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Carrier state studies in *Theileria parva* infected cattle in Zimbabwe

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Thesis submitted for Master of Philosophy degree in Veterinary Science
University of Edinburgh
1997

Dedication

To, Panashe, Mudikani and Catherine

Such little recompense for long hours of loneliness.

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CHAPTER FOUR

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List of Abbreviations

Ab	Antibody
Ag	Antigen
AEC-SA	Associated Chemical Enterprises - South Africa
bp	Base pairs
cpm	counts per minute
CTVM	Centre for Tropical Veterinary Medicine
DNA	Deoxyribonucleic Acid
dNTP's	Deoxynucleotide triphosphates
EDTA	Ethylene Diamine Tetraacetic Acid
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
Fig	Figure
FP	Forward Primer
IFAT	Indirect Fluorescent Antibody Test
ILRI	International Livestock Research Institute
Kb	Kilo bases
Lab	Laboratory
LPG	Left Prescapular Gland
MAb	Monoclonal antibody
Macs	Macroschizonts
Macros	macroschizonts
MEM	Minimum Essential Medium
MM	Master Mix
µl	microlitre
µg	microgram
µM	micro-molar
mins	minutes
ml	millilitre
mM	milli-molar
MoR	Moderate reaction

MR	Mild reaction
n.d.	not done
ng	nanogram
NR	No reaction
-ve	negative
nps	no parasites seen
PBM	peripheral blood mononuclear cells
PBS	Phosphate buffered saline
p.c.	post challenge
piros	piroplasms
+ve	positive
p.i.	post infection
PCR	Polymerase Chain Reaction
p.t.a.	post tick application
RBC	Red blood cell
Ref	Reference
recip.	Reciprocal
REG	Right Ear Gland
rpm	revolutions per minute
RP	Reverse Primer
RPG	Right prescapular Gland
SDS	Sodium dodecyl sulphate
SR	Severe reaction
Taq	*Thermus aquaticus*
TAE	Tris-acetate EDTA buffer
TBE	Tris-borate EDTA buffer
t.e.	tick equivalent
TE	Tris EDTA buffer
Temp	Temperature
VRL	Veterinary Research Laboratories

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SUMMARY

Theileriosis caused by the protozoan parasite *Theileria parva,* transmitted by *Rhipicephalus appendiculatus* ticks, is an important animal disease for the livestock industry in Zimbabwe. To characterise Zimbabwean *T. parva* parasites, susceptible calves were infected by 3 methods; (a) subcutaneous inoculation with three *T. parva* ground-up-tick stabilates, (b) application of ticks collected from the field and (c) field exposure. The aim of the study was to characterise the duration of parasitaemia following infection by the 3 methods, and to compare the sensitivity of the Polymerase Chain Reaction (PCR) assay with conventional methods for detection of carrier status.

All the animals seroconverted following infection or tick exposure, as detected in the Indirect fluorescent antibody test (IFAT) for antibodies to *T. parva,* were demonstrated to be carriers of infection by PCR for the duration of experiment, and immune to *T. parva* Avery challenge after 522 days under tick free conditions. In order to demonstrate a carrier status clean *R. appendiculatus* nymphs were fed on the recovered animals on 4 dates up to 456 days post infection and allowed to moult to adults and examined for infection.. Ten out of 14 animals had a carrier status that resulted in tick infections.

Cultures of *Theileria* infected lymphoblastoid cells were attempted from animals which developed theileriosis at Hunyani estates on field exposure. The monoclonal antibody (MAb) profile indicated infections in each case with *Theileria taurotragi* isolates. However, the clinical and post-mortem examination findings of the calves were typical of *T. parva* infection. Animals which recovered after treatment had persistent piroplasm parasitaemia and high infection prevalence in ticks applied. This implies that onward transmission in cattle occurs with this parasite, with important implications for causation of severe theileriosis in cattle. Tick infections did not occur following feeding on calves recovered from buffalo derived *T. parva* Bally Vaughan isolate or with *T. parva* Avery isolate. Infections with field parasites were found to result in more efficient transmission of *Theileria* parasites to ticks than the Bolvac® vaccine stock; the former could be associated with the more persistent piroplasm parasitaemia in the recovered animals and the severity of the *Theileria* clinical

reactions. PCR using p104 primer sequences for rhoptry / microneme antigens of *T. parva* was the most sensitive indicator of carrier status. Clinical signs upon infection were not correlated with persistence of infection; carriage of parasite DNA occurred irrespective of initial severity. The IFA test was validated using sera from the carrier animals and naive animals pre-infection. The IFAT titre required to detect all infected animals was 1:160, at which the test had a specificity of only 64.63%. An IFAT cut-off titre of 1:320 resulted in a sensitivity and specificity of 90.49.% and 88.06% respectively. The current cut-off used in Zimbabwe (1:640) resulted in 100% specificity, but sensitivity of only 64.63%. The PCR employed provided a highly sensitive method for determining *T. parva* infection, was more sensitive than IFAT (cut-off 1:640) and tick pick up. At least 71.7% of the samples tested post infection were positive by PCR compared to the 44% positives by IFAT at a cut-off of 1:640. Only 15% of the tick application experiments were positive compared to the 80.6% PCR positive tests and 38.9% IFAT positives when the tests were carried out synchronously.

Each of the isolates resulted in a carrier state detectable by PCR and this lasted for at least 500 days, with *Theileria* transmission to ticks recorded up to 456 days post infection. This is longer than recorded in previous studies in Zimbabwe. These results suggest that carrier status is the normal state following infection. Indications are that *T. parva* Boleni is the most suitable immunising parasite stock; although *T. parva* Avery and Bally Vaughan infections did not result in onward transmission to ticks, these parasites are of higher pathogenicity which would be disadvantageous in vaccination.

CHAPTER ONE

Carrier state studies in *Theileria parva* infected cattle in Zimbabwe

INTRODUCTION

Theileria parva the causative protozoan organism of East Coast Fever (ECF) in cattle was introduced into Zimbabwe in 1902 by a shipment of cattle which originated from German East Africa (Tanganyika), (Lawrence and Norval, 1979). The cattle shipment was meant to restock the country after the rinderpest panzootic of 1896-1897 (Thomson, 1985).

To control the disease it was necessary to control its tick vector *Rhipicephalus appendiculatus* and therefore short interval dipping was made compulsory in 1914. East Coast Fever was believed to have been eradicated from Zimbabwe in the 1950's (Lawrence and Norval, 1979; Thomson, 1984), although another form of theileriosis, known as "Zimbabwean theileriosis" or "January disease" continues to be a problem (Cranefield, 1991; Lawrence 1991a, 1991b; Perry and Young, 1995).

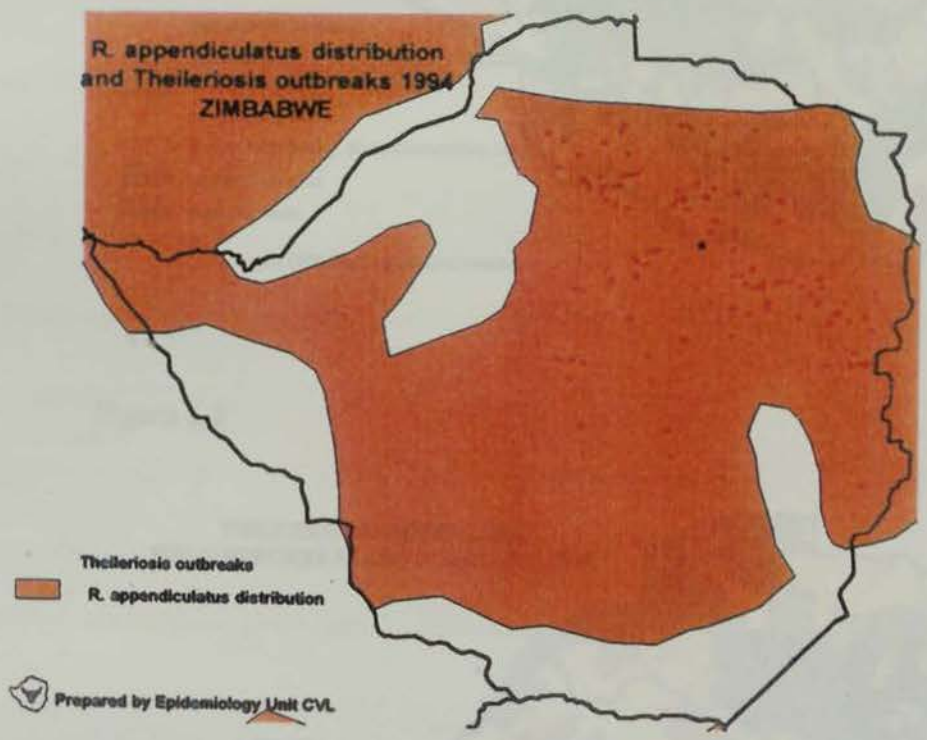
In Zimbabwe, the control of ticks and tick-borne diseases of cattle has been one of the most important factors contributing to the development of the livestock industry, which is of major economic importance to the country. Theileriosis is endemic to certain farms / areas in which a stable to unstable situation appears to prevail, although there is a risk of disease outbreaks because of cattle movement. The government of Zimbabwe spent an estimated US$9 million in trying to control the disease in the financial year 1988/89 (Perry *et al.*, 1990), due to direct mortalities, productivity losses, control costs, extension services, financing, diagnosis, training

and research. *Theileria parva* is a tickborne protozoan parasite transmitted by ticks of the genus *Rhipicephalus* causing theileriosis in cattle in East and Central Africa. In Zimbabwe, theileriosis mainly occurs on the Zimbabwean highveld where the brown ear tick *Rhipicephalus appendiculatus* is found. Figure 1.1(a) shows the distribution of the *R. appendiculatus* ticks in Zimbabwe and the Theileriosis outbreaks of 1994. Figure 1.1(b) and 1.2 shows the theileriosis outbreaks between January and December 1995 and January to September of 1996 respectively. Data was obtained from the Director of veterinary services field report returns and the cases confirmed by Giemsa stained microscopic examination of samples submitted. The outbreaks mainly occur in the commercial farming areas of Zimbabwe which are found along the Highveld of Zimbabwe receiving greater than 400mm annual rainfall, thus an excellent environment for the tick vector.. Theileriosis associated with the presence of buffalo is considered to becoming more significant as a result of the integrated livestock / game farming on the highveld and in the lowveld in areas with the National Parks [Figure 1.1(b)game] (Hove *et al.*, In press).

Bovine theileriosis is one of the most important diseases restricting the development of this farming practice. As a result of theileriosis and other tick-borne diseases it has been necessary to practise weekly dipping during the rainy season, (November to March) when the adult *R. appendiculatus* tick is active (Short and Norval, 1981), and dipping is also carried out fortnightly during the dry season. Government legislation on the control of theileriosis, (Theileriosis Animal Health Regulation of 1976) stipulates that it is a notifiable disease and when outbreaks occur there is temporary suspension of movement of stock to and from infected properties for a period of

twenty-eight days, during which animals are dipped or sprayed on every fourth day and strict tick control is applied. The main disadvantage of intensive tick control is that animals free of ticks will remain susceptible to tick-borne diseases including theileriosis and any breakdown may result in outbreaks of other tick-borne diseases.

Figure 1.1 (a)



* Harare area

Figure 1.1(b)

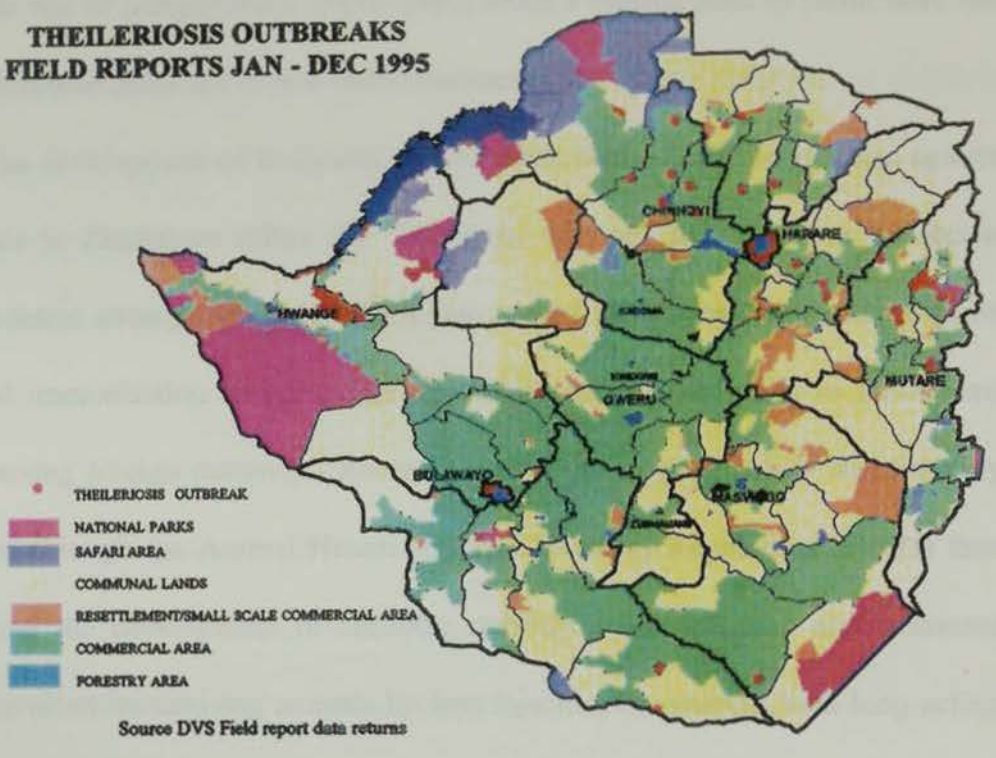
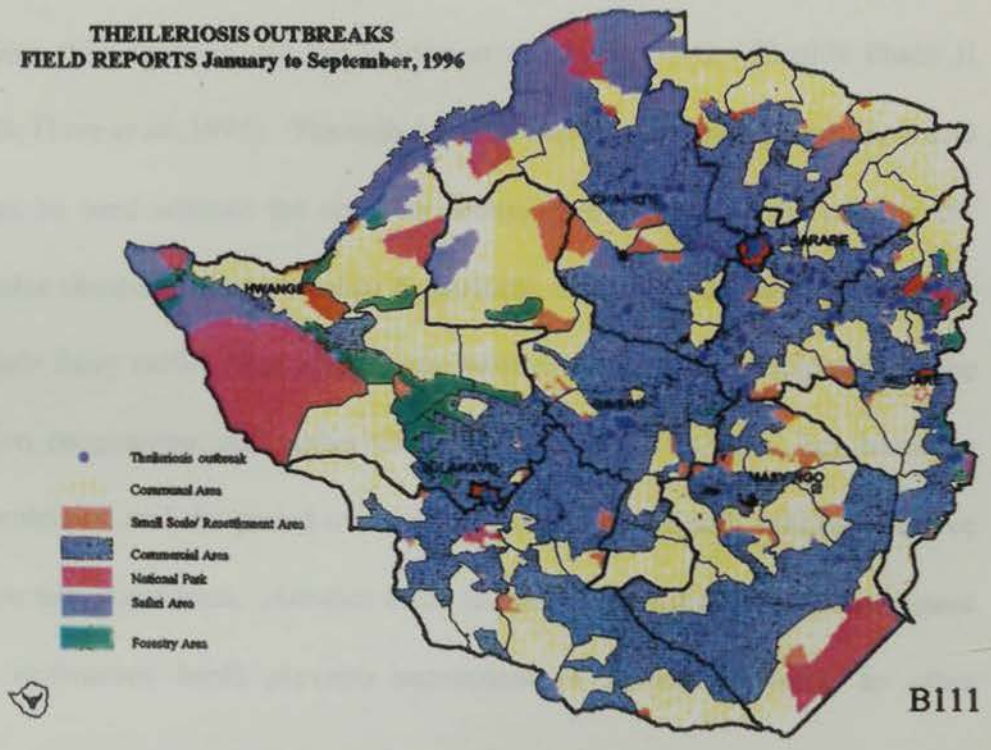


Figure 1.2



* Harare area

Serious disease outbreaks were observed previously (Lawrence *et al.*, 1980), and also during the war of independence (1972-1980) when a million head of cattle were lost in the communal areas due to tick-borne diseases.

The development of treatment and infection method of immunisation against theileriosis in Zimbabwe offers the Veterinary Services an alternative tick-borne disease control strategy which involves less use of acaricides and relies more on controlled immunisation and the development of natural immunity to theileriosis thereby saving foreign currency. Recently a strategic dipping policy was formally introduced through the Animal Health (Cattle Cleansing) Regulations, (1993) thus encouraging the development of enzootic stability. The infection and treatment method involves immunising animals by first injecting the animals with long-acting tetracycline followed by inoculation of the stabilate of *T. parva* (Boleni) sporozoites. In Zimbabwe the *T. parva* Boleni parasite stock was selected for its relatively low virulence and ability to confer immunity against several local stocks and two from eastern Africa (Uilenberg *et al.*, 1982; Irvin *et al.*, 1989; FAO / Danida Phase II report, 1993; Hove *et al.*,1995). Through use of a lower immunising dose, Bolvac® stabilate can be used without the need for tetracycline treatment thus making the whole exercise cheaper and much easier to perform, although treatment is necessary for high grade dairy cattle. One of the main worries of farmers participating in the immunisation programme, and authorities concerned in the exercise, has been the length of protection and the period over which immunised animals remain infective to the vector tick population. Another issue is that at present the cattle movement policy for immunised herds prevents movement of immunised cattle to other

properties except to the abattoir. This implies that immunised animals pose more of a threat, regarding the spread of the parasite than naturally infected animals that might also remain as reservoirs of disease. Animals that are reservoirs of *Theileria parva* infection may present a risk of spread of the disease to other disease free areas where the vector exists (Figure 2.1). The theileriosis team at Harare already has evidence suggesting that some herds remain free of reported disease in theileriosis endemic areas but are moving cattle freely and might have significant numbers of *Theileria* "carrier" animals (T. Hove, personal communication).

This presents the Zimbabwe Veterinary Services Department with a new scenario that may require changes in tick-borne disease control policy and legislation governing *Theileria* infected herds as these may provide foci of infection. Yet, another issue to be studied and established is the ability of the carrier animals to infect ticks resulting in high enough tick infection prevalence to achieve endemic stability in their subsequent hosts. A serological survey of cattle (Norval *et al.*, 1985) found that *T. parva* positive reactors occurred throughout Zimbabwe, including areas in which no outbreaks of theileriosis had been recorded. *Theileria taurotragi* has been isolated in Zimbabwe (Lawrence and Mackenzie, 1980; Uilenberg *et al.*, 1982) and is known to show some cross-reaction with the *T. parva* group on the IFAT (de Vos and Roos, 1981; Uilenberg *et al.*, 1982) and this could have affected the results as the test is very subjective. There is therefore a need to develop a sensitive test for the detection of parasites in *Theileria* carrier animals in order to detect animals which have had previous exposure to the parasite and hence understand the true epidemiology of the disease in Zimbabwe.

An improved test is required for the detection of bovine theileriosis, as the sensitivity and specificity of current antibody detection tests and stained blood smear examinations are inadequate. The current serological techniques used to diagnose theileriosis may not detect carrier animals and do not distinguish between *T. parva* and closely related parasites, such as *T. taurotragi.* Positive serological reactions to *Theileria* parasites may simply indicate a previous exposure to theileriosis, current infection or the presence of antibodies against other non-pathogenic *Theileria* parasites. A false negative reaction observed in serological tests may result from rearrangement of the antigens due to harsh treatment during the process of extraction and storage or the reduction in antibody levels to schizonts and piroplasms with time as seen in the immunofluorescent tests. False negative results can result from natural or induced tolerance, antibiotic induced immunoglobulin suppression, incomplete or blocking antibody, non-specific inhibitors like anticomplementary serum or tissue culture toxic substances or the test could be very sensitive (Thrusfield, 1995). Mackenzie and Lawrence (1979) demonstrated that an animal with a non-specific schizont antibody titre could transmit a lethal infection. This results in difficulties in the selection of non-exposed and therefore susceptible animals for experimental purposes, and also in inaccuracies in epidemiological surveys and live animal exports to countries in the region.

