,* 1986; Naessens *et al.,* 1997) was produced as mouse ascitic fluid, clarified by centrifugation and filtered through a 0.2 μm filter.
3) Mouse anti-bovine monoclonal antibody IL-A105; isotype IgG2a specific for bovine CD8 ∝-chain was produced as described by MacHugh *et al.,* (1993).
4) Mouse anti-bovine monoclonal antibody IAH-CC15, IgG2 of specificity BoWC1 (Howard *et al.,* 1991) was raised against bovine gamma-delta (γ/δ) T-cell-specific surface antigen and produced as mouse ascitic fluid, clarified by centrifugation and filtered through a 0.2 μm filter.

Table 1: Monoclonal antibodies used in the present study

Monoclonal antibody	*Ig isotype*	*Specificity*	*Reference*	*Dilution*	*Antigen retrieval solution*
IL-A11	IgG2a	BoCD4; T cell subset	Baldwin *et al.*, 1986	1:100	Citrate buffer
IL-A65	IgG2a	BoCD21; B cells	Naessens *et al.*, 1990	1:100	Tris-buffered Saline
IL-A105	IgG2a	BoCD8; T cell subset	MacHugh *et al.*, 1993	1:100	Citrate buffer
IAH-CC15	IgG	BoWC1; γ/δ T cell sub set	Howard *et al.*, 1991	1:100	Citrate buffer

A 100 μL of each of the mAb at optimal dilution in TBS-T containing 10% normal rabbit serum (X0902, DAKO), 1% Bovine Serum Albumen (BSA) and 0.5% NIDO powdered milk (Nestle®) was applied per section in a humidified chamber overnight at room temperature.

The sections were then washed with TBS-T to remove unbound antibodies and artefacts, and then incubated for 90 minutes at room temperature with biotinylated rabbit anti-mouse immunoglobulins (E0413, DAKO) diluted 1:500 in TBS-T containing 10% normal rabbit serum (X0902, DAKO), 1% Bovine Serum Albumen (BSA) and 0.5% full cream NIDO milk.

The sections were washed four times with TBS-T, and then StreptABComplex/HRP (K 0377, DAKO) diluted 1:500 was applied for 90 minutes at room temperature in a humidified chamber and then rinsed with TBS-T.

Peroxidase activity in the sections was visualised using 0.01% H_2O_2 in 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) substrate-chromogenic solution for Horse Radish Peroxidase (HRP) in 50mM TBS for 5 - 15 minutes at room temperature in a humidified chamber. Incubation with the substrate-chromogenic solution was continued until desired color intensity had developed. The sections were counterstained with Mayer's haematoxylin for 10 - 20 seconds (depending on the extent of color intensity by the DAB reaction), rinsed in distilled water, and dehydrated in alcohol baths and then cleared in xylene.

The sections were mounted with DPX (Depex mounting medium, BDH Limited, Poole. England), and dried in oven at 37^{0}C overnight before viewing under light microscope (Olympus BHT/BH-2 microscope) and selected photomicrographs recorded in ProFoto 100 film.

For the negative tissue control incubations (Pinkus, 1982; O'Leary, 2001), tissue specimen from lungs of control animals were fixed and embedded in the same manner as the infected tissue specimens, and then stained by the same primary antibodies.

3.2 Specimens from natural ECF infection

Tissue samples of lungs, spleen and parotid lymph nodes were also collected from two ECF naturally infected animals that were slaughtered at the Morogoro Municipal

abattoir. These animals were slaughtered at the life-threatening stage of clinical ECF that was characterized by low rectal temperatures (37^0 — 38^0 C), severe respiratory distress and discharge of frothy fluid from the nostrils. Impression smears from the spleen and lymph nodes were also made and stained by Giemsa.

The lung specimens collected were handled as previously described with the experimentally infected animals.

3.3 Counting of B- and T-lymphocytes

Immunostained lymphocytes in sections of lungs from cattle experimentally and naturally infected with *Theileria parva* were counted under the light microscope at X100 objective in the interalveolar interstitium. This location was purposefully selected in order to avoid peribronchial areas that normally have subepithelial diffuse or dense unencapsulated lymphatic tissues/lymph nodules that are associated with the bronchi and bronchioles (Banks, 1986; Slauson and Cooper, 1990; Jubb *et al.*, 1992). The numerical density and location of lymphocyte populations in the tissue sections were assessed microscopically by counting the positively stained cells in thirty chosen fields of 0.011050 mm^2 each.

The field of 0.011050 mm^2 was obtained by measuring the area of the format reticule in the field of view of the 35-WHK-finder eyepiece 10X/20L of the BHT/BH-2 light microscope against a stage micrometer scale. The final count for each field

was calculated as the mean of the thirty fields counted and expressed as the number of cells per mm^2. The counting of lymphocytes was conducted as follows: - Using the X-Y translational mechanical stage of the microscope, the specimen slide was moved in both the X-axis (right and left) and Y-axis (back and forth) directions (2-dimensional) in order to examine the entire microscope slide in a systematic and uniformly random to sample 30 fields for each mAb. The basic stepping length in either direction was one division of the graduated locator marks positioned on the mechanical portion of the stage. All the $BoCD4^+$, $BoCD8^+$, $BoWC1^+$ and $BoCD21^+$ cell were considered for counting if and when they displayed a brown stained cytoplasm and unambiguously visible nucleus and also whether the cells were completely or partly inside the field area.

3.4 Statistical analysis

Student two sample *t*–test (Kirkwood, 1988) employing software Statistix 7.0 was used to determine the significance difference ($*P<0.05$) in haematological parameters between the control and infected animals and also the lymphocyte cell types infiltrating lung tissue sections between artificially and naturally infected animals.

CHAPTER FOUR

4.0 RESULTS

4.1 Clinical observations

4.1.1 Pre-infection period

During the nine days of acclimatization none of the animals showed any sign of illness and the mean values of the rectal temperatures, total RBC and WBC counts, Hb and PCV parameters that were monitored in the two groups of animals did not differ significantly between the groups (Table 2).

Table 2: Mean values of rectal temperatures, total RBC and WBC counts, Hb and PCV in groups I and II of experimental animals and their overall mean values ($\overline{X}$).

Group	Parameter				
	Temp. (^{0}C)	RBC (X 10^{12} /L)	Hb (g/L)	PCV (L/L)	WBC (X 10^9 /L)
I	38.39	6.60	90.25	0.26	8.44
II	38.63	6.93	87.5	0.27	7.39
$\overline{\mathbf{X}}$	38.51	6.77	88.88	0.27	7.92

The overall mean values of the parameters shown in Table 2 were then considered to be baseline values for all the experimental animals.

4.1.2 Post infection period

4.1.2.1 Clinical response during post infection period

All infected animals (group I) developed clinical theileriosis within two weeks of infection. Pathological events recorded in cattle experimentally infected with *Theileria parva* are summarised in Table 3.

Table 3: Pathological events recorded in cattle (Group I) experimentally infected with *Theileria parva*

ANIMAL NO.	PATHOLOGICAL EVENTS AT DIFFERENT DAYS POST INFECTION				
	LYMPH NODE ENLARGEMENT	ONSET OF FEVER	APPEARANCE OF MACROSCHIZONTS	COUGHING	DYSPNEA
21479	6	8	8	13	-
21481	6	8	7	10	-
21497	6	9	7	13	16
21498	7	9	8	12	15
Mean ± s.e.	6.25±0.25	8.5±0.29	7.5±0.5	12.0±0.71	15.5±0.5

From Table 3 it is seen that the average time to the detection of macroschizont in the infected animals was 7.5±0.5 days, and the fever onset was observed at 8.5±0.29 days post infection when rectal temperature had risen to ≥ 39.5^{0}C. Lymph node enlargement was detected as early as 6.25±0.25 days.

Coughing was observed in all four steers by day 12.0±0.71. Two steers out of the four became dyspnoeic by day 15.5±0.60, while the remaining two steers did not become dyspnoeic.

The uninfected control animals (Group II) did not show any clinical sign of disease up to the 16th day of the experimental period.

4.1.2.2 Rectal temperatures

The trends of mean rectal temperatures in all the experimental animals are shown in Table 4 and plots in Fig. 1.

Table 4: Mean rectal temperatures (°C) in *T. parva* infected animals (Group I) and in the uninfected control animals (Group II)

Day(s) Pre/post-infection	Rectal Temperature °C	
	GROUP I	GROUP II
-9	38.65±0.60	38.70±0.20
-6	38.38±0.13	38.50±0.50
-3	38.28±0.14	38.75±0.15
0	38.25±0.14	38.55±0.65
3	37.90±0.67	38.45±0.15
6	38.40±0.10	38.40±0.00
9	40.13±0.31	38.90±0.60
12	40.63±0.35	38.45±0.15
15	41.00±0.00	38.30±0.20

The mean rectal temperatures of group I animals rose to ≥ 39.5^0 C at 8.5±0.29 day post infection marking the onset of fever. From that time, the rectal temperatures continued to rise reaching a maximum of 41^0 C when the animals were slaughtered. The mean rectal temperatures of the control animals remained at a mean normal of 38.5 ± 0.1^0 C. Moreover, the mean rectal temperatures of group I animals was

significantly higher (*P<0.05) than the mean rectal temperatures of group II animals by day 9 post infection.