Identification of *T. parva* carriers by subinoculation of blood into susceptible animals and tick feeding is not considered to be feasible because of the number of animals that would be involved and the labour and expense required. The recent development of specific DNA probes and nucleic acid hybridisation protocols to

detect parasites directly in blood from cattle has several advantages over conventional microscopic, serologic and subinoculation techniques. The polymerase chain reaction (PCR) is an *in-vitro* method of nucleic acid synthesis by which a particular segment of target deoxy-ribonucleic acid (DNA) can be specifically replicated. It involves two oligonucleotide primers that flank the parasite DNA fragment to be amplified and repeated cycles of heat denaturation of the DNA, annealing of primers to their complementary sequences, and extension of the annealed primers with *Thermus aquaticus (Taq)* DNA polymerase. The PCR assay could prove to be useful in epidemiological studies in detecting very low parasitaemias in carrier animals because the method has resulted in the highest level of sensitivity for the detection of many organisms, including the piroplasms of *Babesia bovis* (Fahrimal *et al.*, 1992). Specificity can be manipulated by the choice of primers to gene sequences that are conserved or variable between related organisms, if such sequence information is available. Primers can be selected which are specific for *T. parva* and will not amplify the more distantly related *T. taurotragi* or *T mutans* DNA. The test takes less than twenty-four hours , requires less expensive equipment than serology by fluorescent antibody or enzyme linked immunosorbent assays and is easy to perform. This study is aimed at developing an improved method of detecting *T. parva*-carriers based on the PCR and comparing it with other methods of parasite detection in infected / immunised recovered cattle.

The objectives of this study were to compare a PCR assay with other methods of assessing *T. parva* infection or carrier status in animals infected with *T. parva* Boleni and other Zimbabwean parasites. The other methods used for assessing

infection or carrier status were microscopy, serology, nymphal *R. appendiculatus* feeding and dissection of the subsequent adults to determine mean prevalence of infections.

The first described PCR assays for the detection of *T. parva* used primer sequences based on the p67 gene (Bishop *et al.*, 1992). The p104 primer sequence (based on work by R.Skilton, ILRI) was utilised after comparing the results of the two PCR assays. At the CTVM, Edinburgh the sensitivity of the PCR assay using the p67 primers was assessed using *T. parva* Muguga blood stabilate of known amounts and piroplasm DNA. The assay's specificity was performed on 1 ng aliquots of DNA of other *Theileria* species and other haemoparasites including *Babesia bigemina, Babesia bovis, Trypanosoma congolense, Cowdria ruminantium, and Anaplasma marginale.* Other methods of DNA extraction were tested in-order to develop an easy reproducible method for preparing samples for PCR.

The sensitivity and specificity of the p67 PCR was assessed by infecting calves with *T. parva* Boleni and Marikebuni parasites which are known to cause carrier status (Koch, 1990; Bishop *et al.*, 1992). The sensitivity of the polymerase chain reaction for the detection of *T. parva* parasites was compared with the Giemsa stained blood smears, and indirect fluorescent antibody test. Using primer sequences designed by Bishop *et al.*, (1992) for the *T. parva* p67 sporozoite antigen which is thought, on the basis of the absence of observed restriction fragment length polymorphism to be conserved among *T. parva* stocks. The following haemoparasite stocks were tested by PCR; *T. parva* (Muguga), *T. parva* (Boleni), *T. taurotragi, Theileria mutans, T. parva* Lawrencei, *Theileria annulata and Theileria sergenti.*

Other haemoparasite DNA material including *Babesia bigemina* and *Babesia bovis, Anaplasma marginale, Cowdria ruminantium* and *Trypanosoma congolense* were tested to confirm the specificity of the assay.

At the Veterinary Research Laboratories, Harare, calves were infected with different *T. parva* stocks isolated in Zimbabwe. Clean nymphal *R. appendiculatus* were fed on the *Theileria* recovered animals and the subsequent adult ticks had salivary glands dissected out and stained with the Feulgen stain to determine *Theileria* infections.

Results from the adult ticks fed as nymphae on infected animals suggest that *T. parva* infection can be maintained by carriers and it supports the earlier assumption that recovered cattle can set up new foci of disease (Koch, 1990). It has long been accepted that the protozoan parasite *Theileria parva*, causing January disease in Zimbabwe, produces a carrier state in recovered animals (Mackinnon,1953; Neitz, 1957). This conclusion was not based on experimental evidence, but was deduced from epizootiological observations where recovered cattle were assumed to be the source of new foci of infection when moved to farms where tick control was inadequate and the tick vector and disease were present (Brocklesby and Barnett, 1966a). The FAO team in Harare also established that *Theileria* recovered animals remained carriers for a period over 18 months (FAO phase II Epidemiology and Immunisation report ,1993) by transmission to susceptible cattle. *Theileria* recovered animals were also shown to be infective to the tick population at 7 and 12 months post infection (Koch, 1990). The causative *T. parva* parasites are remarkably homogeneous and usually of relatively low virulence to those causing

ECF, and it was also demonstrated in studies by Koch (1990), that these parasites have a well developed carrier status. Matson (1967) recorded a great variation in pathogenicity of *Theileria* infections over a period of two seasons, ranging from as severe as *T. parva* to inapparent. Mixed infections of *T. taurotragi* are to be expected as both parasites use the same tick vector and also are identical parasitologically in the vertebrate and arthropod host (Norval *et al.*,1992). This has complicated sero-prevalence studies as high antibody titres have been observed in areas where theileriosis outbreaks have not been reported, and needs to be considered in the planning of tick and tick-borne disease control programmes. This problem has been attributed to cross-reactions between *T. parva* and *T. taurotragi* (Koch, *et al.*, 1986).

The theileriosis situation in Zimbabwe is also complicated by the wide distribution of both *T. parva* and *T. taurotragi* as revealed by anti-schizont MAbs and RFLP analysis using a *Theileria* extra-chromosomal element probe (Bishop *et al.*, 1994), and the presence of wildlife providing alternative hosts for the tick and hence parasites.

The PCR assay using p104 primer sequences for *T. parva* microneme / rhoptry antigens can be utilised in the field for species differentiation where it is difficult to distinguish *T. parva* from other *Theileria* species morphologically or serologically. Identification of *T. parva* carriers, confirmation of *T. parva* outbreaks and understanding of the epidemiology of disease as the situation in Zimbabwe is complicated by the occurrence of *T. taurotragi* and *T. mutans*. It is difficult to define stocks on the basis of virulence or pathogenicity as the *Theileria* stocks vary

considerably in their virulence to cattle. Mildly pathogenic stocks of *T. parva* have been described from Kenya and Zimbabwe (Barnett and Brocklesby, 1966b; Brocklesby, 1969; Koch., *et al.*, 1988). Diagnosis of the disease has been based on the presence of macroschizonts in the stained lymph node biopsy smears, but although this is the most important diagnostic test it is impossible to differentiate the schizonts of *T. parva* and *T. taurotragi*. New outbreaks of Theileriosis have occurred in areas where the tick vector is present, and this has been attributed to the movement of cattle from properties which have experienced pathogenic *T. parva* outbreaks, thus further complicating the disease situation.

This was an observational study aimed at surveying the nature and importance of carrier state of *T. parva* in Zimbabwe in an effort to improve diagnostic methods of the disease. It is very important to advise farmers that *T. parva*-carriers do occur, and they should take this into consideration when buying new stock and for disease control purposes.

Objectives of study were to:

(a). Develop a polymerase chain reaction assay for the detection of Zimbabwean *T. parva* including the *T. parva* Boleni (Bolvac®) infections in carrier / recovered cattle using p67 and p104 gene primer sequences.

(b). Assess the sensitivity of p67 PCR assay using dilutions of *T. parva* infected blood with known parasitaemias.

(c). Assess the specificity of the P67 and p104 PCR assays using other *Theileria* species, and haemoparasites including *Babesia bovis, Trypanosoma congolense, Cowdria ruminantium and Anaplasma marginale.*

(d). Compare the p104 PCR assay with other methods of assessing *T. parva* infection or carrier status in infected / recovered animals; eg. microscopy, serology, nymphal tick feeding on recovered animals and examination of stained adult tick salivary glands.

CHAPTER TWO

LITERATURE REVIEW

2.1 Identification and classification of *T. parva.*

The genus *Theileria* are protozoan parasites, classified in the phylum Apicomplexa, class Sporozoa, subclass Piroplasmia, order Piroplasmida, and family Theileriidae (Levine *et al.*,1980). The classification and features are shown in the Table 2.1

Table 2.1

Classification and features of *Theileria* (Levine *et al.*, 1980).

Subkingdom	:Protozoa; single celled eukaryotes.
Phylum	:Apicomplexa; apical complex present in some stages; reproduce sexually by syngamy.
Class	:Sporozoa; sporogonic stage producing sporozoites.
Subclass	:Piroplasmia; piriform, rod shaped or amoeboid; parasite in erythrocytes and sometimes other cells.
Order	:Piroplasmida; asexual and probably sexual reproduction, vectors are ticks.
Family	:Theileriidae; schizont stage in lymphocytes.
Genus	:*Theileria*; piroplasm stage in erythrocytes lacks pigment.

East Coast fever (ECF) is alleged to have been eradicated in the 1950's, and the disease does not occur in Zimbabwe (Matson, 1967; Lawrence and Mackenzie, 1980). However, when strains of the formerly designated subspecies, *T. parva*

lawrencei or *T. parva bovis*, occur the possibility of classical ECF re-emerging spontaneously can not be ruled out (Uilenberg *et al.*, 1982; Norval *et al.*, 1985). *T. parva* (Boleni) has been reported as a strain of *T. parva* with characteristics intermediate between those of classical "cattle-derived *parva* type" and those of the "buffalo associated *lawrencei*-type" (Corridor disease) (Uilenberg *et al.* 1981). However recent genetic studies have revealed that cattle-derived *T. parva* isolates are relatively homogeneous on the basis of reactivities with monoclonal antibodies (MAbs) and restriction fragment length polymorphisms (RFLPs) detected using *T. parva* repetitive and ribosomal DNA probes (Bishop *et al.*,1994).

2.2 Life Cycle and Transmission.

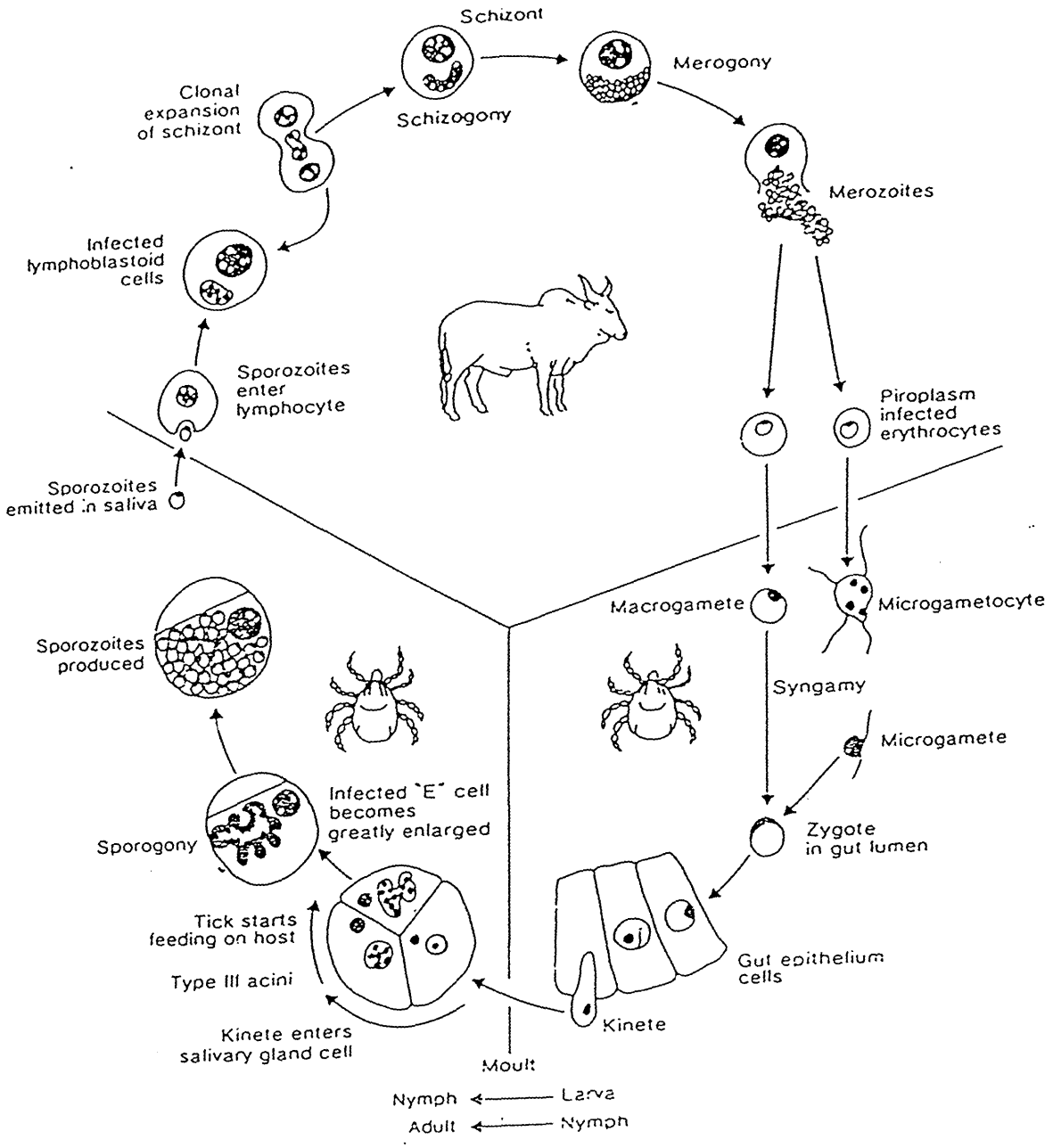
The life cycle of *Theileria* is complicated as shown in figure 2.1.

Several authors have reviewed the life cycle of *T. parva* (Barnett, 1968; 1977; Irvin *et al.*, 1981; Melhorn and Shein; 1984; Morrison *et al.*, 1981). *R. appendiculatus* larval and nymphal ticks become infected with *T. parva* when they feed on infected cattle or wild animals and they ingest erythrocytes which contain the piroplasm stage of the parasite. The infected erythrocytes are lysed within the tick gut releasing piroplasms which differentiate into male and female gametes which then fuse to produce zygotes. The zygotes subsequently invade the cells in the gut wall of the tick several days after the tick has completed feeding and detaches itself from the host (Melhorn *et al.*, 1978; Young and Leitch, 1981a). Motile kinetes are released into the tick-haemolymph during and following the period when the tick is moulting (Young and Leitch, 1980; Melhorn and Schein; 1984). The kinetes then migrate to the salivary glands where they invade the "e" cells of the type III acini glands and

undergo sporogony (Fawcett *et al.*,1981a & b; 1982a, b & c; 1985). Sporogony usually does not start until the tick (now a nymph or adult) commences feeding, but it can be induced by incubation of the tick at 37°C (Young *et al.*,1979; Ochanda *et al.*, 1988). Occasionally it may occur before feeding under natural conditions when there is high ambient temperature. When the kinete invades the tick salivary glands it proliferates as the sporoblast stage throughout the host cell. The sporozoites are finally liberated into the saliva and injected into the mammalian host during the feeding process of the tick. The development of the sporozoites in the adult tick is completed 3 to 5 days after commencement of feeding at which time infective sporozoites appear in the tick saliva (Purnell and Joyner, 1968). It has been postulated that a single kinete can result in as many as 40 000 sporozoites (Fawcett *et al.*, 1982a) or more in *T. taurotragi* infection (Fawcett *et al.*, 1985). Young and Leitch (1980) have suggested that susceptibility of ticks and infectivity of *Theileria* parasites are the factors that control the infection prevalence and levels of *Theileria* parasites in the ticks. There is however a marked variation in the level of infection between individual ticks and within a batch of ticks fed on the same animal, and this range may encompass no infection in a tick to greater than 250 infected salivary gland acini (Young and Leitch, 1980).

Figure 2.2

Life cycle of *T. parva*



The infection of the host animal commences with the feeding of the tick when the infective stage of *Theileria*, the sporozoite, is injected with the saliva into the host during feeding 3 to 4 days after attachment (Purnell *et al.*, 1974; Young *et al.*, 1975). The attachment site is generally infiltrated by a variety of inflammatory cell types including lymphocytes because the tick remains attached for several days before initiation of the rapid feeding phase, during this time the sporozoites are released into the host tissue (Walker, 1990). The sporozoites of *T. parva* have an apical complex, micronemes, rhoptries and a surface coat, and resemble sporozoites of *Plasmodium*. These sporozoites are capable of penetrating a susceptible lymphoid cell within a few minutes or seconds (Fawcett *et al.*, 1982) resulting in multiple infections of up to eight schizonts in a single host cell although these cells may eventually die (Stagg *et al.*, 1981). The parasite then develops into a schizont and the host cell is transformed into a lymphoblastoid cell which produces two infected daughter cells resulting in a clonal expansion with a ten-fold increase of infected cells every three or so days (Jarrett, *et al.*, 1969; Irvin, *et al.*, 1982). Within 12-14 days all the lymphoid tissues of the bovine host are infiltrated and the schizonts start undergoing merogony (Melhorn and Schein, 1984; Shaw and Tilney, 1992). The host cell plasma membrane ruptures on completion of merogony liberating the mature merozoites which penetrate erythrocytes and develop into piroplasms. Under the microscope the giemsa stained blood smear shows the piroplasms as comma or spherical shapes lying freely within the erythrocyte cytoplasm. Thus the piroplasm waits for another tick to feed for the life cycle of *T. parva* to continue.

2.3 Pathogenesis of Theileriosis

Susceptible cattle usually die within 2 to 4 weeks of infection with the parasite and the disease is characterised by large numbers of parasitised lymphoid cells found throughout the lymphoid system and associated with extensive lymphocytosis (Morrison *et al.*,1981). The course of infection in cattle may be divided into three stages: a prepatent stage; a stage of lymphoid proliferation; and a stage of lymphoid disorganisation and depletion. The prepatent period stage covers the period between the inoculation of sporozoites by the tick and the appearance of schizonts in the draining lymph node. The sporozoites invade lymphocytes at the site of inoculation and develop into recognisable schizonts after two to three days. The schizonts stimulate proliferation of both infected and non-infected lymphocytes, possibly by stimulating production of a T-cell growth factor similar to interleukin-2 (Brown and Logan, 1986). Lymphocyte proliferation starts at the site of inoculation, but from five days after infection parasitised cells begin to appear in the draining lymph node.

The appearance of parasitised cells in the lymph nodes coincides with the onset of fever and stimulates active lymphoid proliferation and the release of large numbers of infected and non-infected lymphoblasts into the efferent lymph. After a further two to three days, more distant lymph nodes in the chain become infected and hyperplastic and parasitised and non-parasitised lymphoblasts enter the peripheral circulation. Parasitised cells also infiltrate the non-lymphoid tissues, particularly the gastrointestinal tract and the lungs which results in pulmonary oedema. These parasitised cells can also establish in other lymph nodes and lymphoid tissue (thymus, spleen) as well as in many parenchymatous organs (notably liver, kidneys,

lungs, myocardium, adrenals), bone marrow, and sometimes in the brain. It is the schizont stage of the parasite which accounts for the pathology of theileriosis.

Four to five days after the initial appearance of schizonts, the process of lymphocyte proliferation is succeeded by a process of lymphocyte destruction, and lymphoid organs and lymphoid foci in parenchymatous organs may show evidence of necrosis and depletion of lymphocytes. Both parasitised and non-parasitised lymphocytes are destroyed and this appears to be the result of activation of specific protective cytotoxic T-lymphocytes and non-specific cytotoxic T-lymphocytes, ['natural killer cells'] (Emery *et al.*, 1981). Lymphocyte destruction causes a fall in serum immunoglobulin levels (Spooner *et al.*, 1973) and immunosuppression (Wagner *et al.*, 1975), and this may enhance progress of the disease and facilitate secondary respiratory infections.

In non-fatal cases, development of specific protective cytotoxic T-lymphocytes (which destroy parasitised lymphoblasts) and helper T-lymphocytes terminates schizont proliferation (Eugui and Emery, 1981; Baldwin *et al.*, 1987). Recovery from infection is thought to depend on the survival of a sufficient number of effector cells over the first 14 days and on their capacity to achieve a specific protective response (Emery *et al.*, 1981). Re-exposure of recovered animals stimulates activation of memory T-lymphocytes which, in turn, stimulate activation of cytotoxic T-lymphocytes which are thought to eliminate the infection (Morrison *et al.*, 1989). Although some development of schizonts and piroplasms may occur and cause a mild clinical response.

A proportion of recovered animals remains unthrifty and unproductive, and foci of infected lymphoid cells may be found persisting in a variety of organs (Dolan, 1986). Immunosuppresssion may predispose such animals to secondary infections and eventually prove fatal.

Lymphocyte proliferation and destruction are responsible in a number of ways for the lesions which characterise East Coast fever. Focal or diffuse infiltration of lymphocytes and lymphoblasts may cause degenerative changes in liver, kidneys and brain, while focal infiltration in the mucosa of the gastrointestinal tract is responsible for necrosis and erosion of overlaying tissue. Activation of the coagulation cascade leading to an accumulation of fibrin degradation products suggests that disseminated intravascular coagulation may be an important component of the terminal stages of disease (Maxie *et al.*, 1982; Shitakha *et al.*, 1983).There is also activation of the complement cascade, and vasoactive components are released as a result of lymphocyte disintegration in the lungs. These may be responsible for the pulmonary oedema which is often final cause of death.