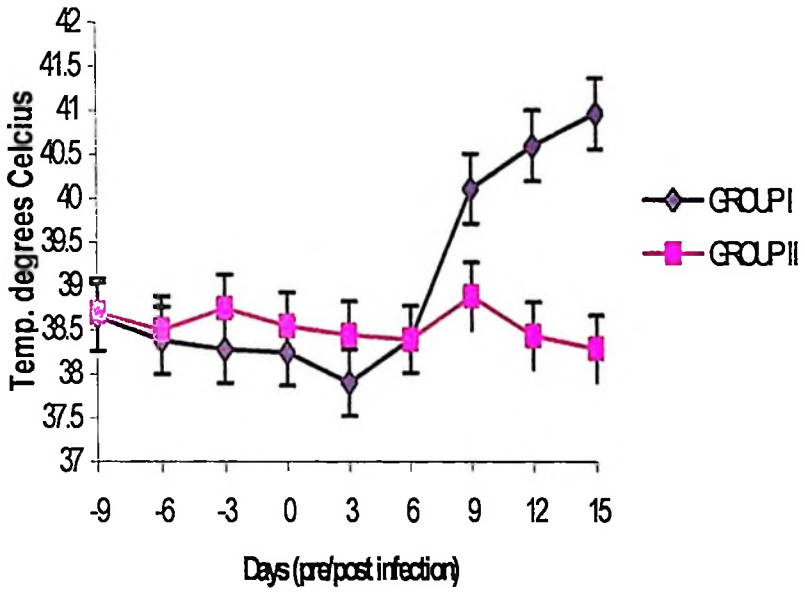

Fig. 1: Mean rectal temperatures in animals experimentally infected with *T. parva* (Group I) and in the uninfected control animals (Group II).

4.1.2.3 Hematological findings

4.1.2.3.1 Red blood cells count

The mean RBC counts in the two groups of the experimental animals are shown in Table 5 and the plots are shown in Fig. 2.

Table 5: Mean RBC count (X 10^{12} /L) in experimentally infected (Group I) and in the uninfected control animals (Group II)

Days pre/post infection	Total RBC count (cells X 10^{12} /L)	
	GROUP I	GROUP II
-9	6.83±0.17	6.75±0.21
-4	6.88±0.16	7.19±0.52
0	6.10±0.23	6.86±0.12
4	6.84±0.49	7.84±0.41
8	6.53±0.66	8.40±0.40
11	6.91±0.25	8.26±0.66
15	5.93±0.59[a]	7.52±0.24

[a] Significantly lower (*P<0.05) than pre-infection mean value.

The RBC counts of the experimentally infected animals (group I) were not significantly different (*P< 0.05) from RBC counts of the uninfected control animals (group II) till day 11 post infection. However, a significant drop in the RBC count (*P< 0.05) in the infected animals was observed by day 15 post infection.

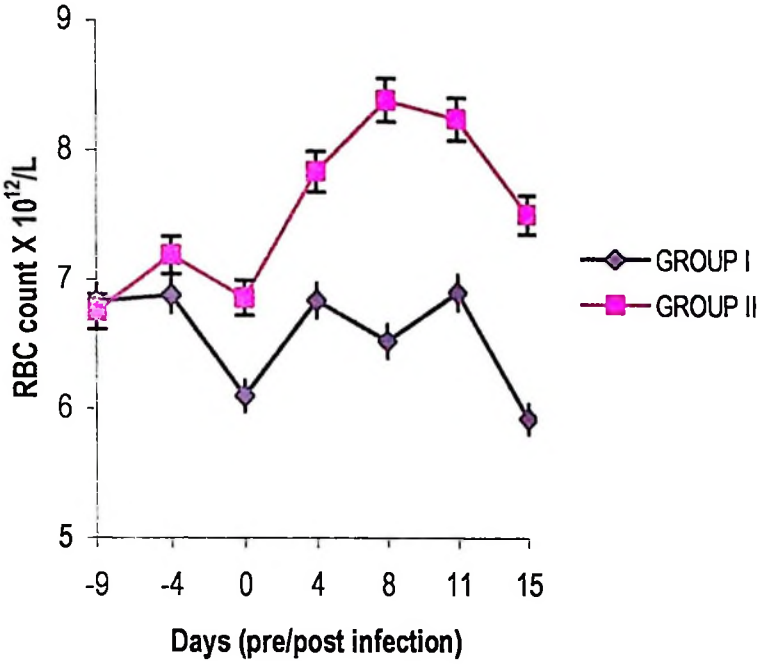

Fig. 2: Total RBC count in animals experimentally infected with *T. parva* (Group I) and the uninfected control animals (Group II).

4.1.2.3.2 Haemoglobin concentration

The mean Hb in the infected animals (Group I) and in the uninfected control animals (Group II) are shown in Table 6 and their plots are shown in Fig. 3.

Table 6: Mean Hb (g/L) in animals experimentally infected with *T. parva* (Group I) and the uninfected controls (Group II)

Days pre/post infection	Haemoglobin concentration (g/L)	
	GROUP I	GROUP II
-9	90.00±1.68	88.00±1.52
-4	90.25±1.32	87.50±1.16
0	90.50±0.96	87.00±2.21
4	89.00±3.37	90.50±1.53
8	87.58±2.27	91.00±2.03
11	84.50±1.99	93.00±1.48
15	80.50±2.05[a]	92.50±1.34

[a] Significantly lower from pre-infection mean value.

Fig. 3 shows that the Hb in the experimentally infected animals (group I) started to decline by day 4 post infection, and by day 15 post infection the Hb was significantly lower (*P<0.05) than the pre-infection mean value.

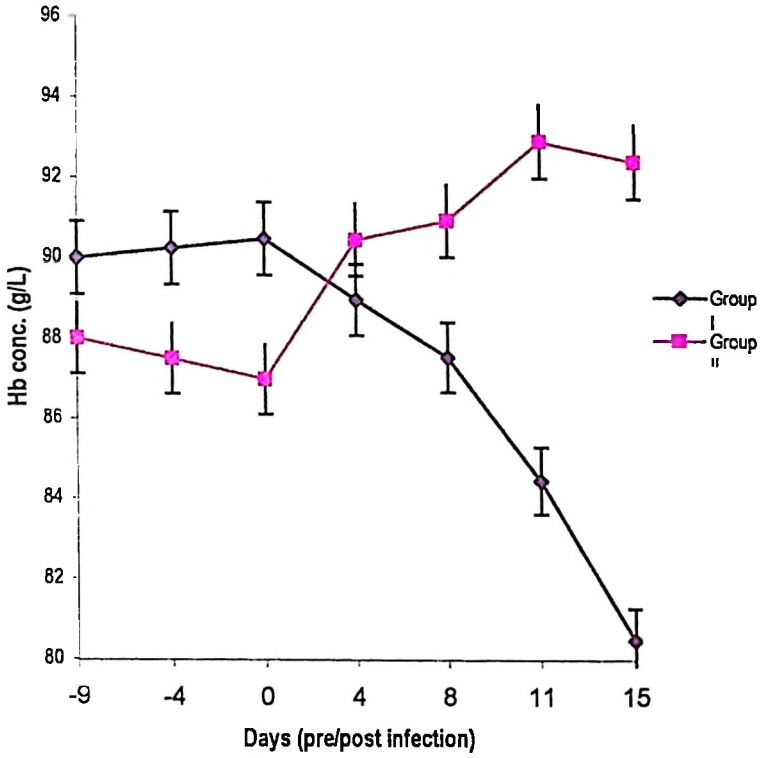

Fig. 3: Haemoglobin concentration in animals experimentally infected with *T. parva* (Group I) and of the uninfected control animals (Group II).

4.1.2.3.3 Packed cell volume

The mean PCV values in infected animals (group I) and in the unifected control animals (group II) are shown in Table 7 and their plots are illustrated in Fig. 4.

Table 7: Mean PCV (L/L) in animals experimentally infected with *T. parva* (Group I) and of the uninfected controls (Group II)

Days pre/post infection	Packed cell volume (L/L)	
	GROUP I	GROUP II
-9	0.26±0.01	0.27±0.01
-4	0.27±0.00	0.27±0.01
0	0.26±0.00	0.26±0.00
4	0.28±0.01	0.29±0.00
8	0.28±0.02	0.27±0.01
11	0.22±0.02	0.27±0.00
15	0.22±0.02	0.31±0.02

There was no significant difference (*P<0.05) between pre-infection and post-infection Hb values in the infected animals.

However, the plots in Fig. 4 shows an apparent fall in PCV values in the experimentally infected animals (Group I) at day 8 post-infection which was not significantly different (*P<0.05) from PCV of Group II animals.