Within the erythrocytes piroplasms can undergo merogony to produce further merozoites (Melhorn and Schein, 1984; Conrad *et al.*, 1986). The invasion of erythrocytes by piroplasms does not have any pathogenic effect. In *T. parva* intraerythrocytic division of piroplasms does not occur although in *T. annulata, mutans, sergenti,* and *taurotragi* it is the main mechanism of maintenance of infection (Conrad, 1983; Conrad *et al.*, 1985).

2.4 Characterisation of *T. parva* stocks.

Following the realisation that different immunological strains in *T. parva* stocks exist and with the development of the infection and treatment method of immunisation, it became critically important to characterise *T. parva* stocks.

The following definitions of terms below were taken from Irvin *et al* (1983) Irvin (1987) and Anon (1989a).

1) *Isolation* :Viable organisms, isolated in experimental hosts or culture systems or prepared as a stabilate on a single occasion from a field sample.

2) *Stock* :All the populations of a parasite derived from an isolate without any implication of homogeneity or characterisation including cell lines and tick stabilates, and subsequent parasite preparations derived from them.

3) *Line* :A laboratory derivative of a stock maintained under defined physical conditions, such as in a culture of parasitised bovine lymphoid cells.

4). *Strain* :A population of homogeneous organisms possessing a set of defined characteristics. Unambiguous characterisation of a strain can only be assured if the population of organisms was initiated from a parasite clone.

5) *Stabilate* :A sample of organisms preserved alive (usually in replicate) on a single occasion.

6) *Clone* :Genetically identical organisms derived from a single cell by asexual division (mitosis).

6a) *Parasite clone* :*Theileria* line derived from a single parasite.

6b) *Cell clone* :*Theileria* line derived from a single parasitized cell.

2.4.1 Cross immunity

Cross-immunity tests were developed to characterise the immunological behaviour of *T. parva* which involves immunising cattle with a stock of *Theileria*, usually by the infection and treatment method, and challenging the immune animals with different *Theileria* stocks (Mutugi *et al.*, 1988b). In Zimbabwe *T. parva* Boleni was selected for use in immunisation on the basis of cross immunity tests and displays a wider dose range over which mild reactions can be induced making it an attractive candidate for wide scale immunisation against ECF (Irvin *et al.*, 1989). This means that large numbers of animals have to be used in cross-immunity tests for stock characterisation making the testing very expensive.

2.4.2 Infectivity

Another way of characterising *Theileria* stocks has been the measurement of clinical and parasitological parameters after infection with a standard dose of sporozoite stabilate. The clinical and parasitological parameters to be measured would be the duration of parasitosis and fever; the time to appearance of piroplasms; and the time to death or recovery.

Additional features which can be measured are the level of schizont parasitosis, piroplasm parasitaemia and the reduction in white blood cell numbers. These parameters define the infectivity of a particular stock for a particular breed and age of animals (Norval *et al.*, 1990).

The behaviour of stocks varies considerably. For example some stocks kill animals despite showing low parasitosis and parasitaemia. Others produce extended clinical reactions accompanied by a high number of schizonts and piroplasms resulting in severe clinical reactions. There is also stock variation in drug sensitivity resulting in the need to characterise the different stocks for selecting candidate vaccines for infection and treatment immunisation. Several stocks produce biphasic febrile responses, possibly due to the heterogeneous nature of the stocks used (Morzaria, 1989).

2.4.3 Drug testing

For stock characterisation a drug sensitivity test is whereby a group of highly susceptible Taurine breeds are immunised by the infection and treatment method using a different doses of stabilate and assessing the reactions (Mutugi *et al.*, 1988b; Morzaria 1989a). This also assesses the suitability of a particular stock to be used in immunisation at the same time considering the use of chemotherapy in case of severe reactions in infected cattle since different stocks have been demonstrated to vary in their sensitivities to drugs.

2.4.4 Monoclonal antibody profiles (MAbs)

Monoclonal antibodies raised against different *Theileria* parasites can be useful in the identification of *Theileria* spp, and when directed against a specific parasite epitope can give identification of a species (Williamson *et al.*, 1990).

A panel of *T. parva* anti-schizont monoclonal antibodies was generated for stock characterisation and used in an IFA test against *T. parva* schizont infected cells derived from *in-vitro* culture (Pinder and Hewett,1980; Minami *et al.*, 1983; Conrad

et al., 1987b; 1989b; Maritim *et al* 1989b). Most cattle derived *T. parva* stocks isolated from Zimbabwe do not react with MAb-7 (Koch *et al.*,1988), and this has been a useful tool in differentiating Zimbabwean parasites in the region. Monoclonal antibodies are particularly useful to determine whether there is contamination with another parasite, they demonstrate antigenic differences in the characterisation of *T. parva* stocks (Pinder and Hewett, 1980). Disadvantages include lack of sensitivity and the selection of certain parasites during the establishment of cultures (Norval *et al.*,1992). The latter was shown by the dominant types surviving in culture when there are mixed parasite populations.

2.4.5 Protein analysis.

Attempts have been made to use isoenzyme analysis to differentiate stocks of *Theileria* (Musisi *et al.*, 1981; Melrose *et al.*, 1980; Allsopp *et al.*, 1985). Glucose phosphate isomerase has been found to be of value in analysis of *Theileria annulata.* Another approach to identify proteins which are parasite antigens is to probe western blots of relevant antigens with a panel of monoclonal antibodies. Shapiro *et al.*,(1987) showed that MAb5 identifies a polymorphic schizont antigen in *T. parva* stocks thus showing that certain anti-schizont MAbs may therefore identify stock specific markers. Another technique used to characterise infection specific proteins of various *T. parva* stocks is two-dimensional gel electrophoresis (Sugimoto *et al.*, 1989). Stock specific differences have been detected, although the technique is difficult to perform and interpret. It can be a useful laboratory tool in stock identification in research.

2.4.6 DNA analysis

Conrad *et al.*,(1987a) and Allsopp and Allsopp (1988) described *T. parva* repetitive (*Tpr*) DNA probes capable of revealing extensive polymorphism among *Theileria* stocks.

In order to detect any changes that may occur when stabilates are passaged through ticks, cattle or other hosts such as buffalo or waterbuck, it is important to differentiate stocks genetically. It is necessary also to characterise any breakthrough parasite populations during immunisation to investigate the failure or otherwise of vaccination trials in relation to parent bulk stock. DNA probes capable of revealing extensive polymorphism among *Theileria* stocks, have been described and recently probes such as ribosomal and telomeric sequences (Bishop *et al.*,1994) and *Sfi* I digestions of *T. parva* DNA on pulsed field gels (Morzaria *et al.*,1990a), which also detect polymorphism have been developed. By sequencing cloned PCR products derived from the amplification of specific portions of the *Tpr* sequences, it has been possible to select and synthesize oligonucleotide probes capable of differentiating specific *T. parva* stocks or groups of stocks (Allsopp *et al.*, 1988; 1993; Bishop *et al.*,1994). These markers reveal the existence of a sexual cycle in *T. parva* and also whether immunisation stocks are transmitted to previously uninfected ticks following vaccination by the infection and treatment method. The PCR technique results in amplification of very small amounts of DNA to a detectable level (Allsopp *et al.*,1989), and allows the detection of *Theileria* carrier animals and it might be able to identify the parasites at species and strain level (Williamson *et al.*, 1990).

DNA probes could be used to identify and quantify the DNA from different *Theileria* species in ticks and also in the mammalian host (Stiller, 1990).

Restriction enzymes cleave DNA at specific nucleotide sequences and fragments produced can be resolved on agarose gels by electrophoresis and visualised under ultra violet-illumination following ethidium bromide staining. Through selection of restriction enzymes such as *Sfi* I and *Not* I cut *Theileria* DNA at infrequent intervals, and separating the digested DNA in pulsed field gel electrophoresis, unique and characteristic banding patterns of *T. parva* and *T. mutans* have been detected (Morzaria *et al.*,1990a).

In Africa the two most important *Theileria* species, *Theileria parva* and *T. annulata* do not overlap in their distribution, except possibly in southern Sudan. The distribution of their tick vectors controls the distribution of these parasites, along with the distribution of the mammalian hosts they infect. Due to the lack of infection in the tick by *Theileria* parasites or lack of suitable conditions for parasite development within the tick, the distribution of the disease may not follow that of the major vector, *Rhipicephalus appendiculatus*. The schizonts of *T. parva* and *T. annulata* are indistinguishable from each other on morphological grounds as are those of *T. taurotragi*, except when they develop multiple infections of cells. In cultures of *T. parva*, *T. taurotragi* and *T. annulata*, the host cell may adopt a different morphology (Stagg *et al.*, 1976). *T. parva* has been shown to infect cattle and buffalo and other wildlife species. The virulence of species of *Theileria* may be variable depending on the stock of parasite, the dose of parasite, and the type of mammalian host.

Further to the identification of *Theileria* parasite repetitive DNA sequences, *T. parva* stocks have been used as radiolabeled probes to hybridise specifically to *T. parva* DNA but not that of *T. mutans, T. annulata and T. taurotragi* (Conrad *et al.*,1987a: 1989a; 1989b; Allsopp and Allsopp, 1988).

2.5 Immunity to *T. parva*.

As regards immunity to *Theileria* the relative speed at which parasite sporozoites enter lymphocytes (Fawcett *et al.*,1982); the intracellular synchronous multiplication of the macroschizont within the host cell (Hulliger *et al.*,1964) and the fact that piroplasm development occurs relatively late in the course of infection makes immunity to *Theileriosis* a complex matter. It has been demonstrated (Burridge *et al.*,1973a) that antibody titres to both schizont and piroplasm stages of the parasite can go below detectable levels after about six months in the absence of further challenge in infected cattle or artificial immunisation, although such animals remain immune to lethal homologous challenge (Burridge *et al.*,1972a; Irvin and Morrison, 1987). Among *Theileria* species there is no evidence of cross protection. For example, cattle immunised against *T. annulata* are fully susceptible to *T. parva* and *vice versa* (Sergent *et al.*, 1945; Neitz, 1957). The cross-immunity evaluation is particularly useful in areas where *T. parva* and *T. taurotragi* co-exist (Mutugi *et al.*, 1990a). It is of interest to note that all *Theileria* species which parasitize cattle can occur concurrently in a host animal because there is no cross-immunity between species, and carrier states from these concurrent infections can develop (Young, 1981).

The proper establishment of infection by *T. parva* confers immunity to the disease (Morrison *et al.*,1987). Attempts to confer immunity by passive transfer of serum from immune to naive cattle (Muhammed, *et al.*, 1975) were unsuccessful. There was no protection against challenge with infected ticks despite the strong antibody responses after inoculation of schizont and piroplasm antigen of *T. parva* (Wagner *et al.*, 1975b; Emery *et al.*, 1981). These results implied that immunity to *T. parva* is cell mediated, and this was supported by further experiments which showed that immunity could be transferred adoptively with thoracic duct lymphocytes from *T. parva* immune chimeric twins to their susceptible partners (Emery, 1981; Mckeever and Morrison, 1990).

The study of cellular immune mechanisms has been made possible by the success of *in-vitro* infection of bovine lymphocytes (Brown *et al* 1973). Potent peripheral blood lymphocytes (PBL) were stimulated by macroschizont infected cells grown *in-vitro* (Pearson *et al*, 1979), and they eventually showed that the growth of cells resulted in the generation of cytotoxic cells which killed infected cells. Cells acquired from lymphoid tissues of cattle undergoing lethal infection with *T. parva* were shown to induce proliferative responses into normal autologous PBL which had been collected and preserved prior to the initiation of infection, thus demonstrating that the proliferative response was not directed against antigens acquired in culture. It has also been demonstrated that these cytotoxic T-cells do not kill cells from unrelated cattle infected with the same parasite. Further studies have shown that these genetically restricted cytotoxic cells, which are generated in *Theileria* immune cattle are T-lymphocytes which recognise cell surface antigens of macroschizont

infected cells in conjunction with self-class major histocompatilbility (MHC) antigens (Morrison *et al.*, 1987). It is therefore currently necessary to protect animals against theileriosis using live parasites although low grade carrier state results from this (Dolan,1981; Young *et al.*, 1981a), because in an immune animal the cell-mediated responses against the schizont act in concert with antibodies against sporozoites (Musoke, et al., 1984) Also, it has been suggested that when sporozoites are deposited by ticks into the skin of immune animals they are killed by sporozite antibody-mediated processes, and that those escaping the latter proceed to invade target cells and develop into schizonts which are then eliminated by CTLs (cytotoxic T-cells). Immunity to homologous challenge has been shown to last for 3.5 years in animals that recover spontaneously from the disease (Burrridge *et al.*, 1972).

2.6 Diagnostic methods for *T. parva* infection

2.6.1 Clinical signs

The first clinical signs are fever and increases in pulse and respiration rates. There may be a sharp decline in milk production. After a few days the animals becomes depressed and lethargic. The temperature continues to rise, often to 41-42°C. Lachrymation commonly occurs together with oedema of the eyelids, and may be accompanied by photophobia. The animal is often constipated. There is a generalised enlargement of the superficial lymph nodes; the prescapular and precrural nodes become very prominent (Norval *et al.*, 1992).

Zimbabwe theileriosis exhibits the same clinical features as East Coast fever except that the course is often shorter and death may occur three to four days after the first onset of signs. *T. parva* infections are characterised by pyrexia, enlargement of

several lymph nodes particularly those draining the area head, severe pulmonary oedema and wasting. On development of pyrexia, a lympho-destructive phase is initiated which is normally associated with a severe leucopenia. In fatal cases anaemia is not present, nor develops in terminal cases (Norval *et al.*, 1992). The predominant sign observed clinically is acute difficulty in breathing and frothing at the nostrils and at post mortem is pulmonary oedema in terminal cases. Pyrexia occurs seven to ten days following infection and persists through-out the clinical course. Sometimes one of the presenting signs is blindness associated with corneal opacity. The incubation period is generally about 15 days from the time of attachment of the infected tick, but may range from eight to 25 days. The period and the course of disease become shorter as the challenge is increased (Jarrett *et al.*, 1969).

The disease usually progresses over a period of 15 days, but may terminate after five days or be prolonged to 25 days. The fever remains high, although in a small proportion of cases there may be a temporary remission for one or two days. Appetite and rumination become increasingly depressed and there is a severe loss of condition, increasing weakness and ataxia, and frequent recumbency. Constipation is succeeded by diarrhoea and there may be blood and mucus in the faeces.

In the terminal stages of the disease dyspnoea develops, with an increased respiratory rate, a watery cough and the discharge of frothy fluid from the nostrils. Evidence of pulmonary oedema and hydropericardium may be detected on auscultation. Sternal and submandibular oedema may be present. The superficial

lymph nodes begin to regress, the rectal temperature falls to subnormal levels, the animal becomes recumbent and dies in a coma.

A small proportion of animals, usually about 5 per cent , may recover, but convalescence is prolonged and the animals may remain emaciated and unproductive for months.

The disease may assume a less severe form in animals with partial immunity or inherent resistance, but pyrexia and enlargement of superficial lymph nodes remain constant features and in calves may be accompanied by persistent unthriftiness. Mild disease has also been reported occasionally following infection with mild strains of *T. parva* of reduced virulence. The occurrence of the tick vector should be considered in making a diagnosis.

2.6.2 Microscopy

A laboratory diagnosis is affected by the level of parasite as this is determined by the clinical signs as these vary from inapparent, or mild to severe and fatal. Diagnosis of classical East Coast fever is based on the characteristic clinical signs and lesions, and may be confirmed by demonstration of schizonts and in the later stages of disease, piroplasms in Giemsa-stained lymph node smears. The only difficulty with this method is that one cannot distinguish the schizonts of *T. annulata*, *T. parva* and *T. taurotragi* from one another morphologically. However it should be noted that *T. annulata* does not occur in the same geographical areas as *T. parva* and *T. taurotragi*, except where their distributions overlap in southern Sudan (Norval *et al.*, 1992). The examination of stained blood smears for piroplasms which appear 5-8 days after the detection of schizonts in new infections can be of not much use without clinical data

and lymph node biopsy, as it may merely detect the carrier status in clinically normal animals. Also the fact that piroplasms appear intermittently post-recovery and are variable in *Theileria* cases means that they are of no significance to the field veterinarian or clinician.

2.6.3 Antibody Detection.

Retrospective diagnosis of theileriosis can be made by demonstration of a rising titre to *T. parva* schizonts using indirect immunofluorescence. Although several serological tests have been used to identify antibodies to *Theileria* infection e.g. capillary agglutination test, indirect haemagglutination test, complement fixation test, indirect fluorescent antibody test and micro enzyme-linked immunosorbent assay (ELISA), only the IFA test has been used as a routine test (Young 1986; Morzaria 1989). Antibodies persist on average for a period of 30 weeks with a range of 12 to 73 weeks (Burridge and Kimber, 1973). However, there are cross reactions in this test between *T. parva, T. mutans, T. annulata* and *T. taurotragi* (Burridge *et al.*, 1974a; 1974b; Grootenhuis *et al.*, 1979), but the test can be useful in identifying animals undergoing infections with these species under experimental conditions and using appropriate controls (Young 1986; Irvin 1987; and Morzaria 1989).

2.7 Control of Theileriosis and other Tick-borne diseases

Tick-borne diseases constitute a major constraint towards cattle production and the expansion of the dairy industry in East and Central Africa. Since East Coast fever had the greatest impact on cattle production among the tick-borne disease complex, past control measures for tick-borne diseases were therefore primarily tailored towards the control of this disease. Thus, the familiar reliance on continuous and

intensive dipping, was designed to render cattle free of the three host brown ear tick, (*R. appendiculatus*), and thus interrupt ECF transmission. The control of theileriosis in Zimbabwe has basically been by tick control, chemotherapeutic treatment and recently by immunisation of cattle on properties at risk of epidemic outbreaks. Intensive tick control (averaging 42 treatments per year) has been practiced for several decades since the introduction of East Coast fever in the country. The department of Veterinary Services has realised that control of ticks and tick-borne diseases can no longer rely on the use of acaricides as demonstrated by the disaster following breakdowns in the systems (Lawrence and Norval, 1979) during the war of independence (1972-1980) when almost a million cattle were lost (Norval, 1979). On a nation-wide scale it has become very costly for the Zimbabwean government to maintain the structure thus a move towards the eventual attainment of endemic stability to tick-borne diseases by immunisation coupled with reduced or strategic dipping has been recently encouraged by the department of Veterinary Services (1993). However this case has to be approached with caution as three other tick-borne diseases occur namely anaplasmosis, babesiosis and cowdriosis, and studies have to be carried out in-order to determine safe acceptable levels of tick challenge in the different geographical regions of the country. Thus in Zimbabwe there are projects involved mainly in applied research on the development of vaccines and related field trials, and research into heartwater is also being carried out.

The use of acaricide chemicals to destroy ticks is a measure beset with numerous problems. These range from the ever escalating acaricide costs, the non availability of acaricides in rural areas due to poor distribution, poor construction of

dip tanks, availability of water, low number of dip-tanks, the potential problem of tick resistance to acaricides (Nolan, 1981; Keating, 1983; Young *et al.*, 1988), to a recently public awareness on issues dealing with pollution of the environment and residues in animal products like milk.

Another issue which has been studied is the development of resistance in cattle to *R. appendiculatus* ticks. The resistance is caused by repeated exposure and probably also affects the transmission of *T.parva* by impairing the feeding of the ticks (Chiera *et al.*, 1985; Fivaz *et al.*, 1989). Infection rate in the ticks feeding on cattle with a high degree of resistance are likely to be low and the severity of the clinical disease in resistant cattle exposed to infected ticks is likely to be reduced (Young *et al.*, 1986). The benefits of allowing tick infestations to a certain level on cattle can be difficult to explain to farmers.

Nevertheless, tick control has remained as the chosen method despite the above mentioned set backs and has contributed to the livestock development in the region as well as the eradication of East Coast fever.

2.7.1 Chemotherapy

There are currently drugs available on the market for the treatment of theileriosis but these are very expensive and usually beyond the reach of the communal farmer. Clexon (parvaqoune) and Butalex (buparvaqoune) are now available and have had good results (McHardy *et al.*, 1985) and still a good diagnostician is required to prescribe timely treatment to avoid any loses. The drugs are effective against both schizonts and piroplasms, but treatment does not achieve a parasitological cure, and recovered animals may remain carriers and may take several months to return to

normal productivity (Dolan, 1986). Both drugs have the disadvantage of being relatively expensive and this reduces their application in the field, but they can be used as a back-up to prevent mortality if other forms of control prove ineffective.