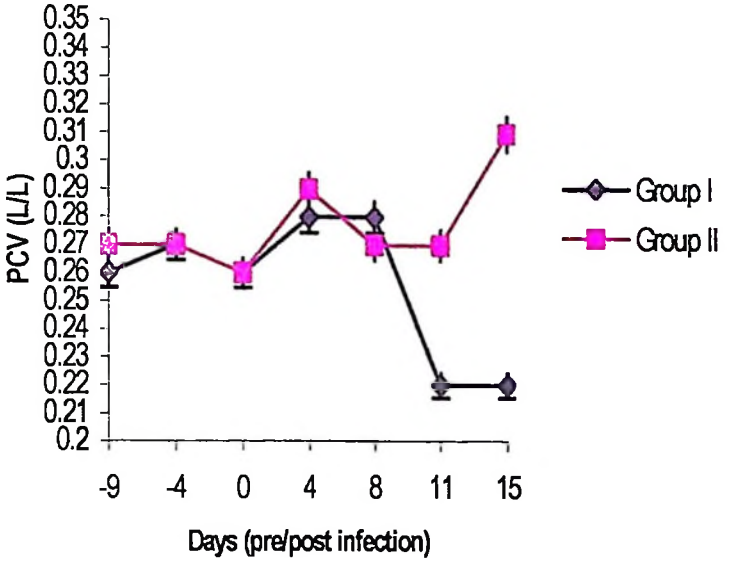

Fig. 4: PCV values of animals experimentally infected with *T. parva* (Group I) and of the uninfected control animals (Group II).

4.1.2.3.4 Total White blood cell counts

The mean total WBC counts in the infected and non infected animals are shown in Table 8 and the plots of the mean counts are shown in Fig. 5.

Table 8: Mean total WBC counts (X 10^9 /L) in animals experimentally infected with *T. parva* (Group I) and in the uninfected controls (Group II)

Days pre/post infection	Total WBC count (cells X 10^9 /L)	
	GROUP I	GROUP II
-9	8.15±1.35	5.50±0.70
-4	8.27±1.23	8.03±0.58
0	8.90±0.80	8.65±0.40
4	9.08±1.98	8.28±1.38
8	16.95±0.65[b]	6.10±1.10
11	5.53±0.03	7.43±2.08
15	1.31±1.09[a]	8.90±0.10

[a] Significantly lower (*P<0.05) from pre-infection mean value in the same group.

[b] Significantly higher (*P<0.05) from pre-infection mean value in the same group.

The plots of mean WBC counts in Fig. 5 shows that there was a significant (*P<0.05) increase of total WBC count in the infected animals over pre-infection values at day 8 post infection which however declined steadily to significantly (*P>0.05) lower value than pre-infection values at day 15 post infection.

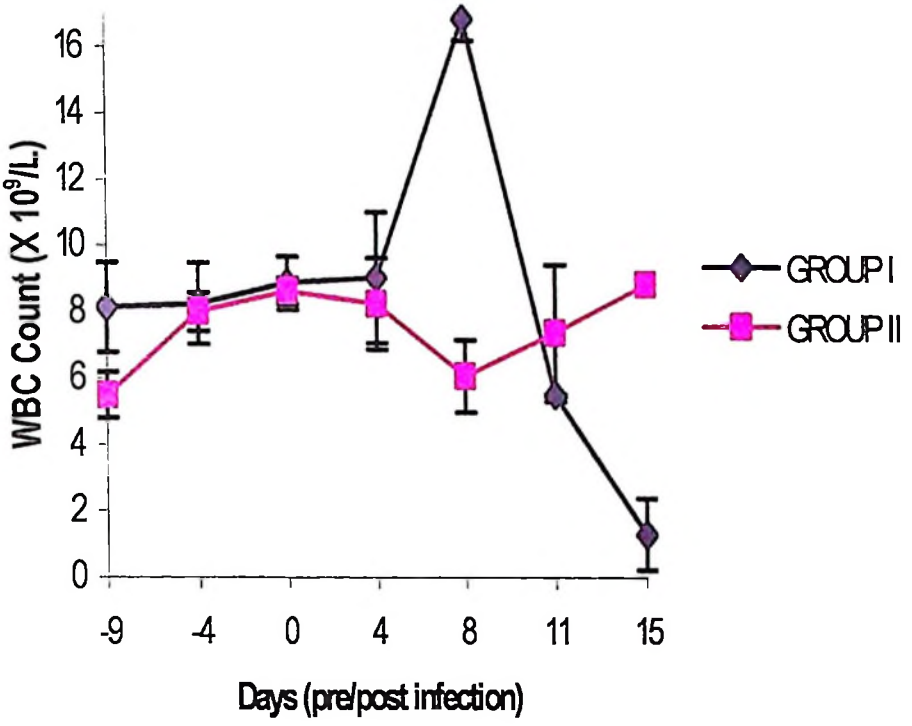

Fig. 5: Total WBC count in the animals experimentally infected with *T. parva* (Group I) and in the uninfected control animals (Group II).

4.1.2.3.5 Differential Leukocyte Count

The mean differential leukocyte counts (%) in the two experimental groups are shown in Table 9 and the plot for the experimentally infected (Group I) is shown in Fig. 6.

Table 9: Differential leukocyte count (%) of animals experimentally infected with *T. parva* (Group I) and of the uninfected control (Group II)

Days post infection	Lymphocytes	Neutrophils	Eosinophils	Basophils	Monocytes
Group I					
-9	72.1±0.94	23.3±1.16	2.5±0.47	0.00	2.1±0.31
-4	70.4±1.12	23.5±0.83	3.6±0.11	0.5±0.17	2.0±0.26
0	68.0±0.71	23.8±0.58	4.2±0.20	0.00	4.0±0.84
4	74.8±0.97	21.2±1.24	2.6±0.57	0.4±0.20	1.0±0.32
8	76.4±1.21	20.8±1.02	1.8±0.80	0.00[a]	1.0±0.45
11	74.2±0.68	22.2±0.86	1.6±0.40	0.4±0.21	1.6±0.68
15	68.8±1.24[a]	24.6±0.81[b]	2.1±0.58	0.5±0.22	4.0±1.14[a]
GROUP II					
-9	70.56±1.0	23.1±0.21	1.1±0.67	0.0	1.5±0.23
-4	67.32±0.57	24.0±1.65	0	0.0	1.0±1.45
0	69.87±1.94	23.4±1.12	1.1±0.07	0.23±0.71	1.1±1.14
4	71.10±0.46	22.9±1.05	0	1.0±0.8	0.0
8	72.51±0.21	22.9±1.0	0	1.5±1.0	1.5±0.84
11	69.84±1.24	21.8±0.95	1.0±.98	1.5±0.65	1.0±0.58
15	70.18±0.69	22.6±0.54	1.0±0.65	0	0.5±0.20

[a] Significantly lower (*P<0.05) from pre-infection mean value in the same group.

[b] Significantly higher (*P<0.05) from pre-infection mean value in the same group.

Table 9 and Fig. 6 shows that the experimental animals (cattle) had higher percentages of lymphocytes followed by neutrophils, and monocytes. In the infected animals (Fig. 6) significant (*P<0.05) reduction in lymphocytes and

monocytes compared to pre-infection values occurred by day 15 post infection. On the other hand significantly higher (*P<0.05) percentage of neutrophils were recorded at day 15 post infection.

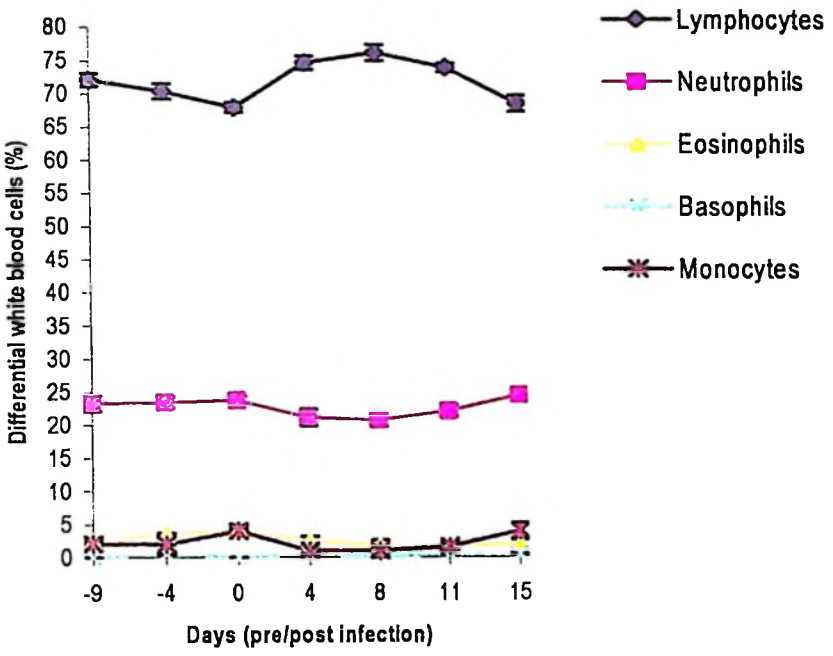

Fig. 6: Differential leukocyte counts (%) in the animals experimentally infected with *T. parva* (Group I).

4.2 Spleen and lymph node smears from animals experimentally and naturally infected with *T. parva*

Smears made from spleen and lymph nodes of experimentally infected animals and from natural cases of ECF from the abattoir showed schizonts which appeared as blue bodies in the lymphocyte cytoplasm best known as Koch's blue bodies (KBB). In the case of experimental ECF infection, about 15-20% of lymphocytes examined under the microscope had macroschizonts, whereas more than 50% of lymphocytes examined in natural ECF infection had microschizonts.