2.7.2 Vaccination

The development of methods of immunisation against East Coast fever has been reviewed by Brown (1985), Norval *et al* (1992) and Mutugi *et al.*, (1988). Protective immunity to *T. parva* persists for at least five years after natural infection (Neitz, 1957). Immunity will develop only in animals in which infection with schizonts has been established. Effective methods of immunisation are achieved by controlled infection of cattle using sporozoites derived from ticks.

In the early years of this century, before the introduction of acaricides, crude, techniques of controlled infection were developed for immunisation. In Kenya, animals from endemic areas which were thought to be immune were exposed to natural infection on heavily infested pastures. The majority which survived could be used as transport oxen through infected areas. In South Africa, cattle were immunised by the intravenous inoculation of preparations of spleen and lymph node from infected animals, and up to 70% were protected against natural challenge (Theiler, 1911).

No further progress was made until 1953, when Neitz discovered that the administration of chlortetracycline (Aureomycin) during the incubation period had a marked suppressive effect on the development of infection and permitted the establishment of a solid immunity. Neitz's observation was followed up by a team of scientists working in Kenya under the auspices of the Food and Agricultural

Organisation (FAO) of the United Nations, and an infection and treatment method of immunisation was developed (Radley, 1978). The method is described in detail in an FAO manual (Anon, 1984). A crude suspension of sporozoites is prepared from adult ticks which have fed as nymphs on infected cattle, have moulted, and have been pre-fed on rabbits for three to four days to allow maturation of the sporozoites. The suspension is mixed with glycerol as a cryoprotectant and stored, deep frozen, at minus 70°C or in liquid nitrogen. Aliquots are titrated in susceptible cattle to determine an optimum dose for immunisation. The stabilate is inoculated subcutaneously into the cattle to be immunised and simultaneously an intramuscular injection of 20 mg/kg of a long-acting oxytetracycline preparation is administered. Infection develops to a mild or subclinical level and the animals are thereafter resistant to challenge, both experimental and natural, although infection may persist and animals may remain asymptomatic carriers. The duration of immunity appears to be comparable to that which follows natural recovery.

The infection and treatment technique provides good immunity against the homologous immunising strain, but is not consistently successful against unrelated strains. On the basis of extensive cross-immunity trials, the Boleni stock has been found to be a suitable vaccine for immunisation in Zimbabwe. Vaccination against theileriosis can be viewed as an attempt at artificially introducing enzootic stability between cattle, *Theileria* parasites, tick vectors, so that susceptible cattle once introduced to the *Theileria* pathogen thro igh immunisation are subsequently able to withstand lethal challenge from these infections. The use of live vaccines even in the control of other tick-borne disease require that trained personnel be available to

vaccinate susceptible herds. Also the preparation of the vaccines can only be done in special laboratories to avoid contamination with extraneous pathogens.

There are six strategies for controlling tick-borne diseases in areas where the diseases are enzootic (Lawrence, 1990) :-

(a) Absolute tick control without immunisation

(b) Absolute tick control with immunisation

(c) Tick reduction with immunisation

(d) Tick reduction without immunisation

(e) Immunisation without tick control

(f) No control

The choice of the most appropriate strategy is dependent on the epidemiological and economic characteristics of the cattle production system targeted for. In the Zimbabwe subsistence livestock economy most of the cattle would be adapted to local ticks and tick-borne diseases and it is currently appropriate to allow enzootic stability to develop naturally. For the high production livestock economy, especially dairy production with highly susceptible animals, the control measures adapted must be effective but also economically justifiable. In order to allow maximum productivity it might be necessary to protect against all the major tick-borne diseases to reduce the cost of acaricides thus rendering the animals to develop enzootic stability. However the whole exercise has to be approached with great caution because of the development of carrier stat is and the possibility of having high levels of parasites in the tick vectois and hosts not forgetting the introduction of the parasites in areas with the tick vector, which do not have the disease.

2.7.3 Recombinant DNA vaccines

The main mechanism of immunity in theileriosis is cell mediated and thus makes the development of genetically engineered vaccines challenging. The schizont stage of the parasite induces antigenic changes on the surface of the infected lymphocyte, which are recognised in conjunction with MHC antigens of the host (Irvin, 1987: Morrison, 1989). Efforts are underway to identify and isolate the parasite genes responsible for the antigenic changes seen in infected cells, with the goal of transfecting such genes into host cells to induce the changes normally stimulated by intact parasites. In addition to the schizont associated cell mediated immunity, humoral responses are induced by *Theileria* sporozoites. Monoclonal antibodies have been demonstrated to neutralise *T. parva* sporozoite infectivity (Musoke *et al.*, 1984). The neutralisation was not strain specific indicating a common antigenic determinant associated with the sporozoite surface coat (Webster *et al.*, 1985). Dobbelaere *et al.* (1985a, b) found the 67kDa protein to be the common antigen.

Williamson *et al.*, (1989) characterised a surface antigen complex *of T. annulata* sporozoites by using monoclonal antibodies that neutralised sporozoite activity in-vitro. The gene encoding for this complex was cloned, and a fusion protein expressed from a fragment of the gene elicited strong neutralising antibodies. Work on potentially protective antigens of *T. parva* has produced promising results. Sera from animals that had recovered spontaneously or from animals in endemic areas identified five major antigens of relative molecular masses 180, 104, 85, 67 and 35 kDa (Musoke *et al.*, 1987). Of these antigens, the 67 kDa and 85 kDa are located on the surface of sporozoites (Dobbelaere *et al.*, 1985). The 104 kDa was shown to

be a microneme / rhoptry antigen (Iams *et al.*, 1990), and the location of the other antigens on the parasite has not been determined. Both the 67 and 85 kDa proteins were recognised by neutralising monoclonal antibodies as well as by colostrum collected from immune dams (Morzaria *et al.*, 1989) The 67 kDa antigen is stage specific being present in sporoblasts and sporozoites while the 85 kDa antigen is found on the surface of sporozoites and schizonts of *T.parva.* The 67 kDa antigen is invariant whereas the 85 kDa antigen varies in relative molecular mass depending on the stock of parasite. The 85 kDa has been demonstrated to vary from 69 kDa for *T.parva* (Marikebuni) to 100 kDa for buffalo derived *T.parva* (the former *T.parva* Lawrencei) (Shapiro *et al.*,1987). Further work on the p67 was done in an attempt to develop an alternative immunisation procedure at ILRI, Nairobi. The gene encoding the p67 was expressed by using the plasmid expression vector pMG1. Previous work found the gene to encode for 709 amino acid residues, a single intron of 29 base pairs and is only transcribed during sporogony. The recombinant p67 sequences were fused to the first 85 amino acid residues derived from a non-structural gene (NSI) of influenza virus A. Protection in 6 out of nine animals on homologous challenge was successful with the partially purified recombinant antigen emulsified in 3% saponin (Musoke *et al.*, 1992).

Partial characterisation of the *T. parva* genome using monoclonal antibodies to screen genomic expression libraries generated from piroplasm DNA inserted into bacteriophage vectors has been done by Guerin-Marchand and Lambiotte (1984). Several clones were identified and characterised and found to encode sequences

having molecular weights ranging from 18.5 to 25.5 kDa. Antisera to these sequences neutralised the infectivity of sporozoites *in vitro.*

Although the prospects of developing effective recombinant vaccines for *Theileria* spp. seem promising, particularly if the parasite DNA can be introduced in a vaccinia expression vector, such vaccines are not likely to be available soon for field use (Irvin and Morrison, 1989).

2.8 Carrier State

In a study in Zimbabwe, *T. parva* was demonstrated to produce a carrier state in cattle, 7-12 months after infection (Koch , 1990), by transmission of disease using adult ticks fed as nymphs on healthy theileriosis recovered cattle.

Investigations into *T. parva* Boleni carrier state were performed in the FAO / Danida programme for Tick and Tick-borne disease control (Phase II). Three tick pick-up / transmission attempts were carried out using *T. parva* Boleni-carrier animals at 6, 10, and 18 months post immunisation. None of the 15 tick batches fed as nymphs on the carrier animals showed infection in the salivary glands. However, infectivity of the tick batches to susceptible cattle was achieved with 10 out of 15 batches. After 18 months, transmission of the carrier parasites was achieved in four of the six cattle.

Successful PCR amplifications were performed using DNA from 2 cattle infected with *T. parva* Boleni 3 months post infection, tick infections were negative 6 weeks post infection of experimental animals (Bishop *et al.*, 1992)

In Kenya, Kariuki *et al.*, (1995) showed that uninfected *R. appendiculatus* nymphs applied to naturally recovered Zebu and exotic cattle kept under tick free conditions

in the laboratory could transmit fatal theileriosis as adult ticks to susceptible cattle at 16 and 17 months respectively. It was also possible to demonstrate that cattle immunised by the infection and treatment method to be carriers of *T. parva* by examination of the salivary glands of ticks applied to them and by tick transmission. In Kenya, uninfected *R. appendiculatus* nymphs were applied to cattle from a *T. parva* endemic area over a period of 13 months after transferring the animals to Muguga Veterinary Institute, and 18 out of 23 batches transmitted *T. parva* infection to cattle. Infection intensities in the tick batches were very low, with one (1) salivary gland acinus infected per tick.

A carrier state lasting for 19 months was demonstrated by Dolan (1981; 1985) in cattle experimentally infected with *T. parva* isolates after treatment with curative drug, parvaquone (Clexon, Welcome Foundation).

The carrier state of *Theileria* has been defined as the ability of an infected and recovered host to infect ticks, which are then able to transmit the parasite to susceptible animals (Levine, 1973; Schwabe *et al.*, 1977; Young *et al.*, 1986). Stages which maintain infection are:

1) slow proliferation of schizont-infected lymphocytes, some of which undergo merogony to produce merozoites infective to the erythrocytes (Maritim *et al.*, 1989b; Kariuki, 1991); this stage can occur in *T. parva* infections of cattle and buffalo.

2) by the regular division of intra-erythrocytic piroplasms, this occurs in all *Theileria* species, with the possible exception of *T. parva.* Recent work by Conrad *et al.*, (1987) has confirmed that limited division of intraerythrocytic stages of *T. parva*

may occur. Establishment of cell lines from carrier cattle (Kariuki *et al.*,1995) infected with *T. parva* have been successfully demonstrated but the methods are very laborious and can be very insensitive especially in field situations where there might be mixed parasite populations. However the methods can be applied successfully in research and in special cases of vaccine breakthroughs.

CHAPTER THREE

Infection of experimental animals with *Theileria parva* parasites

3.1 INTRODUCTION

The Zimbabwe Theileriosis vaccination programme using the live Bolvac® vaccine is targeted at areas of high disease incidence and properties with theileriosis problems. The present Zimbabwean *Theileria* vaccine stabilate Bolvac® was confirmed to confer immunity against several stocks and two from eastern Africa. Partial immunity was demonstrated against another stock from northern Malawi (Hove *et al.*, 1995). The control of theileriosis in Zimbabwe is now moving away from absolute reliance on intensive dipping, to vaccination against the disease with the aim of attaining endemic stability of cattle through strategic seasonal tick control, coupled with immunisation. Indigenous cattle in enzootic areas are generally resistant to theileriosis and undergo mild infections from which they recover, but may remain chronic carriers. In contrast, exotic cattle are highly susceptible and undergo severe infections that may be fatal, consequently immunisation is targeted at these improved breeds. It is these vaccinated herds which could provide further foci of infection (Koch, 1990), as it has long been accepted that *T. parva* produces a carrier state in recovered animals (MacKinnon, 1953; Neitz, 1957). The cost of acaricides to control ticks and anti-theilerial drugs have been a major drawback to cattle producers. Cattle of improved breeds which recover from pathogenic *Theileria parva* infections may fail to develop their full productive potential and are less economic to maintain (Pegram *et al.*, 1996). Several factors which affect infection in the mammalian hosts include the number of ticks infesting cattle, tick infection rates, success of

transmission, antibody prevalence, case fatality rates, disease incidence and "carrier state". Hence the need to cautiously introduce vaccination in the cattle populations at risk.

In order to obtain accurate epidemiological data it is important to develop new diagnostic methods for *T. parva* to determine if the animals are carriers of infection and also to investigate apparent vaccine failures or breakthroughs. The situation in Zimbabwe is further complicated by the fact that animals which could be *T. parva* carriers are being moved from place to place at cattle sales thus resulting in the spread of the parasite to areas where the vector is present.

In order to compare conventional methods of detecting carriers of *Theileria parva* by PCR, calves were infected and exposed to infected ticks in order to establish carrier states of *T. parva* with Zimbabwean isolates including the immunisation vaccine candidate Bolvac®. The animals were kept in tick-proof facilities and monitored until 40 days after challenge with *T. parva* Avery. Burridge *et al.*, (1972) demonstrated that cattle recovered from East Coast fever are almost invariably immune to challenge with *T. parva*, and this immunity has been demonstrated to last for at least 3.5 years. Hence in this study it was very important to establish *T. parva* infection in order to achieve a carrier state of the disease and immune status.

3.2 An overview of the experiments

Preliminary studies were carried out at the CTVM, Edinburgh to test the PCR assay. Two susceptible calves were infected one with *T. parva* Boleni (Zimbabwe) and the other *T. parva* Marikebuni (Kenya). Both parasites are known to induce a carrier

state. The clinical, parasitological parameters and serology were monitored in the calves and blood taken for PCR assay.

Different Zimbabwean *T. parva* stocks including the *Theileria* vaccine (Bolvac®) were used to infect susceptible Friesian calves at the Veterinary Research Laboratories (VRL), Harare. Clinical and parasitological parameters were measured intensively until recovery from primary infection and then intermittently until the calves were challenged 522 days, later. In this study 5 groups of experimental calves were infected with different stocks of *T. parva* (as described in Table 3.1). Due the unavailability of cattle pens and calves to infect by the application of infected ticks only two calves could be used for the Ayrshire and Chikeya tick applications (Group IVa and b). The vaccine stock *T. parva* (Boleni) was used to infect group I experimental calves and the clinical reactions compared to other Zimbabwean parasite stocks.

At Hunyani estates there was a risk of a mixed *Theileria* infection because of the presence of wild animals e.g. tsesebbe, impala and sable. As a result it was very important to make cell culture isolates from the calves for characterisation using a panel of anti-schizont monoclonal antibodies in order to correctly identify any other *Theileria* species that may be present. The use of MAbs is particularly useful in differentiating *T. taurotragi* from non-pathogenic stocks of *T. parva* because these parasites are difficult to distinguish morphologically and in their common tick vector or in cattle, clinical reactions may be similar. In addition cross-reactions between parasites in routine IFA testing can occur (Grootenhuis *et al.*, 1979; Minami *et al.*, 1983). Those animals surviving initial infection were challenged with *T. parva*

Avery, which is known to be a virulent isolate (Koch, 1990). Calves in this study were challenged on days shown in Table 3.1 and calves in group II had homologous challenge.

This chapter describes the response of these calves to infection and challenge. Tick feeds were performed and material was generated for *in vitro* analysis in the subsequent chapters. Later chapters will describe studies on the persistence of the parasites in the calves as measured by microscopic examination, the feeding of clean nymphal *R. appendiculatus* ticks, PCR and serology. In this chapter the persistence of infection was determined by the examination of weekly blood smears, Giemsa stained to check for piroplasms.

3.3 Materials and methods

3.3.1 Experimental calves and maintenance

Calves for preliminary experiments at CTVM for infection with *T. parva* Marikebuni (calf 818) and *T. parva* Boleni (calf 805) were Friesian bulls of about 6 months of age and were kept on straw in tick-proof facilities. Calf 805 was challenged with a lethal dose of *T. parva* (Muguga) sporozoite stabilate 63 days post infection to determine if the Boleni stock protected against heterologous stock.

In Zimbabwe, Friesian bull calves of about 6 months old were bought from Lanark Farms situated 40 kilometres south of Harare. This farm is known to have no history of pathogenic theileriosis and strict tick control is practised from birth. Calves were shown to be seronegative for *T. parva* antibodies by the Indirect Fluorescent Antibody Test (Burridge and Kimber, 1972) and no parasites were seen in Giemsa-stained blood smears taken from the ear vein. Animals were maintained

on concrete floors in tick-proof facilities at VRL and were fed on low protein dairy meal for the whole experimental period until the final 35 days when they were allowed to graze in a paddock and received acaricidal treatment. Table 3.1 details the experimental calves used and parasite infections. Two groups (Va and Vb) of susceptible calves were transported to Hunyani estates and exposed to ticks in a paddock previously grazed by the beef herd on the farm. The calves were exposed on two separate occasions a year apart.

Table 3.1
Experimental design for primary animal infections performed in Zimbabwe.

Experiment calf group	**Calf number**	**Source of infection**	**Mode of infection**	**Interval between infection and challenge**
I	W70 W73 W74; W75	*T. parva* Boleni-vaccine stock	Subcutaneous inoculation	522 days
II	1442 1454	*T. parva* Avery stabilate	Subcutaneous inoculation	522 days
III	1447 1459	*T. parva* Bally Vaughan stabilate	Subcutaneous inoculation	522 days
IVa	1456	Ayrshire farm ticks	Tick application	564 days
IVb	1437	Chikeya farm ticks	Tick application	not done
Va	1372 1376 1451 1458	Hunyani Estates (1st)	Field exposure	566 days
Vb	E1, E2, E3, E4, E5	Hunyani Estates (2nd)	Field exposure	182 days

3.3.2 *T. parva* parasite stocks

The *T. parva* stocks were stored in liquid nitrogen as cryopreserved tick stabilates in Minimum Essential Medium (MEM) (Gibco) (Cunningham *et al.*, 1973) containing 7.5% glycerol, 200 units / 200 μg per millilitre of penicillin / streptomycin (Gibco) and 3.5% bovine plasma albumin-Fraction V (Sigma). Details of *T. parva* stocks are shown in Table 3.2.

(a) The original *T. parva* (Boleni) stock, when isolated at Boleni Farm in Arcturus in 1979 was found to be pathogenic (Lawrence and Mackenzie, 1980; Uilenberg *et al.*, 1982). From 1984, the pathogenicity of the parasite has been found to be mild in Friesian calves (Koch *et al.*, 1988; Irvin *et al.*, 1989). The stabilate *T. parva* (Boleni 86-1), which was derived from *T.parva* (Boleni 85-4), proved to be even milder than the original stocks. Examination of the *T. parva* (Boleni GU79-1) stabilate, derived from the original *T. parva* (Boleni GU79), in Friesian calves also resulted in less severe reactions than the original stabilate (FAO / DANIDA Programme Phase II, 1993). Group I calves were infected with the vaccine stock.

(b) Adult *R. appendiculatus* ticks were collected from a paddock at Bally Vaughan farm, 45 kilometres north-east of Harare Zimbabwe, shared by both cattle and wild ungulates including buffalo. The adult ticks were fed for four days on rabbit ears and a stabilate was prepared (Cunningham *et al.*, 1973). The parasite was characterised at The International Livestock Research Institute (ILRI, Nairobi) as buffalo derived *T. parva* formerly known as *T. p. lawrencei* (Bishop *et al.*, 1994). Group III calves were infected with the stock by subcutaneous inoculation.

(c) The Avery stock was isolated at a dairy farm near Gweru owned Mr. W. J. Avery. During a theileriosis outbreak, a sick Holstein-Friesian cow was brought to the Veterinary Research Laboratory, Harare and clean nymphal *R. appendiculatus* ticks were applied to pick up the infection (Koch, 1990). The salivary glands of the moulted adult ticks were dissected out, and stained using Methyl-green pyronin and had a mean intensity of infection of 133.3 infected acini per tick. The ground-up-tick stabilate was prepared as per method described by Cunningham *et al.* (1973). This stock was used to infect group II calves. (Table 3.2).

Table 3.2 *T. parva* stocks used for infection

	Boleni	Avery	Bally Vaughan
Isolation site	Boleni farm	Avery farm	Arcturus
Isolation date	Jan 1979	Jan 1986	Feb 1991
Method of Isolation	Clean *R. append.* ticks fed on field case	Clean *R. append* ticks fed on field case	Questing field ticks
Reference	Lawrence & Mackenzie, 1980	Koch, 1990.	FAO, 1991
MAb characterisation	Cattle derived *T. parva*	Cattle derived *T. parva*	Buffalo derived *T. parva*
Stabilate used	Gu79-3	Avery	BV 17
Stabilate number	24	23	17
Passage number	3	2	1
% infected ticks	63.5	58.2	1.8
Tick equivalent / ml	10	10	10
Volume used per calf (ml)	1	1	1

R. append. *Rhipicephalus appendiculatus*

Group I, II, and III calves were infected with *T. parva* Boleni, Avery and Bally Vaughan stabilates described above respectively.

3.3.3 Infection of experimental animals with ground up tick stabilates

At least four 2.0 ml Nunc cryotubes of any one stabilate were thawed rapidly in a 37°C water bath, pooled and allowed to equilibrate at room temperature for twenty minutes. Each experimental animal received 1.0 ml of a particular stabilate by subcutaneous inoculation in front of the right parotid lymph node using a tuberculin syringe with a one-inch 18-gauge needle.

3.3.4 Infections by application of adult *R. appendiculatus* ticks

Group IVa (calf 1456) was infected by applying 150 adult *R. appendiculatus* ticks to ear bags. The adult ticks were picked up by hand from grass-tops at the beginning of February 1994 from a paddock at Ayrshire Farm 90 kilometres north of Harare. The property is known to have had outbreaks of pathogenic theileriosis and cattle on the farm have been immunised against theileriosis since 1990. A sample of 29 adult ticks from the infection batch had an infection prevalence of 20.68% when they were dissected to determine the *Theileria* infection rate. The ticks collected from Ayrshire Farm had a total of 21 infected acini in the 6 infected ticks after partial feeding for 3-4 days on rabbits' ears:

Group IVb (calf 1437) was infected by applying 150 adult *R. appendiculatus* ticks in ear bags, which had been questing on grass in paddock at Chikeya Farm 100 kilometres north of Harare. The property is known to have had outbreaks of pathogenic theileriosis. A sample of 30 adult *R. appendiculatus* ticks were dissected and the infection intensity of 16.7%. Out of the 30 Chikeya Farm adult ticks collected 25 *Theileria* positive acini were detected in the 5 ticks that were infected.

The mortality records due to theileriosis are shown on Table 3.3 for the period 1986 to 1991 as reported to the Director of Veterinary Services Harare for the two above mentioned properties (DVS Monthly reports 1986 - 1991).

Table 3.3

Mortality due to Theileriosis on Ayrshire and Chikeya farms

Farm	Herd Census	DEATHS*				
		1986-87	1987-88	1988-89	1989-90	1990-91(δ)
Ayrshire	600	?	?	18	22	20(+35∞)
Chikeya	410	18	74	100	70	15(+16∞)

* DVS/Owner - manager/ FAO staff reports

(δ) FAO phase II Theileriosis project tracer animals put to graze in the same paddocks with farm owner's animals.

∞ severe infections treated with Clexon® (Coopers).

? Data not available

3.3.5 Field exposure of calves at Hunyani estates

Hunyani Estates, a property 70 kilometres south east of Harare, was identified for exposure of susceptible calves to theileriosis infection. The proprietor had reported four cases of pathogenic theileriosis in the 1992 rainy season and the FAO Theileriosis team at VRL had immunised cattle on the property in October 1993. The farmer practised weekly dipping of cattle from the months of November to June

and twice per week from July to October in Amitraz. Neighbouring farmers had also lost animals due to theileriosis when they had strayed through broken fences onto the Hunyani estates.

A group of four *Theileria* negative calves purchased from Lanark Farms (Group Va, numbers 1372, 1376, 1451, and 1458) were allowed to graze in a paddock at Hunyani Estates known to be heavily infested with adult *R. appendiculatus* ticks in February 1994. Questing grass top *R. appendiculatus* adult ticks were picked up by hand in the paddock when the susceptible calves were exposed, and had a prevalence of infection of 10.7%. A second group (Group Vb calves E1, E2, E3, E4 and E5) were moved to Hunyani Estates in February 1995, into the same paddock as the previous group for field exposure to ticks, in order to pick up infection and attempt tissue culture isolation of the *Theileria* parasite. Both groups were seen daily by an animal attendant on the farm and monitored once a week by Mr. K. Kanhai (FAO) and the author. They were visited immediately if there were any reports of sick animals.

3.4 Monitoring and assessment of Theilerial infections during primary reaction

At the CTVM the calves used for the preliminary studies were monitored daily by Dr. S. Williamson and the author according to the monitoring regime detailed below. The calves exposed at Hunyani estates (group V) were monitored once a week by visits to the estates and closer monitoring was achieved when they were transferred to the VRL-Harare post recovery and challenge with the rest of the experimental

animals. Calves in groups I - IV were monitored from the day of infection (day 0) by Drs. T. Hove, D. Munodzana and the author for 35 days as follows:

(a) Clinical examination

Rectal temperatures were recorded daily from day 0 of primary infection or challenge. A temperature of 39.5°C and above was recorded as a febrile response. During the course of infection the clinical signs of the disease including pyrexia, depression, inappetance, weakness, lachrymation, petechial haemorrhages and lymph node enlargement were noted. A check list was used on each occasion. Post mortem examinations were carried out on all animals which died and smears were prepared from the prescapular, parotid, precrural and mesenteric lymph nodes, the heart muscle, spleen, liver, kidney, lung and brain.

(b) Parasitological examination

(i) Lymph nodes-: The presence of schizonts (macroschizonts/microschizonts) and hyperplastic cells in the draining lymph nodes were monitored by examination of Giemsa stained needle biopsy smears. These were prepared daily when they were enlarged, starting on day 5 post inoculation of sporozoite stabilate, when the lymph nodes were first found to be enlarged. Lymph node biopsy smears from animals which had ticks applied on them were made after having palpation to check for enlargement due to infection. The biopsy sampling was continued until schizonts were no longer detected on three consecutive days of sampling. Lymph node biopsy smears were air dried, fixed in absolute methanol (AEC-SA) for one minute and stained in filtered 10% Giemsa stain (Merck) in Giemsa buffer (Appendix 1) for 20 minutes. The smears were rinsed in tap water, air dried and examined under oil

immersion at 500 X and 1000 X magnification using a Labolux D microscope (Leitz, Germany). The degree of schizont parasitosis was evaluated as:

Mac+ 1% cells containing schizonts (1 on the macroschizont index).

Mac++ 1-5% of cells containing schizonts (2 on the macroschizont index).

Mac+++ > 5% of cells containing schizonts (3 on the macroschizont index.

(ii) Blood smears

The presence and level of parasitaemia was monitored by the examination of thin blood smears, using blood taken from the ear veins taken on day 0 and from day 12 until no parasites were seen for three consecutive days, following which blood smears were taken weekly. Blood smears were stained as described above, and the degree of parasitaemia was recorded as the percentage of infected red blood cells. The parasitaemia was noted after 50 high power fields (HPF) using a X100 objective had been examined. The smear was recorded as "no parasites seen" if 100 high-power fields (approximately 35 000 RBC) were examined and no piroplasms observed.

(c) Tissue culture

Jugular blood was taken into vacutainer tubes (Becton Dickinson, California, USA) containing the di-sodium salt of ethylenediaminetetra-acetic acid (EDTA - Sigma) in order to isolate *Theileria* infected cells from calves undergoing a clinical reaction after exposure at Hunyani estates. Details of tissue culture attempts are given in section 3.3.

(d) PCR

Blood was taken into EDTA vacutainer tubes for use in the PCR as detailed in Table 3.5.

(e) Serology

Blood was taken in plain vacutainer tubes (Becton Dickinson, Meyland, Cedex, France) a day prior to infection or exposure of calves and one day before challenge. Calves were also sampled for serum as indicated in Table 3.3. The blood was left to clot at room temperature, incubated for 1 hour in a 37°C water bath and then left to stand overnight at 4°C. The following day, serum was removed and centrifuged at 400 xg for 20 minutes at room temperature. The serum was aliquoted and stored at -20°C until use.

3.5 Monitoring between recovery and challenge

Blood for serum and PCR was taken according to the sampling protocol shown in Table 3.4. Rectal temperatures were recorded daily. Blood smears were also taken weekly from all the animals for the duration of the experiment and Giemsa stained for microscopic examination.

3.5 (a) Treatment

During the primary infection and the challenge, animals which underwent severe reactions were treated with buparvaquone (Butalex, Coopers, England) at a dose of 2.5 mg/kg, intramuscularly on a single occasion.

3.5 (b) Evaluation for *T. parva* reactions

The criteria used to define calf reactions to the infection were as described by Anon, (1989):

NR no reaction - when no parasites were detected on the Giemsa-stained blood smears and no clinical signs were apparent.

MR mild reaction - when the animal was clinically normal, with no fever or a fever persistent for less than 4 days, a low schizont parasitosis and the animal recovered.

MoR moderate reaction - when the animal showed a mild and transient clinical reaction which eventually disappeared with a schizont parasitosis and a febrile reaction of 5 to 9 days.

SR severe reaction - when schizonts were detected, the febrile reaction persisted for 8 days or more and the animal was clinically ill.

Some of the experimental animals at Hunyani Estates died despite treatment when they had severe theileriosis.

Post mortem examinations were carried out on all animals which died and smears were prepared from the prescapular, parotid, precrural and mesenteric lymph nodes, heart muscle, spleen, liver, kidney, lung and brain. Smears were fixed and Giemsa stained as described above in order to confirm the cause of death as theileriosis.

Table 3.4 Sampling protocol for IFAT and PCR for Group I, II, III experimental calves

Day of experiment*	Serum for IFAT	EDTA blood for PCR Giemsa-stained blood smear
0	+	+
5	-	+
7	-	+
9	-	+
14	-	+
25	-	+
40	+	+
74	+	+
95	+	+
109	+	+
137	+	+
179	+	+
207	+	+
218	+	+
263	+	+
305	+	+
361	+	+
456	+	+
493	+	+
522	+	-
563	+	-

* The calves were sampled on the same dates, thus the days post infection differed depending on the method of infecting the animal applied, (post infection with stabilate, post tick application or post exposure at the Hunyani Estates).

Blood smears were done on each animal weekly for Giemsa staining. The PCR and IFAT are described in the later chapters 4 and 6 respectively. The group IV, Va and Vb experimental calves were sampled at the same time as the other calves post tick application and exposure, thus they had different days post infection samplings.

3.6 Characterisation of the Hunyani isolate with Monoclonal Antibodies (MAbs)

In order to identify the *T. parva* stock at Hunyani Estates a panel of eleven monoclonal antibodies (MAbs) raised against *Theileria* macroschizont antibodies supplied by ILRI (Nairobi) were used to analyse the cell lines established from the animals exposed at Hunyani estates. Infected lymphoblastoid cells isolated from the animals (Tag numbers E1, E3, E4, and E5) exposed at Hunyani Estates were tested with the panel of MAbs, *T. parva* Muguga (Kenya) and *T. parva* Boleni (Zimbabwe) schizont antigens. All the antigens were prepared using the formaldehyde fixation method as described in the preparation of macroschizont antigen (section 6.2.1). The anti-schizont MAbs 1-4, 7, 10, 12, 15, and 20 used in this study have been described before (Minami *et al.*, 1983; Conrad *et al.*, 1987). MAbs 21 and 22 have also been described by Conrad *et al* (1989). Pearson *et al* (1979) prepared MAb 23 using mice inoculated twice with cells of a *T. taurotragi* schizont -infected lymphoblastoid cell line, which gave positive reactions with *T. taurotragi* schizonts but not with *T. parva* schizonts. The ten well slides used in the MAb -IFAT had three MAbs applied per slide at dilutions of 1 in 50 and 1 in 200. Twenty µl of MAb at the specified dilution was used and the remaining wells had PBS pH 7.2 added. After incubation in a moist chamber for 30 minutes at room temperature the slides were washed

thoroughly two times for 15 minutes with gentle agitation in ice cold PBS. Twenty µl of conjugate was added to each well after the washes and incubated in a moist chamber at room temperature for 30 minutes. The conjugate used was a sheep anti-mouse IgG-Fluorescein isothiocyanate (FITC) conjugate (Serotec, Denmark) at a dilution of 1:60 in PBS containing 0.01% (w/v) Evans Blue. Following incubation with the conjugate the slides were given a further wash as before and mounted under 50% dilution buffered glycerol (Microscopic glycerol-BDH) in PBS(1:1) with a 50 mm x 22 mm cover slip and were ready to be read under a Leitz fluorescent microscope. The results were recorded as the percentage of fluorescing cells and the intensity of fluorescence as follows:

+ >90% fluorescence

(+) 50% fluorescence

$(+)^{w}$ weak fluorescence

- no fluorescence

3.7 RESULTS

3.7.1 Reactions to primary infection

The reason for carrying out *T. parva* infections at the CTVM was to determine whether the type of experiment could be carried out successfully in Harare. That is, the infection of cattle with laboratory *T. parva* stocks, monitoring of parasites microscopically, determining of presence of parasites indirectly by PCR, serology and the optimisation of the PCR assay. The experiments at CTVM were successful and indicated that similar studies could be carried out in Zimbabwe using local

stocks of *T. parva*. Clinical reactions and microscopy results are described in this chapter and the PCR, tick infections and serology in subsequent chapters.

A summary of calf reactions to primary infections with *T. parva* Boleni and Marikebuni at the CTVM is given in Tables 3.5 and 3.6 respectively. Calf 805 infected with neat 1 ml of the Bolvac® vaccine had a severe theileriosis reaction and did not require treatment since we intended to validate the PCR assay for the detection of *T. parva*.

The clinical and parasitological reactions for individual calves (groups I, II, III, IV and V) for the first 30 days post infection are summarised in Tables 3.7 and 3.8. Two calves in Group I (W74 & W75) were lost in accidents in the cattle pens at VRL Harare and calf 1437 had to be humanely killed due to a bacterial infection in its urinary bladder.

3.7.2 Reactions to *T. parva* Boleni and Marikebuni preliminary infection studies

Calf number 818 infected with *T. parva* Marikebuni underwent a mild clinical reaction. The calf had macroschizonts detected in the right and left prescapular glands on three days post infection. The febrile episode persisted for less than four days and the animal recovered without any treatment as detailed in Table 3.6.

The *T. parva* Boleni infected calf (805) had a severe clinical reaction with schizont parasitosis detected for 16 successive days and had 12 days of febrile reaction as detailed in Table 3.6. Piroplasm parasitaemia in this calf lasted for 20 successive days from day 14 with a maximum of 0.6% on day 19 post infection. The calf recovered without treatment and was challenged with a lethal dose of *T. parva*

(Muguga) sporozoite stabilate 63 days post infection to determine if the Boleni stock protected against heterologous stock.

PCR assays using the p67 primer sequences for *T. parva* on samples from the calves 818 and 805 were successful and therefore allowed us to carry out further experiments in Harare. (PCR results are given in Chapter 4).

3.7.3 *T. parva* Boleni infection

Calves in group I (W70, W73, W74 and W75) underwent moderate reactions and did not require treatment. Calf W73 did not demonstrate a rectal temperature greater than 39.5°C and had a macroschizont parasitosis and piroplasm parasitaemia of less than 0.1% for 23 days. The other calves in the group had rectal temperatures above 39.5°C as shown in summary of primary infections in Table 3.7. Calves in this group showed piroplasm parasitaemias of not more than 0.1% during the 30 days post infection. The piroplasm parasitaemia persisted for an average period of 28 days in all 4 calves (range of 22 to 35 days). The piroplasms in the *T. parva* Boleni infected animals appeared intermittently during the 522 day period before heterologous challenge. The group also had low macroschizont parasitosis for 8 to 10 days.

3.7.4 *T. parva* Avery infections

Of the two calves in Group II, animal 1442 had no parasites detected in the draining lymph nodes. Calf 1442 showed a piroplasm parasitaemia of less than 0.1% on days 179 and 193 post infection although it did not show evidence of infection during the initial 30 days. The second calf in group II (calf 1454) had a severe *Theileria*

reaction and had to be treated on day 15 post infection and it did not show any piroplasm parasitaemia.

3.7.5 *T. parva* Bally Vaughan infections

Calf 1459 showed no evidence of *Theileria* reaction, no febrile reaction and no piroplasm parasitaemia throughout the period before heterologous challenge as shown in Table 3.7. The other animal infected with the *T. parva* Bally Vaughan stabilate (calf 1447) had a moderate clinical *Theileria* reaction as shown in Table 3.7. A piroplasm parasitaemia of less than 0.1% was detected from day 17 to 25, and on a single occasion, day 367 post infection in the Giemsa stained blood smear (calf 1447).

3.7.6 Infections from tick applications

(a) Naive calf 1456 which had application of Ayrshire Farm ticks showed a moderate *Theileria* reaction and macroschizont parasitosis for 8 days and piroplasm parasitaemias of less than 0.1% for 11 days. The calf recovered without treatment and had persistent piroplasm parasitaemia for up to day 321 post tick application (Table 3.7).

(b) Calf 1437 had the Chikeya ticks and underwent a moderate clinical reaction with macroschizonts detectable by microscopy for 12 days post tick application. The piroplasm parasitaemia was 0.1% for 11 successive days from day 17 post tick application. (Table 3.7)

3.7.7 Hunyani field exposure

3.7.7 (a) Three calves in group Va (Tags 1451; 1376; 1458) died from severe theileriosis on day 14 following field exposure at Hunyani Estates even after

buparvaquone treatment. Post mortem confirmed theileriosis as the cause of death and macroshizonts were seen in brain smears of two calves (1451 and 1376). Calf 1372 survived following treatment and maintained a piroplasm parasitaemia of between 0.1% and 2% during the 522 days prior to heterologous challenge. Details of clinical reactions to primary infection are shown in Table 3.7.

3.7.7 (b) Group Vb calves (E1 to E5) exposed to ticks in the field at Hunyani Estates a year later (February 1995) suffered severe clinical theileriosis despite buparvaquone treatment, two calves died (E2 and E3) after transportation to the tick proof facilities at VRL - Harare. Post mortem examinations confirmed theileriosis as cause of death.

The piroplasm parasitaemias in the surviving calves E4 and E5 were 1% when the animals were undergoing Theileria clinical reactions and decreased to 0.1% post recovery. Piroplasms could be seen in the Giemsa-stained blood smears on every seventh day up to 80days post exposure. Piroplasms were seen more frequently in the Hunyani Estates exposed calves than in the other experimental groups (I, II ,III, IVa and b).

3.7.7 (c) The results for the reactivities of *Theileria* schizont-infected bovine lymphocyte culture isolates are presented in Table 3.9. Isolates from Hunyani estates gave positive reactions with the *T. taurotragi* - specific MAb 23 and also MAbs 12 and 10. Reactions with MAb 10 which was raised against *T. parva* (Kiambu-Kenya), and MAb 22 which is characteristic of *T. parva* showed 50% fluorescence (Table 3.9) The *T. parva* Boleni and Muguga controls gave negative and positive reactions with MAb 7 respectively Table 3.9).

MAbs 1-4, 7, 10, 12, 20, 21, 22 show a pattern characteristic of *T. parva* (Minami *et al.*, 1983; Conrad et al., 1989). Zimbabwean isolates have been known not to react with MAb 7 (Koch *et al.*, 1988). Isolates E1, E2, E3 and E4 gave strong positive reactions with the *T. taurotragi*-specific MAbs 23 and 12, but weak reactions with MAb 10. There was also some weak binding with MAb 22 in cell culture isolates E1 and E2 at 1:50 dilution of the MAb.

3.8 Reactions to *T. parva* Avery challenge

A summary of clinical reactions to *T. parva* Avery challenge is given in Table 3.8 for the first 40 days post challenge. The experimental animals were immune to *T. parva* Avery challenge although two calves exposed at Hunyani estates (E4 and E5) had macroschizont parasitosis for 4 and 3 days, at 14 and 16 days post challenge respectively.

The *T. parva* Boleni infected / recovered calves did not have a febrile reaction, no macroschizonts but a piroplasm parasitaemia of less than 0.1% was seen in the Giemsa stained blood smears 21 and 24 days post challenge in calves W70 and W73 respectively. The piroplasms persisted for 5 days in calf W70, and 2 days in calf W73 post challenge.

In the group II calf (1454) had a piroplasm parasitaemia of less than 0.1% on day 23 only post challenge and the other calf had no parasites seen in the Giemsa stained blood smear. The other calf (1442) which received a homologous challenge had a rectal temperature above 39.5°C on day12 post infection, did not have macroschizont parasitosis and piroplasm parasitaemia.

The *T. parva* Bally Vaughan (group III) infected / recovered calf 1442 had no parasites seen in the Giemsa stained lymph node and blood smears post challenge, but calf 1459 had a piroplasm parasitaemia of less than 0.1% on day 21 only post challenge with *T. parva* Avery.

Calf 1456 infected and recovered after tick application had no clinical *Theileria* reaction and a piroplasm parasitaemia of less that 0.1% was seen in the blood smears for 4 days from day 23 post heterologous challenge.

The control calves had severe reactions and calf 4855 died on day 30 post infection. A prolonged macroschizont parasitosis was seen in the lymph node smears for 10 days in calf 4853, and 11 days in calf 4855 post infection.