4.3 Post mortem findings in the lungs

4.3.1 Gross findings

All steers in Group I had moderately enlarged and oedematous lungs. The caudal lobes were more enlarged, puffy, and non-collapsible with rounded edges and widened interlobular septa when compared to the cranial lobes. Moderate, slightly yellowish edema fluid oozed from cut surface of the lungs on compression. There was moderate amount of fluid in the airway passages. Mild ascites and hydrothorax consisting of serous/straw-coloured fluid were seen in two of the four steers. Subcutaneous edema was marked around the inoculated lymph node and also observed in the ventral neck region. Furthermore, one animal had pale raised nodules on kidney surfaces. Lymph nodes were enlarged, oedematous and hemorrhagic. Nothing significant was seen in the steers in Group II (control animals).

In the naturally infected cattle, the lung airways were filled with copious frothy fluid. The lungs were reddened, puffy and non-collapsible. The lymph nodes were slightly enlarged, edematous and hemorrhagic.

4.3.2 Histological findings

The lung sections from the experimentally infected (Group I) animals revealed moderate to severe mononuclear cell infiltration in the interstitial tissue. They revealed widening of the interlobular septa due to edema and mononuclear cell infiltration. The interalveolar septa were 6 – 8 cells thick. Also there was dilation of lymphatic vessels and light eosinophilic staining material in the majority of alveolar spaces. The blood vessels were dilated and congested. Hyaline microthrombi were seen in some of the blood capillaries. Nothing significant was seen in the control animals (Group II).

In addition to the histological changes described above, the lung sections from the naturally infected cattle presented an intensive mononuclear cell infiltration and widened interalveolar septa. Disintegrating lymphocytes were seen in the interalveolar septa.

4.4 Immunohistochemical visualization and counting of lymphocytes infiltrating the lungs

In this study it was possible to visualize BoCD21$^+$ BoCD4$^+$ BoCD8$^+$ and BoWC1$^+$ lymphocytes cell subpopulations. Positive cells in tissue sections were seen having golden-brown staining cytoplasm (Figures 7 – 14). For clear visualization, the background staining was reduced by use of 0.25% Triton®X and 0.5% full cream milk NIDO - Nestle® in the blocking solution and in the diluents for the primary and secondary antibodies. Also by counterstaining with hematoxylin, the unstained cells that included mononuclear cells were clearly seen. These cells were counted.

Table 10 shows the mean number of stained (positive) lymphocytes and unstained (negative) mononuclear cells (MNC) per mm^2 of lung tissue at X 100 objective against the four different mAbs used in this study (Table 1). There were no positive cells in the lung sections of the control animals. The negative mononuclear cells included lymphocytes and macrophages.

Table 10: Mean number of positive lymphocytes and negative mononuclear cells (MNC) per mm^2 in lung of experimentally and naturally infected animals with *T. parva*.

Type of infection	Animal number	Number of leukocytic mononuclear cells (per mm^2) in lung tissue section.							
		Stained by							
		mAb IL-A65		mAb IL-A11		mAb IL-A105		mAb IAH-CC15	
		$CD21^+$	Negative MNC	$CD4^+$	Negative MNC	$CD8^+$	Negative MNC	$WC1^+$	Negative MNC
Experimental infection									
	21479	44	3338	45	2845	43	2607	25	2466
	21481	39	3341	49	2796	44	2602	29	2387
	21497	38	3362	49	2825	42	2651	28	2412
	21498	47	3339	49	2802	39	2588	26	2303
	Mean ± s.e.	42 ± 2.12	3345 ± 5.70	48 ± 1.00	2817 ± 11.23	42 ± 1.08	2612 ± 13.61	27 ± 0.91	2392 ± 33.94
Natural infection									
	21489	19	2842	11	3192	138	4299	13	2454
	24902	17	2798	13	3124	146	4365	17	2336
	Mean ± s.e.	18 ± 1.00	2820 ± 22.00	12 ± 1.00	3180 ± 34.00	142 ± 4.00	4332 ± 33.00	15 ± 2.00	2395 ± 59.00

$CD21^+$ = B-lymphocytes stained by monoclonal antibody IL-A65

$CD4^+$ = Helper (T_H) lymphocytes stained by monoclonal antibody IL-A11

$CD8^+$ = Cytotoxic (T_C) lymphocytes stained by monoclonal antibody IL-A105

$WC1^+$ = Gamma-delta (γ/δ) lymphocytes stained by monoclonal antibody IAH-CC15

There was no significant difference (*$P<0.05$) between the number of positive lymphocytes in the experimentally infected and naturally infected animals. Similarly the number of negative MNC in the two groups did not differ significantly (*$P<0.05$) from each other.

4.4.1 Mean number and ratio between positive lymphocytes and negative MNC in the lung sections

The mean numbers of positive B- lymphocytes (BoCD21$^+$) and T-lymphocytes (BoCD4$^+$, BoCD8$^+$ and BoWC1$^+$) and the ratios between these positive lymphocytes and negative MNC in lungs of cattle experimentally and naturally infected with *Theileria parva* are shown in Table 11. The photomicrographs of the same are shown in Figures 7 - 14.

Table 11: Mean number and ratio between positive lymphocytes and negative MNC in bovine lungs with ECF

Type of MNC	Number of mononuclear cells (Per mm^2)		Ratios of positive to negative MNC	
	Experimental Infection	Natural Infection	Experimental Infection	Natural Infection
BoCD21$^+$	42	18	1	1
Negative MNC	3345	2820	76	156
BoCD4$^+$	48	12	1	1
Negative MNC	2817	3158	58	262
BoCD8$^+$	42	142	1	1
Negative MNC	2612	4332	62	31
BoWC1$^+$	27	15	1	1
Negative MNC	2392	2395	88	158

From table 11 it is observed that there are more: -

a) BoCD21^{+} B-lymphocytes in the lung sections of experimentally infected animals than in the naturally infected animals.

b) BoCD4^{+} T-lymphocytes in the lung sections of experimentally infected animals than in the naturally infected animals.

c) BoCD8^{+} T-lymphocytes in the lung sections of naturally infected animals than in the experimentally infected animals.

d) BoCD4^{+} T-lymphocytes in the lung sections of experimentally infected animals than in the naturally infected animals.

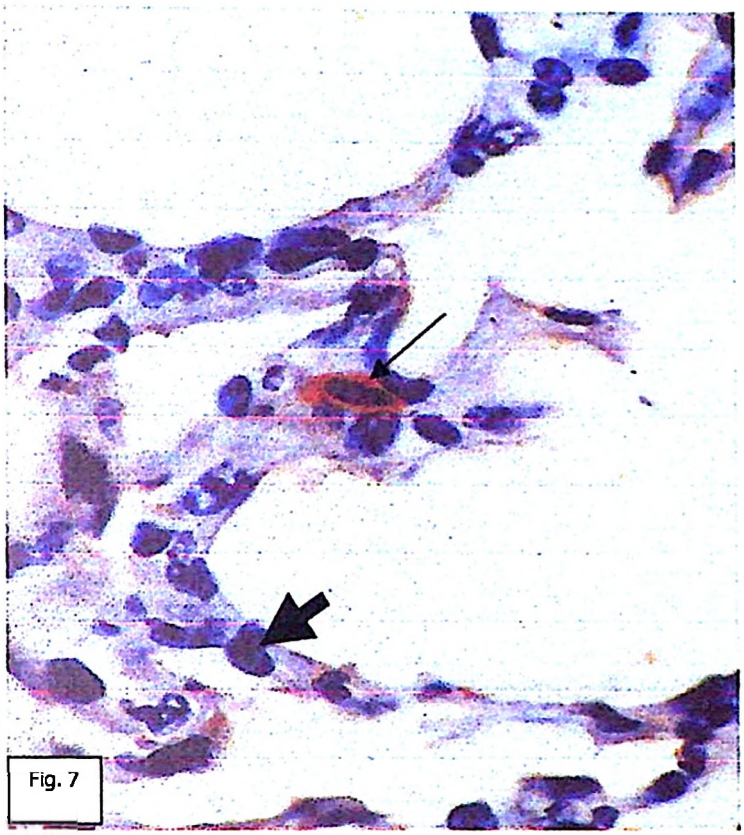

Fig. 7: Positive BoCD21 B-lymphocyte (thin arrow) and negative MNC (thick arrow) in interalveolar tissue of lungs from cattle with experimental ECF. X 1000.