Table 3.5 *T. parva* Marikebuni infected calf No:818 febrile reaction and parasitological evidence of infection

Day p.i	Rectal temp. °C	RPG smear	LPG smear	Blood smear
0	39.0	ns	ns	nps
1	38.9	ns	ns	nps
2	39.2	ns	ns	nps
3	39.0	ns	ns	nps
4	39.5	nps	ns	nps
5	39.2	nps	ns	nps
6	39.2	nps	ns	nps
7	38.8	nps	ns	nps
8	39.6	nps	ns	nps
9	38.7	mac +ve	ns	nps
10	39.3	nps	nps	nps
11	39.1	nps	nps	nps
12	39.1	nps	nps	nps
13	39.4	nps	nps	nps
14	39.2	nps	nps	+ 1piro
15	38.7	mac +ve	nps	nps
16	39.0	nps	nps	+<0.1%
17	39.1	mac +ve	mac +ve	+<0.1%
18	38.9	nps	nps	nps
19	38.8	nps	nps	+<0.1%
20	39.0	nps	nps	+<0.1%
21	39.0	nps	nps	+ 0.1%
22	39.2	nps	nps	nps
23	38.9	nps	nps	+ 0.1%
24	38.7	ns	ns	nps
25	38.9	ns	ns	+<0.1%
26	38.5	ns	ns	+<0.1%
27	38.1	ns	ns	ns
28	38.4	ns	ns	+<0.1%
29	38.9	ns	ns	+<0.1%
30	38.6	ns	ns	ns

temp. :Temperature
nps :no parasites seen#
RPG :right prescapular gland
LPG :left prescapular gland
mac +ve :macroschizont positive
ns :not sampled
+<0.1% :piroplasms observed (less than 0.1% parasitaemia)

Table 3.6: *T. parva* Boleni infected calf No:805 febrile reaction and parasitological evidence of infection

Day p.i	Rectal temp. °C	RPG smear	LPG smear	Blood smear
0	38.6	ns	ns	nps
1	38.6	ns	ns	nps
2	38.7	ns	ns	nps
3	38.6	ns	ns	nps
4	38.6	nps	ns	nps
5	38.8	nps	ns	nps
6	38.5	nps	ns	nps
7	38.8	nps	ns	nps
8	39.7	mac +ve	ns	nps
9	39.2	mac +ve	ns	nps
10	39.1	mac +ve	mac +ve	nps
11	40.6	mac +ve	mac +ve	nps
12	41.2	mac +ve	mac +ve	nps
13	40.9	mac +ve	mac +ve	nps
14	40.4	mac +ve	mac +ve	+<0.1%
15	40.7	mac +ve	mac +ve	+<0.1%
16	40.2	mac +ve	mac +ve	+<0.1%
17	40.4	mac +ve	mac +ve	+ve0.1%
18	39.7	mac +ve	mac +ve	+ve0.3%
19	39.5	mac +ve	mac +ve	+ve0.6%
20	39.6	mac +ve	mac +ve	+ve0.3%
21	39.6	mac +ve	mac +ve	+ve0.4%
22	39.4	mac +ve	mac +ve	+ve0.2%
23	39.2	mac +ve	mac +ve	+ve0.2%
24	39.2	nps	nps	+ve0.2%
25	39.0	nps	nps	+ 0.1%
26	38.7	nps	nps	+<0.1%
27	38.7	ns	ns	+<0.1%
28	38.7	ns	ns	+<0.1%
29	38.6	ns	ns	+<0.1%
30	38.6	ns	ns	+<0.1%

temp. :temperature
nps :no parasites seen
+<0.1% :piroplasms seen (less than 0.1% parasitaemia)
RPG :right prescapular gland
LPG :left prescapular gland
mac +ve :macroschizont positive
ns :not sampled

Table 3.7: Summary of reactions of calves in the first 50 days to primary infection with *Theileria* parasites

GROUP	INFECTION	CALF NO.	DAYS TO and DURATION			*T. parva* IFAT reciprocal titre		*T. parva* reaction	Buparvaquone Treatment	Died/Survived
			Temp >39.5°C	Macs	Piroplasms	Day0	Day 40 p.i.			
I	*T.parva* Boleni	W70	12(3)	8(8)	14(35)	160	640	MoR	nil	Survived
		W73	-	9(7)	14(33)	80	1280	MoR	nil	Survived
		W74	8(5)	8(10)	13(23)	80	2560	MoR	nil	Survived
		W75	7(2)	7(10)	13(22)	160	1280	MoR	nil	Survived
II	*T.parva* Avery	1454	9(8))	10(7	nps	160	5120	SR	Day 15	Survived
		1442	-	nps	nps	160	320	NR	nil	Survived
III	*T.parva* Bally	1447	13(3)	10(8)	17(8)	160	2560	MoR	nil	Survived
	Vaughan	1459	-	nps	nps	160	1280	NR		Survived
IV(a&b)	Ayrshire ticks	1456	12(3)	9(14)	16(11)	160	640	MoR	nil	Survived
	Chikeya ticks	1437	11(8)	11(12)	17(11)	80	1280	MoR	nil	Survived
Va	Hunyani 1st	1372	-	11(13)	19(6)	160	1280	SR	Day 14	Survived
	exposure	1376	nd	10(4)	died	80	died	SR	Day 14	Died day 14 p.i
		1451	nd	12(2)	died	160	died	SR	Day 14	Died day 14 p.i.
		1458	nd	10(2)	died	160	died	SR	Day 14	Died day 14 p.i.
Vb	Hunyani 2nd	E1	9(5)	9(12)	17(5)	80	1280	SR	Day 14	Survived
	exposure	E2	9((5)	10(4)		160	died	SR	Day 14	Died day 14 p.i.
		E3	9(5)	8(6)		80	died	SR	Day 14	Died day 14 p.i.
		E4	10(5)	10(8)	14(13)	160	1280	SR	Day 14	Survived .
		E5	9(5)	14(7)	21(7)	80	640	SR	Day 14	Survived

Key: MoR :Moderate reaction NR :no reaction
SR :severe reaction macs :macroschizonts
nd :not done p.i. :post infection
Temp. Rectal temperature
( ) :figures refer to number of days positive for macroschizonts or piroplasms, or had febrile reaction

Table 3.8: Summary of cattle reactions to *T. parva* Avery challenge

GROUP	INFECTION	CALF NO.	Days post primary infection	DAYS TO and DURATION( ) Temp. >39.5°C	Macs	Piroplasms	*T. parva* IFAT recip titre Day 0 &	40 p.c.	*T. parva* reaction
I	*T. parva* Boleni	W70	522	-	nps	21(5)	320	640	NR
		W73	522	-	nps	24(2)	640	1280	NR
		W74 &75	Died						
II	*T. parva* Avery	1454	522	-	nps	23(1)	640	1280	NR
		1442	522	12(1)	nps	nps	640	1280	NR
III	*T. parva* Bally	1447	522	-	nps	nps	1280	1280	NR
	Vaughan	1459	522	-	nps	21(1)	640	640	NR
IV(a&b)	Ayrshire ticks	1456	564	-	nps	23(4)	640	1280	NR
		1437	Died						
Va	Hunyani 1st exposure	1372	566	-	nps	21(7)	1280	640	NR
		1376	Died						
		1451	Died						
		1458	Died						
Vb	Hunyani 2nd exposure	E1	182	-	nps	21(4)	640	640	NR
		E4	182	-	14(4)	21(7)	320	1280	NR
		E5	182	-	16(3)	21(7)	640	2560	NR
		E2 &3	Died						
Control		4853		13(10)	10(15)	25(4)	80	2560	SR
		4855		7(11)	7(22)	15(14)‡	320	died	SR

Key: SR :severe reaction nps :no parasites seen macs :macroschizonts
NR :no reaction p.c. :post challenge Temp. :rectal temperature
( ) :figures refer to number of days positive for macroschizonts or piroplasms, or had febrile reaction.

Table 3.9
Results of Hunyani *Theileria* schizont lymphocyte culture isolates (E1, E2, E3, and E4) with anti-schizont monoclonal antibodies. Two control cell lines included.

MAb	Dilution	*Theileria* cell line E1	E2	E3	E4	Boleni	Muguga
1	1:50	-	-	-	-	+	+
	1:200	-	-	-	-	+	+
2	1:50	-	-	-	-	-	+
	1:200	-	-	-	-	-	+
3	1:50	-	-	-	-	-	+
	1:200	-	-	-	-	-	+
4	1:50	-	-	-	-	+	+
	1:200	-	-	-	-	+	+
7	1:50	-	-	-	-	-	+
	1:200	-	-	-	-	-	+
10	1:50	(+)	(+)	(+)	(+)	+	+
	1:200	-	-	(+)	-	+	+
12	1:50	+	+	+	+	+	+
	1:200	+	+	+	+	+	+
20	1:50	-	-	-	-	+	+
	1:200	-	-	-	-	+	+
21	1:50	-	-	-	-	+	+
	1:200	-	-	-	-	+	+
22	1:50	?(+)^{w}	?(+)	-	-	-	+
	1:200	-	-	-	-	-	+
23	1:50	+	+	+	+	-	-
	1:200	+	+	+	+	-	-

MAb :monoclonal antibody
\+ :positive (>90% fluorescence)
(+)^{w} :weak fluorescence
MAb 23 :*T. taurotragi* specific
\- :no fluorescence
(+) :50% fluorescence

3.9 Discussion

The *Theileria* vaccine (Bolvac®) was found to produce a severe *Theileria* reaction in the experiments at the CTVM and a high piroplasm parasitaemia of 0.6% on day 19 post infection was observed although the calf recovered without treatment. However, a moderate reaction was demonstrated in Group I calves at VRL-Harare. Although clinical reactions in the groups of calves showed that the Boleni is an ideal parasite stock for immunisation purposes, it has to be administered with caution especially in dairy herds as calf 805 infected at the CTVM Edinburgh had a severe reaction to the *T. parva* Boleni infection. However, the original *T. parva* Boleni stock, when isolated was pathogenic until the early 1980's when the stock became less virulent (Koch, 1990. The present *T. parva* Boleni GU79-1.3 (Bolvac®) stabilate derived from the original *T. parva* (Boleni GU79) causes less severe reactions than the original stabilate as observed in the Theileriosis immunisation programme (FAO, report).

Although calf 1442 did not have a *Theileria* clinical reaction it was infected as it had a piroplasm seen in the Giemsa stained blood smears post infection. The other calf required treatment as it underwent severe reaction.

Group IV calves (1456 and 1437) which had infected ticks from Ayrshire and Chikeya farms applied to them both had moderate reactions to *Theileria* infection and survived without treatment. This is a definite indication of the presence of *Theileria* parasites in the field as previously discussed the two properties have had Theileriosis outbreaks before (Table 3.3) and they were immunisations with Bolvac® vaccine.

A pathogenic *Theileria* was isolated from the calves exposed at Hunyani estates which was shown to be *T. taurotragi* on the basis of monoclonal antibody results. The observation that two of the cell culture isolate (E1 and E2) antigens partially reacted with the MAb 22 indicates that these isolates may contain mixed parasite populations which differ in their expression of specific schizont antigens. This was also confirmed by the severe theileriosis clinical reactions the calves underwent, *as T. taurotragi* has been reported to cause mild infections (Koch, 1990; Norval *et al.*, 1992). he reaction to MAbs 10 and 12 are similar to results obtained with isolates from Chipinge and Lawfield in Zimbabwe (Koch *et al.*, 1988) and both isolates were considered to be non-pathogenic *T. taurotragi*, based on serological and cross-immunity tests (Lawrence and Mackenzie, 1980; Uilenberg *et al.*, 1982). Also the results were similar to *T. taurotragi* isolates from Kenya (Minaini *et al.*, 1983). The only difference was in the severe clinical reactions in the two groups of susceptible Friesian calves exposed at Hunyani Estates. The results emphasise the value of MAb testing as a means of differentiating *T. taurotragi* from non-pathogenic stocks of *T. parva* and in situations where there are wild ungulates which can be alternative hosts for the vector tick. Hunyani Estates is surrounded by the Chivero National Park and the presence of wild ungulates which could provide alternative hosts for the ticks was inevitable. The calves which survived were immune to *T. parva* Avery challenge and yet the monoclonal antibody profile test was positive for the *T. taurotragi* specific MAb 23. After recovery piroplasms persisted for a long period intermittently and were detectable by direct microscopic examination thus indicating the presence of infection in the animals, although piroplasm parasitaemias of less than 0.1% were

recorded frequently post recovery.. Further characterisation studies are required on the Hunyani isolate.

The calves in groups I, II, III and IV had an average live-weight of 380 kilograms. The Hunyani exposed calves were undersized up to the end of the experiment and were disposed of with average live-weights of 150 kilograms

No clinical reactions were noticed on challenge of the experimental animals except for the control group. One of the control group animals did not react to the infection with *T. parva* Avery (4855) but was infected at a later date and showed a severe reaction.

Piroplasms were seen on day 291 post infection in the blood smears of the calf which had a severe *Theileria* reaction (1454) and was treated with buparvaquone. The levels of parasitosis in individual cattle receiving a particular dose of stabilate and even in those receiving a particular number of infected ticks has been demonstrated to be variable (Norval *et al.*, 1992). This variability has been attributed to the different tick infection prevalences and even with *Theileria* infections induced by tick feeding sporozoites are injected over a period of time, resulting in a low grade inoculation over several days (Purnell *et al.*, 1974; Young, 1977), resulting in severe clinical reactions.

In this chapter calves were successfully infected with *Theileria* parasites to provide material to be used in the subsequent chapters in the determination of infection indirectly by PCR, tick infections and serology. The success of the infections was also demonstrated by the immunity to *T. parva* Avery challenge and each method of infection resulted in immunity.

CHAPTER FOUR

Detection of *T. parva* in carrier / recovered animals using PCR amplification

4.1 Introduction

The main aim of this study was to develop an alternative diagnostic technique for the detection of carrier state of *T. parva* infection since the conventional methods of parasite detection have limitations in their levels of sensitivity and specificity. The classical definition of a carrier of *Theileria* is the ability of an infected and recovered animal to serve as a reservoir of infection for vector ticks which are then able to transmit the parasite to a susceptible host (Levine, 1973; Young *et al.*, 1986). This chapter describes the application of the PCR in the detection of infection or *Theileria* parasites either as macroschizonts or piroplasms in the experimental animals described in Chapter 3.

The ability of a diagnostic tool to be highly sensitive and specific in epidemiological studies is important as this will enable workers in disease control programmes to define prevalences of the carrier state in different cattle populations. In many *Theileria* infected animals, infection persists after recovery and is maintained by two stages of the parasite's life cycle: piroplasm, and macroschizont (Norval *et al.*, 1992). The epidemiology of theileriosis in Zimbabwe is complicated by the existence of non-pathogenic *Theileria* species in cattle, which may correspond to *T. taurotragi* from eland, and can be difficult to distinguish from *T. parva* (Lawrence and Mackenzie, 1980; Koch, *et al.*, 1988). Vaccination against theileriosis has been initiated using the Boleni strain and this might further

complicate the epidemiology of the disease as foci of infection are created because the strain is known to induce a carrier state (Koch, *et al.*, 1992).

A method of demonstrating carrier state is tick application to animals and transmission of infection to susceptible hosts (xeno-diagnosis), this is the definitive method of proving the carrier state of an animal, but it is not practical on a large scale is expensive, laborious and may not always detect actual carriers (Koch, 1990). Currently carrier status is thought to be maintained by the persistence and slow division of macroschizonts giving rise to piroplasms and the PCR offers a way of parasite detection. The IFAT and other serological tests do not always detect carriers whose infection can be confirmed by xenodiagnosis (Dolan, 1986a). Therefore, improved methods for detection of long term *T. parva*-infected animals, capable of transmitting disease to the tick vector then to susceptible cattle are required.

Preliminary investigations at the CTVM were done using the p67 primer sequence to detect *Theileria* parasites in *T. parva* Boleni and *T. parva* Marikebuni infected animals undergoing clinical reactions. At the VRL-Harare *T. parva* p104 primer sequences were used on the samples from the animals undergoing clinical reactions and post recovery after comparing with results obtained with the p67 primers. The p104 primer sequences were found to amplify a product from all *T. parva* stocks, including Boleni and buffalo derived, but nothing was amplified from DNA from *T. mutans*, *taurotragi* or *annulata*. The sensitivity of the PCR assay on *T. parva* DNA was about 100-?00 parasites on ethidium-bromide agarose gel, and about 10 parasites with probing (R. Skilton personal communications).

PCR is an *in vitro* method of nucleic acid synthesis by which a targeted segment of DNA can be specifically replicated. It involves two oligonucleotide primers that flank the DNA fragment to be amplified in repeated three-step cycles of heat denaturation of the DNA, annealing of the primers to their complimentary sequence, and extension of the annealed primers with DNA polymerase. These primers hybridise to opposite strands of the target sequence and are orientated so that DNA synthesis by the polymerase proceeds across the region between the primers. Since the extension products of this reaction are also complementary to and capable of binding the primers, successive cycles of amplification essentially double the amount of target DNA synthesised in the previous cycle. The result is an exponential accumulation of the specific target fragment, theoretically 2^n, where n is the number of amplification cycles performed. The isolation of a thermostable DNA polymerase (Saiki *et al.*, 1988) purified from the thermophilic bacterium, *Thermus aquaticus* (*Taq*) which can survive incubation at temperatures required for DNA strand separation (up to 98° C) enabled automation of the process to occur.

Preliminary investigations at the CTVM using the p67 *T. parva* primer sequence were aimed at trying to achieve a high level of sensitivity of the PCR assay. The high sensitivity of the PCR is due to the exponential amplification of target DNA, and is so sensitive that a single DNA molecule has been amplified successfully (Innis and Gelfand, 1990). Factors such as enzyme, dNTP, primer, magnesium concentrations affect the PCR product yield since the process is cyclic (Xu and Larzul, 1991). Many other factors like number of cycles, annealing and denaturation temperatures also affect the efficiency of the PCR. Although theoretically it is

possible to detect one gene copy, this cannot be achieved in diagnostic applications. After optimisation of the PCR, the sensitivity of detection must be determined, and optimal methods should be developed for the treatment of samples to release target DNA, while minimising inhibition by other components. A lack of sensitivity in the detection of *T. parva* was observed in tick stabilates cryopreserved in glycerol when tested after phenol / chloroform extraction.

The discovery of the sequence from the central region of the small subunit ribosomal RNA (srRNA) has provided specific primers for PCR amplification of DNA from different *Theileria* species (Allsopp *et al.*, 1993). *T. parva* (Boleni) and buffalo derived *T. parva* have been detected in naturally recovered animals by PCR (Bishop *et al.*, 1992). Molecular characterisation of 40 *Theileria* isolates from Zimbabwe has shown that there was no support for the subspecific recognition of *T. parva* using the repetitive DNA probe, pBOLREP1 hybridised to Southern blots of *Eco*R I-digested DNA, as there were similarities in *T. parva* parasites isolated from cattle on seven farms. The *Tpr* restiction fragment pattern of 5 *T. parva* isolates from East Africa were not similar to the predominant *Tpr* genotype of the isolates from Zimbabwe (Bishop *et al.*, 1994). A high degree of sensitivity was demonstrated by the detection of 1 piroplasm in 1µl of blood thereby offering an alternative approach to the detection of carrier animals (Bishop *et al.*, 1992).

In an attempt to create a more universally conserved pair of primers for amplification of DNA from *T.parva* stocks , 20-mer synthetic oligonucleotides were designed to amplify a 233 b.p. fragment of DNA encoding amino acid residues 274-340 of a *T.parva* Muguga sporozoite surface antigen, p67 (Nene *et al.*, 1992). This

gene is thought to be conserved among *T. parva* stocks on the basis of the absence of observed restriction fragment length polymorphism (Bishop *et al.*, 1991).

The 104 kDalton protein, which is localised in the microneme / rhoptry complexes of the sporozoite stage of *T. parva*, has been found to be very antigenic [Nucleotide sequence data reported in this study has been submitted to the Gen Bank™ Data Bank with the accession number M29954] (Iams *et al*, 1990). Also Iams *et al,* (1990), have shown that immunological activity to epitopes of the 104 kDa antigen occur in animals immunised by the infection and treatment method.

At the CTVM the PCR products from the p67 *T. parva* sequences were visualised by polyacrylamide gel electrophoresis (PAGE) and silver staining. Samples from the *Theileria* infected / recovered calves were tested on PCR assay using p167 and p104 primers and the presence of amplified DNA was detected directly on ethidium stained agarose gels or by hybridisation to a labelled probe. The results were compared with conventional methods of parasite detection.

4.2 Materials and Methods

Formulation of solutions and buffers used in this chapter are detailed in Appendix 1. Sterile distilled water was used in the preparation of all solutions used in experiments, glassware used was sterilised and all plasticware was previously unused and sterile. Details of parasites used to infect the animals are shown in Tables in chapter 3.

4.2.1 PCR organisation and precautions

The prevention of contamination with amplified DNA sequences is paramount to the successful application of PCR in any establishment.

Successful amplification via PCR yields at least 1 pico-mole of a desired product or about $6x10^9$ molecules per microlitre. Thus cross contamination becomes evident when amplification occurs in negative controls that do not receive template and should be suspected when more than one band is obtained after electrophoresis of PCR products and staining. Contamination usually occurs with amplified sequences via aerosols generated during sample processing as in mixing by vortex action, opening of micro-centrifuge tubes, pipetting, ejecting pipette tips from micropipettors and centrifuging under vacuum. Since transport by air-borne products is common the following precautions were taken:

(a) Different procedures were allocated different rooms up to and including the set up of amplification reactions both at the CTVM and Harare-VRL. Enhanced containment was also provided by a biosafety cabinet (ESB™ , JBio, Techgen International Ltd), designated for PCR use only in Laboratory 105 (CTVM) in which no theileriosis work was being carried out. The preparation of master mixes was done after UV sterilisation of the cabinet.

(b) A separate room was allocated for the detection of PCR products, ie. electrophoresis of PCR products, and dot blotting. Gloves were worn at all stages, with changes of gloves between laboratories.

(c) A set of pipettes were set aside exclusively for each defined procedure to avoid any cross contamination; a set of pipettes for the following, buffers, deoxynucleotide, primers and enzyme. The setting up of master mixes prior to adding to the samples in PCR reaction tubes, reduced the number of pipetting procedures hence reducing the risk of contamination of stock solutions.

(d) At the VRL, Harare, separate rooms were allocated for the processing of samples and preparation of solutions. The main Theileriosis laboratory was not used and most of the work was performed in the Heartwater Immunology laboratory where *Theileria* culture and routine diagnostic work is not performed.

(e) The rooms for the special procedures were only accessible by authorised personnel to avoid people bringing in materials used for related purposes in the laboratory.

(g) All pipette tips and microcentrifuge tubes were autoclaved prior to use and discarded subsequent to use.

4.2.2 Sample preparation

4.2.2.1 EDTA blood saponin lysis extraction.

The EDTA blood samples were prepared as follows: Two hundred microlitres of jugular EDTA blood was mixed with 800µl of saponin lysis buffer (0.22% sodium chloride, 0.015% saponin (Sigma), 1.0 mM EDTA) and spun at 13000 xg for 60 seconds. The pellet was resuspended in 1000µl of fresh saponin lysis buffer and spun at 13000rpm. The pellet was washed twice more in 1000µl saponin lysis buffer, the supernatant being removed after each wash.

The final pellet was resuspended in 100µl TE (10 mM Tris-HCL, 1.0 mM EDTA pH 8.0) and Proteinase K (Boehringer Mannheim) was added to 2.5µg per ml and the sample was incubated 56°C for 1 hour or overnight at 37°C. The samples were then boiled in a water bath for 5 minutes and stored at -20°C or at 4°C.

4.2.2.2 Peripheral blood mononuclear cells (PBM)

Using aseptic techniques, 8 ml of jugular EDTA blood was mixed with an equal volume of sterile phosphate buffered saline (PBS) pH 7.2. The blood was then layered carefully onto two aliquots of 6 ml Ficoll paque (Pharmacia) and centrifuged at 400 xg for 40 minutes at room temperature. After centrifugation the upper plasma layer was discarded and the lymphocyte layer harvested from the interface into 10 mls sterile PBS (pH 7.2). The mononuclear cells were washed twice in PBS at 2000 xg at room temperature. The final PBM pellet was resuspended in 500 µl TE pH 8.0 and stored at -20°C until DNA extraction.

4.2.2.3 DNA extraction from PBM

SDS and Proteinase K were added to the PBM in 500µl of TE pH 8 to a final concentration of 1% and 2.5 µg/ml respectively. The PBM were incubated at 56°C for one hour or overnight at 37°C. The samples were then boiled for 10 minutes to denature the Proteinase K. An equal volume of equilibrated phenol was added, mixed by inverting several times and separated by centrifugation for 5 minutes at 13000 x g at room temperature. The aqueous layer was transferred into a fresh eppendorf tube and an equal volume of phenol:chloroform (1:1) was added to the aqueous suspension, mixed and centrifuged as above. The aqueous layer was again transferred into a fresh eppendorf tube and an equal volume of chloroform (BDH) added and mixed before centrifuging at 13000 x g for 5 minutes.

Following centrifugation the aqueous layer was carefully removed and retained and sodium chloride was added to a final concentration of 0.1M. Twice the sample volume of ice cold absolute alcohol was added and the sample was chilled at

-20°C overnight or -80°C for an hour. The sample was then centrifuged at 13000 x g for 5 minutes and the resulting pellet washed twice in 70% ethanol. Excess ethanol was pipetted off and the pellet dried. The pellet was finally dissolved in 50µl of TE (pH 8.0) and stored at -20°C until used. In a few circumstances some sample volume was increased to 100µl if the sample was found to be too viscous to use and the volume to the PCR reaction was increased proportionally.

4.2.2.4 DNA extraction from *T. parva* Muguga piroplasms

DNA was extracted from *T. parva* Muguga piroplasms for use in developing of the PCR assay and as positive control material. The piroplasms were supplied by Dr. S. Williamson at the Centre for Tropical Veterinary Medicine, University of Edinburgh. The purified piroplasm pellet was thawed and made up to 3 ml with TE. SDS was added to 1% and the pellet was gently homogenised and made up to 15 ml with TE. Proteinase K was added to a final concentration of 100 µg/ml and the piroplasms were mixed for one hour at room temperature. After incubation for one hour at 56°C, the DNA was extracted with an equal volume of equilibrated phenol followed by centrifugation at 3000 xg for 20 minutes (Beckman-CPKR centrifuge). The aqueous layer was collected and mixed with an equal volume of equilibrated phenol for 20 minutes and centrifuged as above. After centrifugation the aqueous layer was collected and mixed for 20 minutes with an equal volume of phenol/chloroform (2:1) followed by centrifugation at 3000 xg for twenty minutes. The aqueous layer was again removed and mixed for 5 minutes with an equal volume of chloroform and centrifuged at 2000xg for 10 minutes at 15°C. The final aqueous layer was removed and sodium chloride added to a final concentration of 0.1M. The DNA was then

precipitated by the addition of twice the volume of ice-cold absolute ethanol and was placed at -20°C for one hour followed by centrifugation at 3500 xg for 30 minutes. The pellet was washed twice in 70% ethanol and dissolved in approximately 50 µl TE. The RNA was removed by incubation in 20µg/ml RNAse for 30 minutes at 37°C and the DNA was re-extracted using ethanol and precipitated as above and the concentration was estimated using a DNA fluorimeter (TKO).

4.2.2.5 DNA extraction from *T. parva* macroschizont cells

DNA was extracted from cultured lymphoblastoid cell lines infected with schizonts of *T. parva* Marikebuni, *T. annulata* and *T. parva* Boleni. Approximately 10 x 10^7 cultured cells of each parasite stock were washed three times in PBS pH 7.2, centrifuging at 2500 rpm for 10 minutes each time. The final pellets were resuspended in TE pH 8 at a concentration of 0.5 x 10^7 cells per ml. The cells were digested in TE buffer containing 1% SDS; and 100µg/ml proteinase K, by mixing at room temperature for 20 minutes then incubation at 56°C for one hour. This was followed by phenol / chloroform extraction and ethanol precipitation of DNA as described by Ausubel, *et al.*, (1987) for the preparation of piroplasm DNA (section 4.2.2 3)

4.3 Polymerase chain reaction conditions for p67 *T. parva* primer sequence

The method of Bishop *et al* (1992) was used to amplify the gene coding for the p67 primer sequence. The primers were procured from the Oswel DNA service of the University of Edinburgh.

To fully optimise the system, titrations were carried out using varying concentrations of $MgCl_2$, dNTPs and primers. The optimal conditions for the

amplification of template *Theileria* DNA were also determined by increasing the number of cycles in the PCR assay to 40. Serial dilutions of blood with a 21% piroplasm parasitaemia from a calf infected with *T. parva* Muguga were processed by saponin lysis and tested by PCR in the presence of 2.5mM, 5.0 mM and 10mM $MgCl_2$ and either 30 or 40 cycles consisting of 1 minute at 94°C, 1 minute at 60°C and 45 seconds at 72°C. (Figure 4.7).

After the 5 µl volume of samples to be tested had been denatured in boiling water for 5 minutes and chilled on ice, the master mix was added, then sterile mineral oil (Sigma) was overlayed. The final concentrations used were the ones recommended by Bishop *et al.* (1992) and were as follows: The 50µl reactions contained 50 mM KCL; 10 mM Tris-HCL pH 8.3; 1.5 mM $MgCl_2$; 0.1% (w/v) gelatin; 200µM each deoxyribonucleotide triphosphate (dNTPs); 2.5 units of *Thermus aquaticus* (*Taq*) polymerase (Boehringer Mannheim); 1µM each oligonucleotide primer; 5 ng schizont containing DNA or 1ng purified piroplasm DNA. The reaction mixtures were subjected to 30 cycles on a Perkin Elmer or Hybaid thermocycler consisting of: denaturation, 94°C for 60 seconds; annealing, 60°C for 60 seconds; and extension, at 72°C for 45 seconds. Control reactions were set up which contained positive , bovine 1 ng bovine DNA (Sigma) and sterile water (blank) samples for each reaction process with the samples. The fragment produced at the end of the amplification process in positive samples was approximately 234 bp long and was not detected in DNA extracts of other haemoprotozoa, *Babesia bovis, Anaplasma marginale, Theileria taurotragi, Theileria annulata, Theileria sergenti, Trypanosoma congolense, Theileria mutans, and Theileria buffeli.*

Figure 4.1 **p67 *T. parva* primer sequence**

(F054) IL144 5` **TCA GGC GCA GCA TCA ACA GGT** 3`

(F055) IL145 5` **GTT CTT TCC CCT TCA TAT GCC C** 3`

Oligonucleotide primers IL144 and IL145 were designed to amplify a 233 bp fragment coding for amino acid residues 274-340 of a *T. parva* sporozoite surface antigen p67 (Bishop *et al.*, 1992).

4.3.1 Optimisation of PCR conditions using p67 primers

The optimal conditions for the amplification of template *Theileria* DNA was determined using varying concentrations of $MgCl_2$, primer, and dNTPs and cycling parameters of the PCR reaction. In a series of experiments dilutions of *T. parva* Muguga piroplasm infected blood were processed by the saponin lysis method and were used to test effect upon the PCR performance variation of:

(a) dNTP concentration (Figure 4.8)

(b) magnesium chloride concentration (Figure 4.9)

(c) primer concentration (Figure 4.10)

(d) number of cycles (Figure 4.7)

Serial dilutions of *T. parva* Muguga piroplasms DNA and saponin lysis extractions were tested by PCR in order to determine the level of sensitivity.

4.4 Polymerase chain reaction conditions for p104 *T. parva* primer sequence

Dr. R. Skilton at ILRI (Nairobi) kindly supplied the p104 primer sequences and the protocol for PCR amplification. The fragment produced was approximately 497 b.p. long and was not obtained in PCR with *T. taurotragi, T. mutans, B. bigemina,* and *B. bovis* DNA phenol / chloroform extracts. Primers were supplied freeze dried and

were resuspended in sterile distilled water and aliquoted in 100ng/μl stocks. The primer sequences are as shown in Figure 4.2

Figure 4.2 p104 *T. parva* primer sequence

4875 5` **ATT TAA GGA ACC TGA CGT GAC TGC** 3`

4876 5` **TAA GAT GCC GAC TAT TAA TGA CAC C** 3`

The polymerase chain reaction was carried out in 50μl reactions and the final concentrations were: 50mM KCl, 10mM Tris-HCL; 1.5 mM Magnesium chloride; 200μM each deoxy-ribonucleotide triphosphate (dNTP's); 2ng/μl each oligonucleotide primer; 1.25 units of *Taq* DNA polymerase; 5μl of processed material (saponin lysed blood or phenol chloroformed PBL). The master mix was prepared in a clean room and then moved to another where the samples were ready to be added to the master mix. An aliquot of processed sample was denatured in a 0.5ml PCR reaction tube by boiling for 5 minutes and then chilled on ice. The samples were then centrifuged briefly at 7000 xg on a microcentrifuge and the 45μl of master mix was added. The reaction tubes were overlaid with 60μl of fine mineral oil (Sigma) and were put on ice as they were ready to go on the thermocycler. The reaction mixtures were subjected to 94°C for 60 seconds and 40 cycles of : denaturation at 94°C for 60 seconds ; annealing at 60°C for 60 seconds; and extension at 72°C for 60 seconds; and finally held at 4°C.

4.4.1 Sensitivity of PCR assay using p104 primers

Dot blot hybridisation was performed to confirm the identity of the predicted PCR product of the 497 bp as being amplified from the target DNA and compared with p67 results (Results not shown). Ten-fold dilutions of *T. parva* Muguga piroplasm

DNA were run in the PCR assay and 10 microlitres of each was dot-blotted and probed with the ^{32}P labelled probe. For each PCR assay an ethidium bromide agarose gel was run with the positive control, negative control (bovine DNA), and size markers to confirm the size of the band.

4.5 Detection of PCR products .

4.5.1 Polyacrylamide gel electrophoresis

Nucleic acids can be separated by molecular weight using electrophoresis on acrylamide agarose gels. Silver stained acrylamide gels have the advantage of better resolution for smaller DNA or RNA molecules (!0 to 500 bp), and are especially useful in DNA sequencing (Maizels *et al.*, 1991). Polyacrylamide gel elecrophoresis (PAGE) of *T. parva* p67 PCR products was used to visualise the product, based on the method of Sambrook *et al.*, (1989). Acrylamide stock (40% acrylamide/bis) diluted in loening buffer (Appendix 1) was used to make 7.5% acrylamide. Polymerisation was catalysed by 0.1% (v/v) N,N,N,N,-tetramethylenediamine (TEMED) (Sigma), and 0.1% (w/v) ammonium persulphate (APS) (Biorad) and the gels were poured after their addition. A comb was then placed into the acrylamide gel to create wells as the solution polymerised. Gels were cast using the "Mini Protean™" gel system (Biorad) and run at 150volts constant setting. Ten microlitres of PCR product was added to 5 µl 6x bromophenol blue (Appendix 1) loading buffer in a microcentrifuge tube before loading onto the cast gel. One of the wells contained the DNA size markers added in the 6x loading buffer.

4.5.1 (a) Silver staining of Polyacrylamide gels

DNA in polyacrylamide gels was stained after separation using the method of Herring *et al.*, (1982). Gentle agitation was applied at each step of the staining procedure. The gel was fixed in fixative (10% ethanol, 0.5% glacial acetic acid) for 10 minutes, followed by staining in 0.21% of silver nitrate (Sigma) solution for 10 minutes. After washing twice in distilled water, the developer (3% (m/v) sodium hydroxide, 0.75% (v/v) formaldehyde) was added until satisfactory colour development had occurred. The reaction was stopped when the bands were clearly visible by removing the solution and adding 0.75% di-sodium carbonate in distilled water. Co-electrophoresis of a pUC19-Sau3A DNA size markers (Sigma) or ΦX174 Hae III digest (Sigma) permitted the estimation of the band size and this was confirmed by plotting the $\log_{10}$ base pair length against the distance travelled through the gel in relation to the DNA marker bands (pUC19Sau3A). Gels were then photographed using a Polaroid MP4 land camera and were dried on a gel drier (Model 583 gel drier -Biorad).

4.5.2 Agarose gel electrophoresis of DNA

Standard electrophoresis on agarose gels can be used to separate nucleic acids from 100 bp to 50,000 bp (50 kb), and horizontal or flatbed electrophoresis is the mthod of choice because it is easy to set up for routine use. One other advantage is that a number of buffers can be used for DNA electrophoreis. Ethidium bromide-stained agarose gels were run to detect PCR products for the positive and negative controls of *T. parva* p104 primer sequences to check if the assay had worked. The molecular grade agarose (Sigma), 1.6%(w/v) was dissolved in Tris -acetate EDTA buffer (TAE)

pH 8.0. The agarose was boiled in microwave oven and ethidium bromide was added to the molten agarose to a final concentration of 200 ng/ml and poured onto the "sub-cell" to set. DNA loading buffer [0.25% (w/v) bromophenol blue, 0.15% (w/v) Ficoll 400] was added at a ratio of 1:6 to the PCR product samples prior to loading, and electrophoresis was carried out at 5 volts per centimetre in 0.5X TAE buffer until the required resolution was achieved. Co-electrophoresis of a of pUC19-Sau3A (Sigma) or ΦX174 Hae III digest (Sigma) DNA size markers permitted the estimation of the band size.

4.5.3 Preparation of *T. parva* Muguga probe

PCR amplification product using p104 primers of *T. parva* Muguga piroplasm DNA was used as a probe. Following preparative gel electrophoresis using low gelling / melting temperature agarose (Sigma) the gel slice containing the band (approximately 497 b.p.) was cut from the gel being visualised under UV light. This agarose slice was then melted at 56°C for one hour and subjected to phenol extraction (Ausubel, *et al.*, 1987), dissolved in TE and stored at -20°C. In order to estimate the amount of DNA in the sample a 10 µl aliquot was electrophoresed on ethidium bromide agarose gel and the fluorescence was compared with similar sized fragments of known concentration using ØX174 DNA size markers (Sigma).

Because of the hazards associated with radioactivity the materials were handled carefully behind a protective shield, gloves and protective glasses were worn. Labelling of the probe was achieved using a random primed extension reaction in a 20µl volume with 100ng of *T. parva* PCR product DNA (Heartwater project-Harare protocol for DNA probes). After denaturation at 95°C for 10 minutes

the following reaction was set up :1μl of each dNTP (ATP, GTP and TTP) (Boehringer Mannheim); 2μl of reaction mix ; 5μl ^{32}P-dCTP (≡50μCi Amersham International), 1μl Klenow (Boehringer Mannheim labeling kit).

The mixture was incubated at 37°C for 30 minutes, and the reaction terminated by the addition of EDTA to a concentration of 0.04 Molar in 100 μl of TE (pH 8.0). One microlitre was taken to measure the amount of incorporated radioactive ^{32}P on an Ls 8500 counter. The remainder was purified on a Column-sephadex G-50 (Boehringer Mannheim). In order to keep the probe denatured 20μl of 20mg/ml Herring sperm DNA was added to the purified probe.

4.5.3.1 Blot Preparation

Forty microlitres of polymerase chain reaction product for each sample tested was made up to a 100μl with TE pH 8.0 and sodium hydroxide to a final concentration of 0.4 Molar and incubated at 37°C for 30 minutes. The samples were put on ice immediately after incubation. Nylon membranes were soaked in 2xSSC for 15 minutes prior to being assembled in a Dot Blot apparatus (Hybri.Dot Manifold-Gibco). The samples to be tested, including controls, were applied to the individual wells (100 μl per well) in the dot blot apparatus and were left to sit for 10 minutes before a gentle vacuum suction was applied to the apparatus to pull the samples through. The apparatus was then dismantled and the membrane was briefly rinsed with 2xSSC then exposed to UV light (UV Transilluminator) for two minutes to fix the DNA allowed to air dry. After drying the membrane was wrapped in plastic film and stored at 4°C until prehybridization.

4.5.3.2 DNA labelling and hybridisation.

Because of the hazards associated with radioactivity, gloves were worn, protective glasses and the work was done behind a protective shield.

Two membranes were placed in a large hybridisation bottle containing 50mls of prehybridization (Appendix 1) solution and placed in a rolling Hybaid oven at 42°C. These were allowed to prehybridise for at least six (6) hours. The ^{32}P labelled probe (50 µl) was added to the prehybridisation solution to a concentration of 2 x 10^7 cpm ml $^{-1}$ and hybridisation was allowed to occur for at least sixteen (16) hours or overnight at 42°C. After hybridisation the membranes were washed in a special designated room as follows:

Wash #1 15 minutes /room temperature in 2X SSC

Wash #2 15 minutes /room temperature in 2X SSC/0.1% SDS

Wash #3 15 minutes /room temperature in 0.5X SSC/0.1% SDS

Wash #4 15 minutes /room temperature in 0.1X SSC/0.1% SDS

Wash #5 60 minutes /60°C water bath in 0.1X SSC/1% SDS

Wash #6 Repeat wash #5 above (60 minutes /60°C water bath in 0.1X SSC/1% SDS).

After the washes the blots were briefly rinsed at room temperature in 0.1xSSC, blotted with 3MM filter paper, wrapped in plastic film and exposed to X-ray film for two hours at room temperature. The auto-radiographs were photographed using a Polaroid Mp4 land camera.