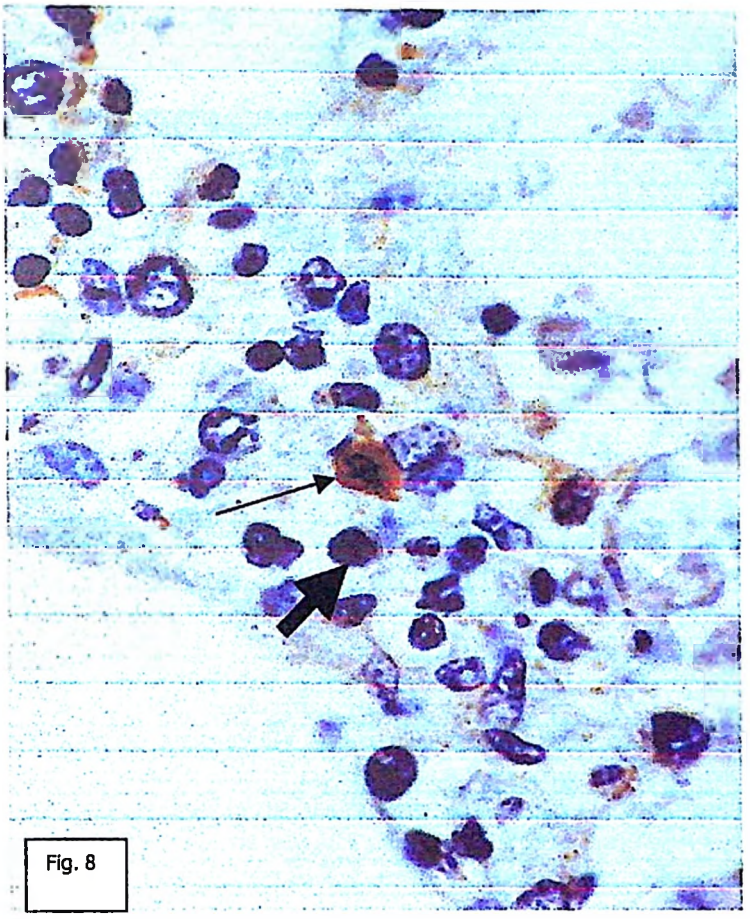

Fig. 8: Positive BoCD21 B-lymphocyte (thin arrow) and negative MNC (thick arrow) in interalveolar tissue of lungs from cattle with natural ECF. X 1000.

Figures 7 and 8 shows BoCD21^{+} B-lymphocyte with brown-stained cytoplasm that was immunohistochemically stained with monoclonal antibody IL-A65 to BoCD21 molecule by the SABC method after heat mediated antigen-retrieval in fixed tissue with TBS-AR.

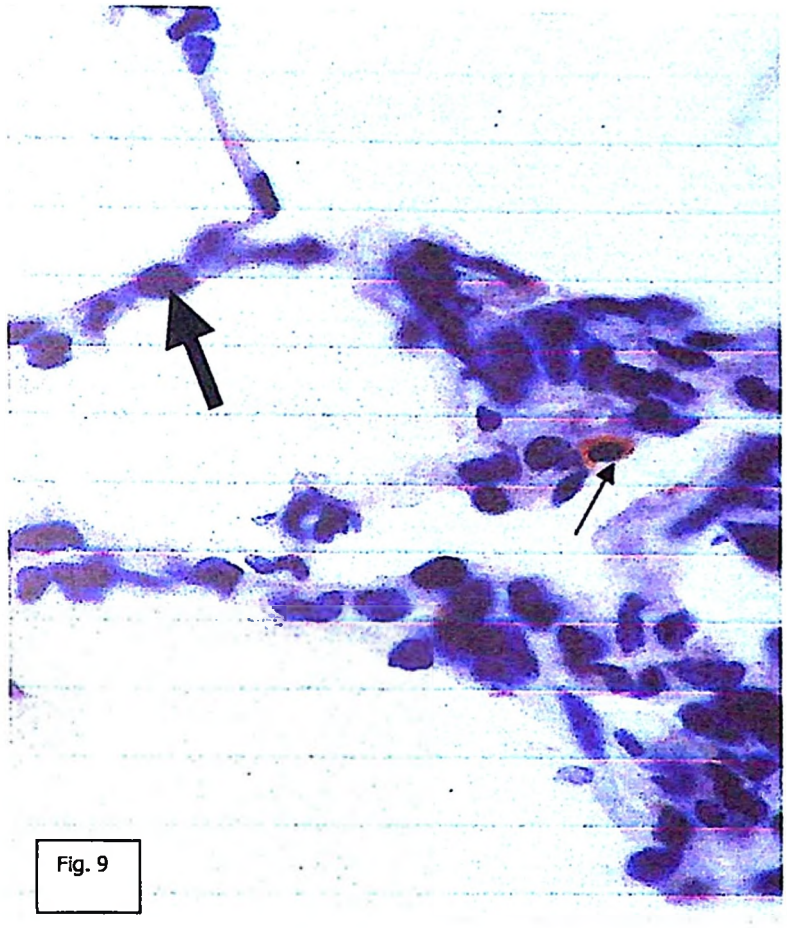

Fig. 9: Positive BoCD4 T-lymphocyte (thin arrow) and negative MNC (thick arrow) in interalveolar tissue of lungs from cattle with experimental ECF. X 1000.

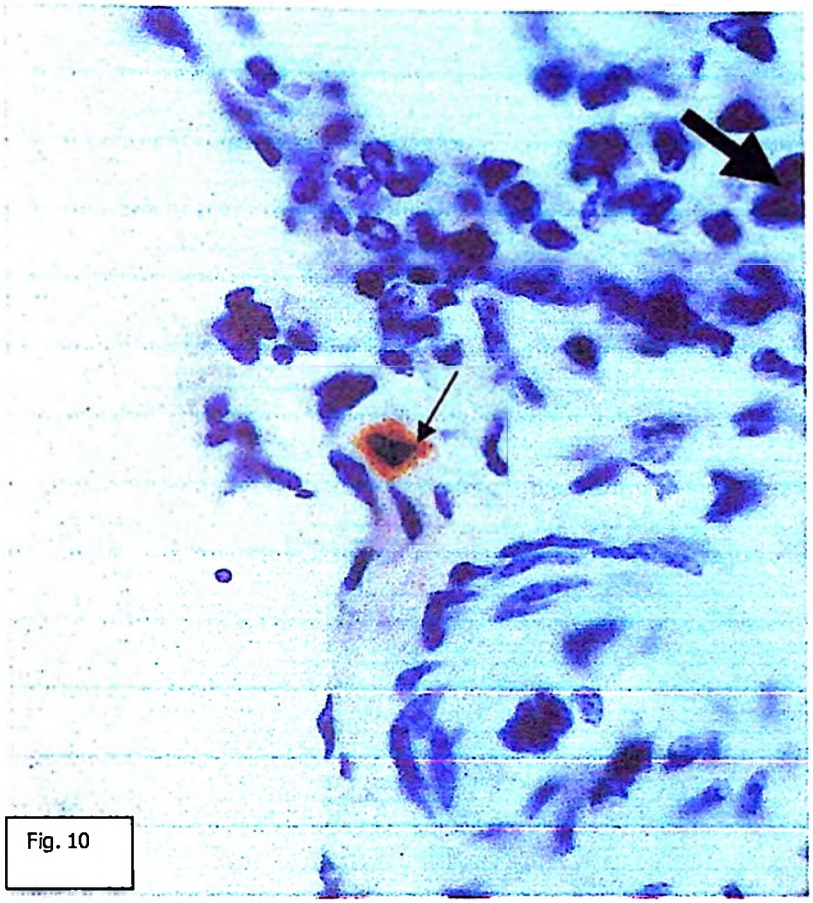

Fig. 10: Positive BoCD4 T-lymphocyte (thin arrow) and negative MNC (thick arrow) in interalveolar tissue of lungs from cattle with natural ECF. X 1000.

Figures 9 and 10 shows positive BoCD4$^+$ T cell with brown-stained cytoplasm that was immunohistochemically stained with monoclonal antibody IL-A11 to BoCD4 molecule by the SABC method after heat mediated antigen-retrieval in fixed tissue with TBS-AR.

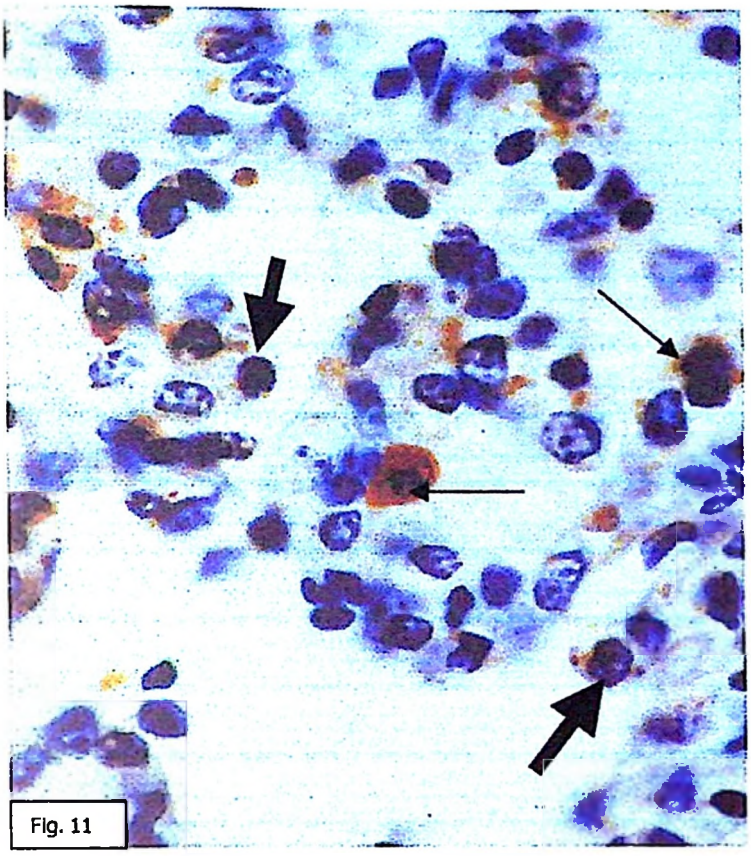

Fig. 11: Positive BoCD8 T-lymphocytes (thin arrows) and negative MNC (thick arrows) in interalveolar tissue of lungs from cattle with experimental ECF. X 1000.

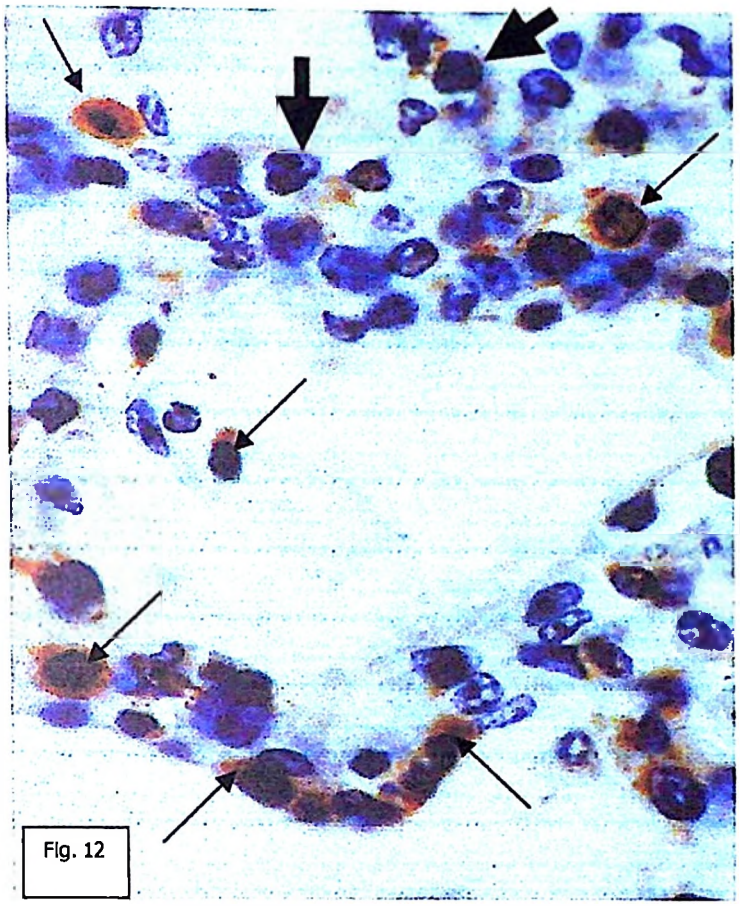

Fig. 12: Positive BoCD8 T-lymphocytes (thin arrows) and negative MNC (thick arrows) in interalveolar tissue of lungs from cattle with natural ECF. X 1000

In Figures 11 and 12 each shows positive BoCD8$^+$ cells with brown-stained cytoplasm that were immunohistochemically stained with monoclonal antibody IL-A105 to BoCD8 molecule by the SABC method after heat mediated antigen-retrieval in fixed tissue with TBS-AR.

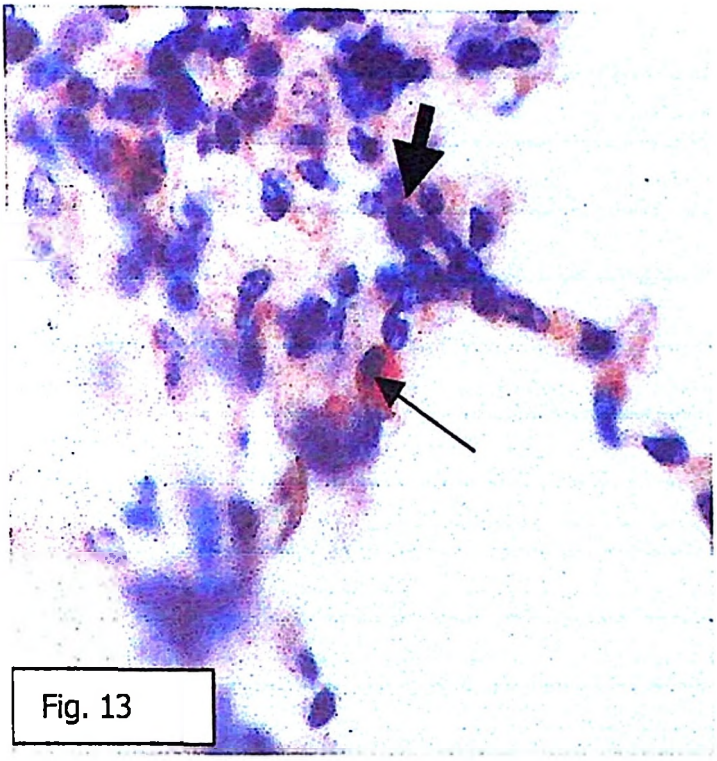

Fig. 13: Positive BoWC1 T-lymphocyte (thin arrow) and negative MNC (thick arrow) in interalveolar tissue of lungs from cattle with experimental ECF. X 1000

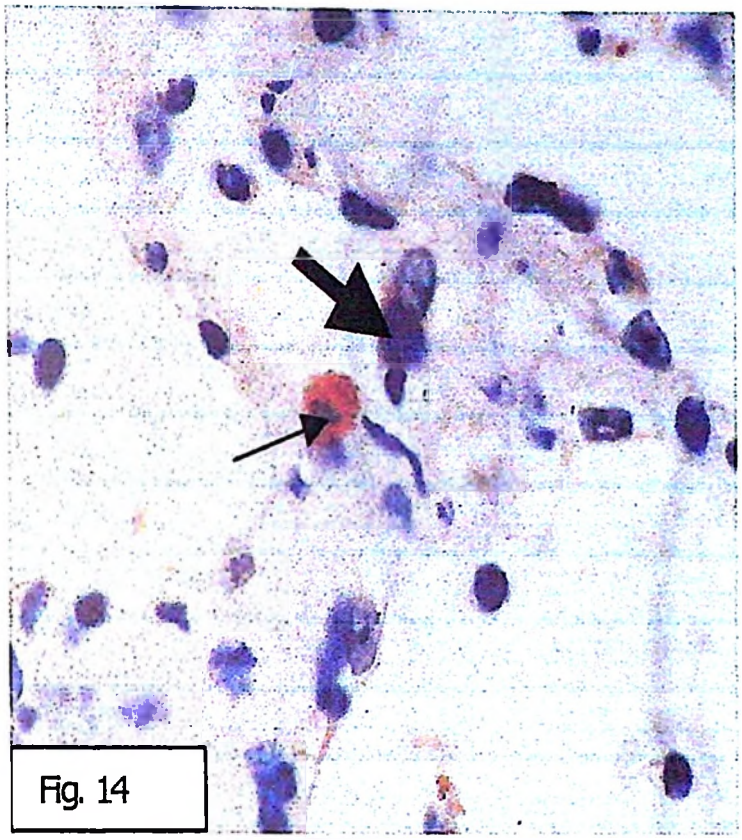

Fig. 14: Positive BoWC1 T-lymphocyte (thin arrow) and negative MNC (thick arrow) in interalveolar tissue of lungs from cattle with natural ECF. X 1000

In Figures 13 and 14 each shows BoWC1^{+} T-cell with brown-stained cytoplasm that was immunohistochemically stained with monoclonal antibody IAH-CC15 to BoWC1 molecule by the SABC method after heat mediated antigen-retrieval in fixed tissue with TBS-AR.

4.4.2 Ratios of lymphocyte types infiltrating lungs of cattle infected with *T. parva*

The ratios of lymphocytes infiltrating bovine lung tissue sections during ECF in experimental and natural infection ranges from 1:1 to 1:9. The different ratios are summarized in Tables 12 and 13.

Table 12 summarizes the ratios of lymphocytes to one another during ECF in experimental and natural infection.

Table 12: Ratios of lymphocyte types infiltrating bovine lungs with ECF

Type of infection	Ratios of lymphocyte type to one another per mm^2					
	$BoCD4^+$: $BoCD8^+$	$BoCD4^+$: $BoWC1^+$	$BoCD4^+$: $BoCD21^+$	$BoCD8^+$: $BoWC1^+$	$BoCD8^+$: $BoCD21^+$	$BoWC1^+$: $BoCD21^+$
Experimental	1:1.07	1:0.56	1:1.36	1:1.56	1:1.27	1:0.64
Natural	1:8.45	1:1.25	1:0.59	1:9.47	1:5.03	1:0.83

The relative ratios of lymphocyte types infiltrating bovine lungs with ECF are summarized in Table 13.

Table 13: Relative ratios of lymphocyte types infiltrating bovine lungs with ECF

Type of infection	Relative ratios of lymphocyte type to one another, per mm^2			
	$BoWC1^+$	$BoCD4^+$	$BoCD8^+$	$BoCD21^+$
[a] Experimental	27 (1:1)	48 (1:1.8)	42 (1:1.6)	42 (1:1.6)
[b] Natural	15 (1:1.3)	12 (1:1)	142 (1:11.8)	18 (1:1.5)

[a] The denominator used to compute ratio is the lowest number of cells/mm^2 in the experimental infection i.e. 27.

[b] The denominator used to compute ratio is the lowest number of cells/mm^2 in the natural infection i.e. 12.

CHAPTER FIVE

5.0 DISCUSSION

While the focus of the study was on characterization of the types of lymphocytes that infiltrate lungs during *Theileria parva* infection, there have been useful observations made with respect to classic pathology that warrant some discussion.

In the experimentally infected animals, the total RBC counts and Hb declined to significantly low levels than the pre-infection levels by day 15 post infection suggesting an anemic situation. However, the PCV did not show significant change between pre-infection and post infection levels. This is in agreement to the findings by Maxie *et al.*, (1982) and Mbassa *et al.*, (1994) that anemia in ECF is usually mild normocytic, normochromic and non-responsive.

There was a marked increase of the total WBC count (leukocytosis) in the experimentally infected animals that started from day 4 pi and rose steadily reaching a peak on day 8 pi coinciding with the onset of fever. Based on the results of differential leucocyte count, the observed leucocytosis was on the expense of neutrophilia that became significantly higher than pre-infection levels by day 15 post-infection. This observation is in contrast to the findings reported by Steck (1928) and Wilde (1966) who observed severe panleukopenia that coincided with the onset of fever. The observed leukocytosis in this study was associated with

lymphocytosis. Leukocytosis declined steadily to precarious levels (1.31 ± 1.09 X 10^9/L) at time the animals were slaughtered. It has been reported that the extensive lympho-destruction that is not confined to parasitized lymphocytes and worsened by the invasion of the bone marrow by the parasitized cells during ECF and perhaps cells infiltrating parenchymatous organs results in a net depletion of circulating leukocytes (Irvin and Morrison, 1987; Mbassa, *et al.,* 1995). Further studies will also be useful in to decipher the peak time for leukopenia and infiltration in the parenchymatous organs.

The noted difference in the percentage of parasitized lymphocytes between the experimental and natural ECF infection seen in the lymph node and spleen smears is perhaps mainly due to time of specimen collection. Whereas in the experimental ECF infection sampling was taken during the midway stage of disease course, in the natural ECF infection sampling was taken in the advanced stage of the disease course. Also, perhaps the amount of sporozoites injected into the animals could also be the reason for the difference. This is attributed to the unique characteristics between the different *T. parva* stocks (Morzaria, 1989).

The most significant gross changes seen in the organs that were examined included pale, slightly raised nodules on kidney surfaces; interlobular emphysema, pulmonary edema and reddening of the lungs; copious frothy exudate in the nasal passages. These findings were more prominent in the natural cases than in the

experimentally infected animals. A moderate straw-coloured fluid was seen in the peritoneal and thoracic cavities of naturally infected animals only. Histologically, the lungs showed widened interalveolar septa infiltrated with mononuclear cells mostly lymphocytes and macrophages. These lesions were reminiscent of interstitial pneumonia that was more prominent in the naturally infected animals than in the artificially infected ones.

The pathomorphological differences between natural and artificial ECF could possibly be due to different stages of infection at which sampling was done. It is possible that in natural infection sampling was done at advanced stages of the disease, whereas in the experimental infection sampling was done at day 15 post-infection which can be considered to be the acute stage of the disease.

Despite the known difficulty to immunostain BoCD4 and BoCD8 epitopes in formalin-fixed paraffin-embedded tissues as alluded to by several authors (Keresztes, 1996; Rathkolb *et al.,* 1996, 1997; Gutierrez, 1999) a successful immunohistochemical protocol was developed for this study on the basis of SABC staining method (Keresztes, 1996; DAKO, 2001) and employing the detergent 0.25% Triton®X to decrease background staining and to enhance antibody penetration (Larson, 1981). Also, the background staining was greatly reduced by including 0.5% full cream milk NIDO - Nestle® in the blocking solution and in the diluents for the primary and secondary antibodies.

The results of immunohistochemical staining in this study have demonstrated that the majority of lymphocyte immunophenotypes infiltrating lungs of cattle experimentally infected with *T. parva* are BoCD4$^+$ lymphocytes at the onset of respiratory distress (i.e. day 15 post-infection the stage when the animals were sampled) and followed by BoCD8$^+$ and BoCD21$^+$ and then by BoWC1$^+$ lymphocytes. It is shown in tables 12 and 13 that in the experimental *Theileria parva* infection, the ratios of lymphocytes infiltrating bovine lungs range from 1:2 and 1:1. BoCD4$^+$and BoCD8$^+$ lymphocytes appear to be in the same ratio. BoCD21$^+$ lymphocytes are slightly more than BoCD4$^+$lymphocytes by about one and one-half times. BoCD4$^+$ lymphocytes are about two times more than the BoWC1$^+$ lymphocytes. BoWC1$^+$ lymphocytes are about one and one-half times more than BoCD8$^+$ lymphocytes. BoCD21$^+$ lymphocytes are about one and one-quarter times more than BoCD8$^+$ lymphocytes. BoWC1$^+$ lymphocytes are about two times more than BoCD21$^+$ lymphocytes.

The study has also showed that the majority of lymphocyte immunophenotypes infiltrating lungs in the naturally infected cattle at the advanced stages of infection are BoCD8$^+$ lymphocytes followed by BoCD21$^+$, BoWC1$^+$ and then by BoCD4$^+$ lymphocytes. From Tables 12 and 13, it is observed that BoCD8$^+$ lymphocytes infiltrating bovine lungs in the natural *Theileria parva* infection are about 8 times more than the BoCD4$^+$ lymphocytes, BoCD21$^+$ lymphocytes are about 5 times more than the BoCD8$^+$ lymphocytes and BoWC1$^+$ lymphocytes are about 9 times mores

than BoCD8$^+$ lymphocytes; BoCD4$^+$ lymphocytes are about 2 times more than the BoCD21$^+$ lymphocytes; BoWC1$^+$ lymphocytes are about one and one-quarter times more than BoCD4$^+$ lymphocytes; BoCD21$^+$ and BoWC1$^+$ lymphocytes appear to be in the same ratio.

Finding of more BoCD4$^+$ (helper T cells) and BoCD8$^+$ (cytotoxic T cells) than BoCD21$^+$ and BoWC1$^+$ in the lungs during ECF suggested these lymphocytes play a dominant role in the local immune response in ECF, while BoCD21$^+$ (B cells) and BoWC1$^+$ (γ/δ cells) play a lesser role.

The high number of BoCD4$^+$ that was observed at the onset of dyspnea in the experimentally infected animals indicated that satisfactory degree of cell-mediated immunity was elicited to control the infection. It is known from other studies, that BoCD4$^+$ release cytokines, particularly TNF-β and IFN-γ, which are responsible for Th1-type response essential for controlling intracellular pathogens such as *Theileria parva* (Taracha *et al.*, 1998). In addition, BoCD4$^+$ are known to produce macrophage-activating cytokines such as IFN-γ. Such activated macrophages produce mediators such as NO, which destroy the intracellular schizont (Ahmed and Mehlhorn, 1999). The TNF-β and IFN-γ cytokines in the case of ECF recruit BoCD8$^+$ which are cytotoxic T cells (CTL). The CTL secrete molecules that destroy the lymphoblasts containing schizonts before they can release fresh crop of merozoites able to infect other lymphocytes (Taracha *et al.*, 1998). The results of this study

have also shown that BoCD4$^+$ decreased while the BoCD8$^+$ increased in the advanced stages of the disease as observed in the cases of natural infection when compared to experimentally infected animals that were slaughtered at day 15 post infection at onset of respiratory distress.

The results of this study showed higher mean number of BoCD8$^+$ in the advanced stage than in the acute stage of *Theileria parva* infection. The apparent higher numbers of BoCD8$^+$ in the advanced stage of ECF infection suggests that they may have more roles to play during the advanced stage than in the acute stage of *Theileria parva* infection.

The increase in BoCD8$^+$ in comparison to BoCD4$^+$ in the advanced stages of the disease suggests an adequate degree of cell-mediated immunity in an attempt to control the infection. In addition, BoCD8$^+$ are known to be responsible for the release of cytokines, particularly IFN-γ, which is responsible for Th-1 type response. Also the CTL cells secrete TNF-β that is directly cytotoxic to the infected cells. It is also shown that IFN-γ and TNF-α are synergistic in promoting NO synthesis that is required in the control of infection, therefore increasing the capacity to fight the infection (Ahmed and Mehlhorn, 1999).

The results of this study showed higher mean number of BoCD21$^+$ (B-lymphocytes) during the acute stage than in the advanced stages of *Theileria parva* infection. The

apparent higher mean number of B-lymphocytes during acute of the course of infection indicates that they may have more roles to play in this stage than in the advanced stages of *Theileria parva* infection.

The scarcity of B cells in the lymphocytic infiltrates in the lungs during the advanced stages of ECF suggests that the local humoral response is of little importance as is with other intracellular pathogens (Su *et al.,* 1997; Yang and Brunham, 1998). Perhaps, B cells as antigen-presenting cells would process the schizont antigens and present them to Th1 helper cells leading to further production of Th1 cytokines.

The results of this study also showed higher mean number of BoWC1$^+$ lymphocytes during the acute stage than in the advanced stage of *Theileria parva* infection. The apparent higher numbers of BoWC1$^+$ during the acute stage of the disease suggests that in this stage BoWC1$^+$ may have more roles to play than in the advanced stage of *Theileria parva* infection. This is in agreement with the obervation made by McKeever, (2001); and Daubenberger *et al.,* (1999) that BoWC1$^+$ have a significant role in control of the disease before the more specific cytotoxic BoCD8$^+$ cells are recruited in substantial numbers to combat the infection.

The high mean numbers of negative MNC observed in the interstitium of alveolar parenchyma in the lungs included lymphocytes and macrophages. Presence of negative lymphocytes in the lung tissue could be as indicated by Naessens *et al.,*

(1985) and Pinder *et al.*, (1981) as due to failure of the cells to express the anticipated surface markers.

Notwithstanding the mechanisms that attract these lymphocytes into the lungs during *Theileria parva* infection, their presence suggests that they locally release cytokines that contribute towards development of inflammation and pulmonary edema. This would happen even before the lymphocytes start to disintegrate during the course of the infection. Lymphocytes are known for their direct and indirect production of pro-inflammatory cytokines some of which are known to have vasoactive influences like IL-1 and TNF-α (Wewers *et al.*, 1997). The disintegrating lymphocytes also release lysosomal substances whose destructive effects on the tissue cells may cascade into vascular changes. Presence of lymphocytes in the lungs, therefore, indicates their role in inducing edema in the lungs. However, the precise function and the cytokine secretion pattern of bovine B-cells and subsets of T-cells in the lungs of cattle infected with *T. parva* remains to be elucidated. The *in situ* examination of the expression of cytokine mRNA in the lungs of cattle infected with *T. parva* in the course of disease could provide valuable information regarding the specific role of B-cells and subsets of T-cells involved in the development of pulmonary edema.

Similarly, vasoactive substances released during disintegration of lymphocytes activate the complement cascade and subsequent formation of anaphylatoxins (C3a

and C5a) are also responsible for the development of pulmonary oedema (Shitakha *et al.* 1983; Lawrence *et al.* 1994).

CHAPTER SIX

6.0 CONCLUSION

1. The presence of such lymphocytes (presumably immunocompetent) in lungs points to local cytokine production and or consumption that could lead to microvascular leakage.

2. The present study has demonstrated and characterised major lymphocyte subpopulations in the lung by the use of immunohistochemistry. The cells characterised are known to secrete various cytokines that directly or indirectly are linked to increase of vascular permeability.

3. The decline in total WBC counts after day 8 is either due to infiltration into parenchymatous organs or lymphocytolysis or both.

4. This study is yet another contribution to the pathogenetic information that is useful in optimisation of regimes in management of ECF and with reference to vascular permeability antagonists particularly in ECF associated pulmonary edema in cattle.

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APPENDICES

APPENDIX I - IMMUNOHISTOCHEMICAL PROTOCOL

DAY 1

1. Deparaffinization in 3 sequential xylene washes, 5 minutes each.
2. Rehydration in alcohol baths:
   a) Absolute alcohol, 3 changes, 5 minutes
   b) 95%, 1 change, 5 minutes
   c) 70% - 1 change, 5 minutes
   d) Three washes in distilled water (dH_2O), 5 minutes
3. Antigen retrieval solution – 20 minutes at $90^0 – 95^0$ C temperature.
   (a) 10mM Citrate buffer pH 6.0 (APPENDIX IV)
   (b) Tris-buffered saline (TBS-ARS) pH 8.2 (APPENDIX V)
   After boiling the slides are cooled slowly in the same retrieval solution for 15 minutes.
   Washes in TBS, 3X 5 minutes.
4. Quenching in 0.3% H_2O_2 in TBS for 30 minutes, room temperature.
   Washes in TBS-T, 3X 5 minutes.
5. Tap off excess liquid and wipe away excess with KimWipe. Slides supported on flat rack in an incubation chamber (enamel tray) with a tight fitting lid (humidity maintained by cloth soaked in TBS).
6. Blocking in 10% rabbit normal serum (Rabbit normal serum, X0902, DAKO,) diluted in TBS-T containing 0.5% milk.
   Colourless nail polish was used to ring sections on slides in order to prevent spread and run of solutions from the slides.
   Blocking done for 1 hour at room temperature in the humidified chamber.
7. Tap off excess serum and wipe away excess with KimWipe. **DO NOT RINSE SECTIONS WITH BUFFER BEFORE APPLYING PRIMARY ANTIBODY.**
8. INCUBATION WITH PRIMARY ANTIBODIES: -
   Incubation of target tissue and negative tissue control with the primary antibody at dilution 1:100 in TBS-T containing 0.5% milk.
   Incubation overnight, room temperature, humidified chamber.

DAY 2

9. Tap off excess antibody
10. Washes 3X 10 minutes in TBS-T
11. Incubation with secondary antibody (2^0 Ab) diluted 1:500 in TBS-T containing 0.25% milk, 1% BSA and 10% RNS.
    Link antibody - biotinylated Rabbit anti-Mouse Immunoglobulins (E0413, DAKO), room temperature; 1.30 hours; humidified chamber.
12. Washes 4X 5 minutes in TBS-T.
13. Apply StreptABComplex/HRP (K 0377, DAKO) dilution 1:500 in TBS.
    The diluted StreptABComplex/HRP was prepared ½ hour before being applied.
    Incubation in humidified chamber; 1.30 hrs; room temperature.
14. Tap off excess liquid
15. Washes 4X 5 minutes in TBS-T.
16. Incubate in fresh substrate-chromogenic reagent solution for HorseRadish Peroxidase (HRP) - 0.01% H_2O_2/0.05% DAB in TBS solution for 30 minutes; room temperature; humidified chamber.
    Apply substrate-chromogen solution and incubate in the dark until desired color intensity has developed.
17. Discard off excess DAB/ H_2O_2_mixture from slide using transfer pipette into a waste hazard container.
18. Rinse in dH_2O 1X 2 minutes to stop the reaction.
19. Washes 2X 2 minutes in TBS-T.
20. Counterstaining in Mayer's Haematoxylin for 10 - 20 seconds, then rinse with distilled water
21. Dehydration of sections in ethanol baths:
    70% ethanol: 5 minutes
    95% ethanol: 5 minutes
    100% absolute ethanol: 3 changes, 5 minutes each
22. Clearing: -
    Xylene: 3 changes, 5 minutes each
23. Covering tissue section
    Applying DPX mounting medium (BDH Limited, Poole. England) and put cover slip.
24. Examined under bright field microscope (Olympus BHT/BH-2 microscope)
    Antigenic sites seen as positive cytoplasm staining brown.

APPENDIX II: COATING SLIDES IN CHROM-GELATIN

(a) Rinse slides in running water.
(b) Rinse in distilled water – 2 changes.
(c) Heat 400-ml dH_2O to 70^0 C in conical flask.
(d) Add 1.5g gelatine (Bactor-Agar, purified) and put magnetic stirrer in the flask at 70^0 C.
(e) Then dissolve 0.15g of chrome alum at 70^0 C (Chromium III potassium sulfate dodecahydrate – $KCr(SO_4)_2.12H_2O$
(f) Filter into jar(s).
(g) Dip slides 5-10 times, 2 minutes in the chrome alum at 60^0 C.
(h) Blot dry.
(i) Dry in oven overnight at 40^0-60^0 C.
(j) Remove and store in closed box at room temperature.

APPENDIX III: 4% NEUTRAL BUFFERED FORMALDEHYDE

1) Sodium phosphate, monobasic dihydrate ($NaH_2PO_4.2H_2O$)	18g
2) Sodium phosphate, dibasic dihydrate ($Na_2HPO_4.2H_2O$)	32.8g
3) Formaldehyde 36%	444g
4) Distilled water	4000g

APPENDIX IV: 10mM CITRATE BUFFER (pH 6.0) - ANTIGEN RETRIEVAL SOLUTION

1) Tri-sodium Citrate dihyadrate (Sigma S-4641)	5.88g
2) 0.2M Hydrochloric acid solution	44.0 mL
3) Distilled water	1956 mL

APPENDIX V: TRIS-BUFFERED SALINE BUFFER (pH 8.2) - ANTIGEN RETRIEVAL SOLUTION (TBS-ARS)

1) Tris (hydroxymethyl) aminomethan, Sigma T-1378	2.42g
2) Sodium chloride	8.77g
3) Distilled water	1000ml

APPENDIX VI: TRIS-BUFFERED SALINE (TBS) BUFFER (pH 7.6) AND TRIS-BUFFERED SALINE CONTAINING 0.25% TRITON X-100 (TBS-T) (pH 7.6)

10X stock Tris (hydroxymethyl) aminomethan, Sigma T-1378	60.55g/L Tris 50mM 73.8 g/L NaCl	Tris pH 7.6 (TBS) Dilute stock 1:10, Adjust the pH to 7.6 with HCl
		Tris-Triton pH 7.6 (TBS-T) Dilute stock 1:10, Add 0.25% Triton X-100 Adjust the pH to 7.6 with HCl,