4.6 RESULTS

4.6.1 Specificity of p67 PCR assay

1.0 ng amounts of *T. parva* Muguga piroplasm and macroschizont DNA extracts were positive in the p67 PCR assay. In contrast *T. annulata* macroschizont bovine DNA, saponin lysed *Babesia bovis* (Lismore), *B. bovis* (Kwanyanga) *B. bovis* (Mexico) piroplasms, and *Anaplasma marginale* (Zimbabwean isolate) did not produce any positive bands. Results shown in Figure 4.3. A 155 bp fragment was amplified from the three isolates indicating that amplifiable *B. bovis* DNA was present (Fahrimal, *et al.*, 1992). Results shown in Figure 4.3 b.When *Theileria buffeli, Trypanosoma congolense, Theileria mutans, T. taurotragi, T. sergenti, T. annulata,* and *T. parva* Muguga piroplasm DNA preparations were tested (1.0 ng each), PCR positive bands were detected only with *T. parva* Muguga. (Figure 4.4). *T. parva* Muguga piroplasms gave positive bands for the saponin lysed blood samples when tested in the p67 PCR at a parasitaemia of 0.0000021%. (Figure 4.5). The saponin lysed *B. bovis* samples were tested by PCR using the BV60 primer sequences known to be conserved in *B. bovis* isolates.

4.6.2 Optimisation results of PCR conditions using p67 primers

One tenth of a picogram and 1.0 pg of *T. parva* Muguga piroplasm and macroschizont DNA was detected respectively in the phenol / chloroform extracts (Figure 4.6) using the standard PCR protocol of 30 cycles.

Thirty cycles with 5.0 mM $MgCl_2$ gave better amplification than 30 cycles with 2.5 mM or 10 mM $MgCl_2$, or 40 cycles with 5.0 mM $MgCl_2$. Amplification of the desired band occurred at lower piroplasm parasitaemias with the former (Figures

4.7 and 4.9). While 30 cycles at 5.0 mM $MgCl_2$ amplified blood dilutions to a detection limit of 0.0000021% piroplasm parasitaemia, 40 cycles at the same $MgCl_2$ concentration amplified dilutions up to 0.00021% piroplasm parasitaemia respectively (100 fold increase in sensitivity at lower cycling conditions) (Figure 4.9). The parasitaemic blood was diluted out in uninfected EDTA blood.

An increase in the dNTP and primer concentration produced extra DNA bands in the PCR product (Figures 4.8 and 4.10). An increase in the $MgCl_2$ concentration lowered the sensitivity of the assay and produced weaker bands of the desired product in lower dilutions of the saponin lysed extracts of infected EDTA blood (Figure 4.9).

4.6.3 PCR results for *T. parva* Marikebuni infected calf (818) using p67 primers.

The optimised PCR conditions using 30 cycles and 2.5 mM $MgCl_2$ were applied for detection of *T. parva* Marikebuni (calf 818) from experimental infection. The PCR products were detected by electrophoresis and silver staining.

In the *T. parva* Marikebuni infected (calf 818) PCR product of 234 base pairs was detected days 10, 12 and 14 post infection in the phenol / chloroform extracted PBM samples (Table 4.1). In the left prescapular gland biopsy material which had been phenol / chloroform extracted, the PCR assay was positive on day 13, 18, 20 and 21 post infection. The calf had been infected by subcutaneous inoculation of stabilate in its left shoulder area. For saponin lysed EDTA extracts positive products were detected from day 15 to 23 post infection. Day 24 extract was not tested but the test was positive on the sample from day 25 post infection. Results shown in Table 4.1.

4.6.4 PCR results for *T. parva* Boleni infected calf (805) using p67 primers

A higher proportion of positive results were obtained with the *T. parva* Boleni infected calf (805) as shown in Table 4.2. PCR positive products were detected in the phenol / chloroform extract of the right prescapular gland biopsy material four days post infection up to day 7 post infection. This calf was infected by subcutaneous inoculation of the stabilate on its right shoulder area. In the phenol / chloroform extracts of peripheral blood mononuclear cells the PCR assay was positive on days 8 to 13, day 15, 18, 21, 22 and 23 post infection.. The right prescapular lymph node biopsy material was negative on day 8, and positive from day 9 to 22. Positive PCR product were also detected on day 24 post infection in the right prescapular lymph node.

The left prescapular lymph node was positive on 13 occasions of the sampling, from day 9 to 25 post infection except for day 12 post infection when it was negative in the PCR assay. Saponin lysed extracts of EDTA blood were positive from day 18 to 25, eight consecutive days post infection in the same PCR assay. The PCR assay was positive on day 18 post infection, 4 days after the piroplasms were seen in the Giemsa stained blood smear (Table 4.2).

4.6.5 Specificity of assay using p104 primers

Products of PCR assay using p104 primers tested with 1.0 ng extracts of *T. mutans, T. taurotragi, Trypanosoma congolense, T. buffeli, T. annulata* and *T. sergenti* did not produce bands (Figure 4.16), and did not hybridise with the p104 probe (Figure 4.17). Positive bands were detectable on the ethidium bromide 1.6% agarose gel for the *T. parva* Muguga (piroplasms) and Boleni (macroschizont) DNA from their

respective PCR products. The p104 probe hybridised with *T. parva* Muguga and Boleni PCR products on the dot blot (Figure 4.17).

4.6.6 Sensitivity of PCR assay using p104 primers

The assay was positive up a dilution of 0.1 ng of *T. parva* Muguga piroplasm DNA on the probed dot blot (Figure 4.18).

4.6.7 Results of tests on carrier recovered animals using dot blot hybridisation

Blood samples taken from the experimental calves on the days shown in the sampling protocol (Table 3.2) were processed and amplification of *T. parva* DNA by PCR attempted and probed with the p104 probe. The hybridisation results are shown in Appendix III for all experimental animals. The controls were analysed by elctrophoresis on an ethidium bromide stained gel before dot hybridisation of the rest of the samples. A summary of results comparing the PCR results with microscopic examination of Giemsa stained blood smears, IFAT and tick infections for each calf including the Hunyani Estates second exposure are shown in Tables in Appendix IV. No PCR products were detected in blank (no template) samples indicating that there was no contamination with extraneous *T. parva* DNA.

4.6.7.1 *T. parva* Boleni infected calves p104 PCR results

Summary of PCR results is shown in Appendix IV.

The *Kappa* statistic is used without assuming that one test is the best, it is possible to assess the agreement between different tests if a gold standard is not available. The logic of using this approach is that agreement between tests is evidence of validity, whereas disagreement suggests that the tests are untrustworthy (Thrusfield, 1995). In this study the *kappa* statistic was used to observe proportions in agreement between

any two methods (test results) on samples taken on the same days post infection or recovery. However, this comparison does not consider agreement that could arise just by chance, so by calculating the *kappa* statistic which takes account of chance agreement the expected proportion (EP) of agreement by chance was calculated.

A definition by Thrusfield (1995) of *Kappa* states that:

it is the excess agreement over that expected by chance, divided by the potential excess; that is:

$$\textbf{\textit{kappa}} = (\textbf{OP} - \textbf{EP}) / (\textbf{1} - \textbf{EP})$$

Kappa ranges from 1 (complete agreement) to 0 (zero) [agreement is equal to that expected by chance], whereas negative values indicate agreement less than is expected by chance. In this study we expected the results between any two methods to be in agreement as the tests were performed post infection in carrier animals. Arbitrary 'benchmarks' for evaluating observed *kappa* values are

>0.81: almost perfect agreement; 0.61-0.80: substantial agreement;

0.41-0.60: moderate agreement; 0.21-0.40: fair agreement;

0-0.20: slight agreement; and 0: poor agreement (Everitt, 1989).

Results for group I calves (W70, W73, W74 and W75): Using the *Kappa* statistic for comparing two tests there was moderate agreement in the PCR results from the group I calves in the PBM phenol chloroformed extracts and saponin lysed EDTA blood extracts (*Kappa* = 0.51), but there was no agreement in between the latter and the results for the microscopic examination of the Giemsa-stained blood smears (*Kappa* = -0.23).

Calf W70: The experimental calves infected with *T. parva* Boleni (Bolvac®) were positive by the p104 PCR assay from day 14 post infection in the PBM extract and sometimes in the saponin lysed extract as detected by dot blot hybridisation.

Calf W70 was positive from day 14 up to the last day tested 493 days, post infection in the PBM sample extracts. In the saponin lysed extracts it was positive from day 40 up to the last day tested (493) except for day 95 when it was negative. In the 15 PBM extracts tested, all were positive in the PCR assay and 12 out of 15 in the EDTA extracts were positive. (Appendix IV-Table 1).

Calf W73: Calf W73 was positive on day 14, 40, 74, 95, 109 and day 493 post infection in the PBM samples. In the saponin lysed EDTA blood the calf was positive on day 14, 40, 109, and 179 up the last date tested (493) post infection. The saponin lysed samples were negative on days 25, 74, 95 and 137 post infection Thus 6 out of a total of 15 PBM phenol chloroformed extracts, and 11 out of 15 EDTA saponin lysed extracts were positive when sampled from calf W73 post infection. (Appendix IV-Table 2)

Calf W74: Calf W74 was tested up to day 305 before it died. Phenol / chloroform PBM cells were positive on the p104 PCR assay day 14, 40, 74, 95 109 and 137 post infection. The EDTA blood extracts were positive on day 25, 40, 74, 109, 137, 179, 218, 221, and 263 post infection. Thus 6 dates tested post infection in the extracted PBM were positive out of 12 tested. Nine out of the 11 dates tested post infection were positive in the PCR assay on the EDTA blood. (Appendix IV-Table 3).

Calf W75.: Calf W75 was positive in both the PBM and EDTA saponin lysed extracts on day 14 post infection, but was negative in the respective samples on days

25 and 40. The PBM samples were again positive on days 74, 95, 109, and 137, and the EDTA blood was positive on days 74, 95, 109, 207, 218, 263, 305, and 361. The EDTA blood sample extracts for days 446 and 493 were negative on the PCR assay. Thus a total of 5 samplings tested in the phenol / chloroform PBM extracts were positive out of 13, and 9 out of 13 of the EDTA saponin lysed extracts were positive when tested post infection with *T. parva* Boleni. (Appendix IV-Table 4)

4.6.7.2 *T. parva* Avery infected calves p104 PCR results

Calf 1442 ; Calf 1442 was positive in the PBM and EDTA sample extracts on day 14 post exposure. The PBM samples were positive on six other dates when they were tested, thus day 25, 74, 95, 109, 137, and 179 post infection. Saponin lysed EDTA blood extracts were positive on days 95, 305 361 and 493 which was the last date tested. Seven PBM samples out of the 15 dates sampled post infection were positive in the PCR assay and 5 out of 15 in the saponin lysed extracts of EDTA blood sampled post infection. Using the *Kappa* statistic for comparing two tests there was no agreement between the results obtained from the PBM phenol / chloroformed extracts and the EDTA blood (*kappa* = -0.34), and even between the latter and microscopic examination of Giemsa-stained blood smears (*kappa* = -0.80). Results shown in Appendix IV-Table 5.

Calf 1454: Calf 1454 was positive in both the PBM and EDTA extracts on days 14, 15, and 25 post infection. The PBM extracts were again positive on days 40, 74, 109, 137, and 361 post infection but negative on days 95, 179, 207, 218, 263, 305 and 456 post infection. More positive results were observed in the saponin lysed EDTA samples on days 74, 109, 137, 179, 218, 305, 361 and 493 which was the last

date to be tested. Eight positive results were obtained out of 17 dates which were tested using the PBM samples which were phenol / chloroform extracted. Eleven positives were seen out of the 15 samples tested post infection in the EDTA blood samples. The p104 PCR results showed fair agreement between the PBM phenol / chloroformed extracts and EDTA saponin lysed blood extracts using the *Kappa* statistic (*kappa* = 0.39), and there was no agreement between the later and Giemsa-stained blood smear results (*kappa* = -0.64). Results shown in Appendix IV-Table 6.

4.6.7.3 p104 PCR results of *T. parva* Bally Vaughan infected calves

Calf 1447: Four out of the 15 dates calf 1447 was sampled were positive in the PBM phenol / chloroform extracts, these were on days 14, 74, 109 and 137. The EDTA saponin-lysed samples were positive in the p104 PCR assay on seven dates, day 14, 109, 179, 207, 218, 305, and 493 out of the 15 dates the calf was sampled and tested post infection. Using the *Kappa* statistic there was no agreement between the PCR results on the two extracts, PBM and EDTA blood extracts, (*kappa* = -0.034), and EDTA blood extracts and blood smear results (*kappa* = -0.75). Results shown in Appendix IV-Table 7.

Calf 1459: Calf 1459 PBM phenol / chloroformed extracts were positive on 3 out of 13 dates that the calf was sampled and tested, day 14, 25 and 95 post infection. In the EDTA saponin lysed samples 9 out the 14 dates sampled produced positive results in the PCR assay post infection on days 14, 74, 109, 204, 218, 263, 305, 361, and 456. A slight agreement was observed n the PCR results obtained in the PBM phenol / chloroformed extracts and the EDTA saponin lysed extracts (*kappa* = 0.00)

and there was no agreement between the later and microscopic examination of Giemsa stained blood smears (*kappa* = -0.85). (Appendix V-Table 8).

4.6.7.4 p104 PCR results of calves infected by application of Ayrshire and Chikeya ticks

Calf 1456 : On only two dates when calf 1456 was tested were the PBM extracts positive on the PCR assay out of 13 dates sampled and tested post infection (days 138 and 180). Six out 13 dates EDTA blood extracts were positive on days 152, 180, 250, 264, 488 and 534 days post tick application. The PCR results in the PBM and EDTA blood extracts were not in agreement but moderate agreement was observed between the later and Giemsa stained blood smears using the *Kappa* statistic (kappa = 0.53). Table of results shown in Appendix V-Table 9.

Calf 1437: Peripheral blood mononuclear cells phenol / chloroformed extracts sampled from calf 1437 were positive on 5 dates out of the 8 dates sampled and tested on the p104 PCR on days 82, 117, 138, 152, and 180 post tick application. The EDTA saponin lysed samples were positive on 3 dates (days 152, 222 and 264 post infection) out of the nine dates that the calf was sampled and tested. There was no agreement observed between the PBM phenol / chloroformed and the EDTA blood saponin lysed extracts (*kappa* = -0.11), and also between the latter and piroplasms seen in the Giemsa stained blood smears (*kappa* = -0.40). (Appendix IV-Table 10).

4.6.7.5 p104 PCR results of calves infected by exposure to ticks at Hunyani Estates (1st exposure)

Peripheral blood mononuclear cells from calf 1372 samples were all negative in the PCR assay after phenol chloroform extraction in the PCR assay. However, the EDTA saponin lysed blood extracts were positive on 10 out of the 14 dates sampled and tested post infection, on days 66, 80, 150, 178, 248, 262, 304, 319, 402 and 535. There was completely no agreement between PBM and EDTA blood extracts when the positive and negative results were analysed using the *Kappa* statistic, and yet there was substantial agreement between the latter and piroplasm s seen in the blood smears (*kappa* = 0.63). Results shown in Appendix IV-Table 11.

4.6.7.6 p104 PCR results of calves infected by exposure to ticks at Hunyani Estates (2nd exposure)

The following days post exposure at Hunyani Estates were tested days tested for the 3 calves were, day 0, 42, 128, and 175. The PCR results for the three calves (E1, E4, and E5) were positive for the p104 *T. parva* PCR assay on days 42. Dot blot hybridisation results of the p104 PCR products are shown in (Appendix IV-Table 12a, b, and c). A comparison of the PCR results obtained in the PBM phenol / chloroform and EDTA blood extracts showed a moderate agreement (*kappa* = 0.47), compared to the complete agreement observed in the later and piroplasms seen in the Giemsa stained blood smears using the *Kappa* statistic (*kappa* = 1.00).

4.7 DISCUSSION

The study showed that PCR using *T. parva* p67 and p104 primer sequences can be used to amplify *Theileria parva* DNA from Zimbabwean carrier animals. The relative advantages of p67 and p104 primers when used in PCR reactions with *T. parva* piroplasm and infected lymphocyte DNA. The primers generated PCR products of the expected sizes respectively (233 bp and 497 bp) and their specificity and high level of sensitivity. The main disadvantage of the PCR assays using these primers was the time spent in extracting the sample DNA to be tested and precautions taken to avoid contamination.

The PCR assay used in the preliminary studies at the CTVM proved to be sensitive and specific for *T. parva* as reported by Bishop *et al.* (1992) and in this study the assay did not amplify DNA from other haemoprotozoa. However the PCR assay using the p67 was found to be affected by the primer, magnesium chloride and dNTP concentrations and the best results were observed using the protocol in Bishop et al. (1992). Parasite detection in the *T. parva* Marikebuni (Kenya) infected calf was possible up to day 48 post infection using the p67 primers and silver staining for detection of the PCR products. The proportion of positive results in the samples from the Boleni infected calf and Marikebuni were different and the clinical reaction in the calf (805) which received the *T. parva* Boleni was severe, and had more positive results with the PCR test (Calf 805). However, it should be noted that the infection prevalence in the ticks used to prepare the *T. parva* Marikebuni stabilate had a lower prevalence and only 6 ticks (3 males and 3 females) were infected out of a total of twenty dissected and examined by Methyl-green pyronin stain.

Dot blot hybridisation of PCR products showed the p104 sequences to be useful in the detection of *T. parva* parasites in infected animals. It was also specific for *T. parva* macroschizont or piroplasm DNA and did not amplify bovine DNA and other *Theileria* species.

The amplification of DNA from animals infected with the three Zimbabwean *T. parva* laboratory stocks, and three infections from field parasites, was possible using the two sets of primers (Bishop *et al.*, 1994; R. Skilton personal communication). Positive PCR results were obtained intermittently and at times which did not necessarily correspond to the occasions when piroplasms were seen in blood smears post recovery. In the Boleni infected calves the positive PCR results did not always agree with the result of blood smear examination. (Appendix V-Tables 1 to 4).

Although the test might not be able to consistently detect carriers in *T. parva* Boleni Bolvac® immunised animals it has shown that it can detect *T. parva* parasites 218, 493 days post infection in healthy recovered animals as shown in the study *T. parva* Boleni infected animals (W70, W73, W74 and W75). PCR amplification of DNA could be used in parallel with the serological and Giemsa stained blood smears so as to increase the overall sensitivity and specificity of the diagnostic system as advised for *T. mutans* (Bishop *et al.*, 1994b). However, where suspect mixed infections occur like the Hunyani Estates exposure it would be advisable to use a panel of MAbs and *T. taurotragi* PCR assay if there is successful isolation in cell culture.

Calves 1442 and 1454 showed intermittently positive PCR reactions but more positive reactions were detected in the latter which was treated on day 15 post infection because of a severe clinical reaction (Appendix IV-Table 5 and 6). The positive PCR results when the calves were going through the clinical reactions were in agreement with the lymph node smear examinations. Positive PCR results did not always agree with the result of blood smear examination post recovery. Similarly PCR results did not agree with the negative results of adult *R. appendiculatus* ticks applied as nymphs on the calves.

In some cases though the PCR did not perform as expected and the results were negative even though piroplasns were detectable in the blood. The explanation for this could be that the samples were not processed to allow the exposure of target DNA. It is also known that negative results may occur due to inhibitors of *Thermus aquaticus* thermo-stable polymerase present in the blood, in particular haemoglobin (Newton and Graham, 1994).

Although a lot of care was taken to remove inhibitors like haemoglobin during the saponin lysis procedure, the proteinase K digestion can also act as PCR inhibitor. To remove the latter, inactivation by boiling for 10 minutes was performed and again before the addition to the PCR master mix.

An advantage of the PCR is its specificity although there could be room for improving its sensitivity by improving the sample extraction methods to expose the target DNA. In many carrier animals piroplasm parasitaemias are intermittent or too low to be detected by the microscopist. In the case of anti-schizont antibodies sometimes very low titres are observed which may only indicate recent exposure to

theileriosis. Antibodies are not considered a useful indicator of prevalence of disease in the carrier population and also are not reliable for speciation of the infection (Norval, *et al.*, 1992).

In blood exhibiting very low piroplasm parasitaemias post recovery the PCR assay was able to detect *Theileria* parasites 18 months post infection in *T. parva* Boleni, Avery, Bally Vaughan, 19 months post tick application of Ayrshire ticks, infected cattle, and 19 months post exposure at Hunyani Estates. Results shown in Appendix III and V.

The positive PCR results were not in agreement with the results of blood smear examination, but were in agreement with the positive results of the adult ticks dissected after having fed as nymphs on the *Theileria* recovered animals.

PCR results on PBM and EDTA blood of calf 1372 did not agree with the blood smear examinations throughout the experiment and this was attributed to the butalex treatment. However, there was substantial agreement between PCR results of the EDTA saponin lysed extracts with the Giemsa stained blood smear results using the *Kappa* statistic (*kappa* = 0.63) as it was observed that this animal had a continuous piroplasm parasitaemia. The tick infection results were in agreement with the Giemsa stained blood smear microscopic examinations. (Appendix V-Table 12). A comparison of all the PCR results on the EDTA blood extracts and microscopic examination of Giemsa stained blood smears indicated that 82% of the results were not in agreement using the *Kappa* statistic (*kappa* = -0.23), with less positive results in the blood smear examinations.

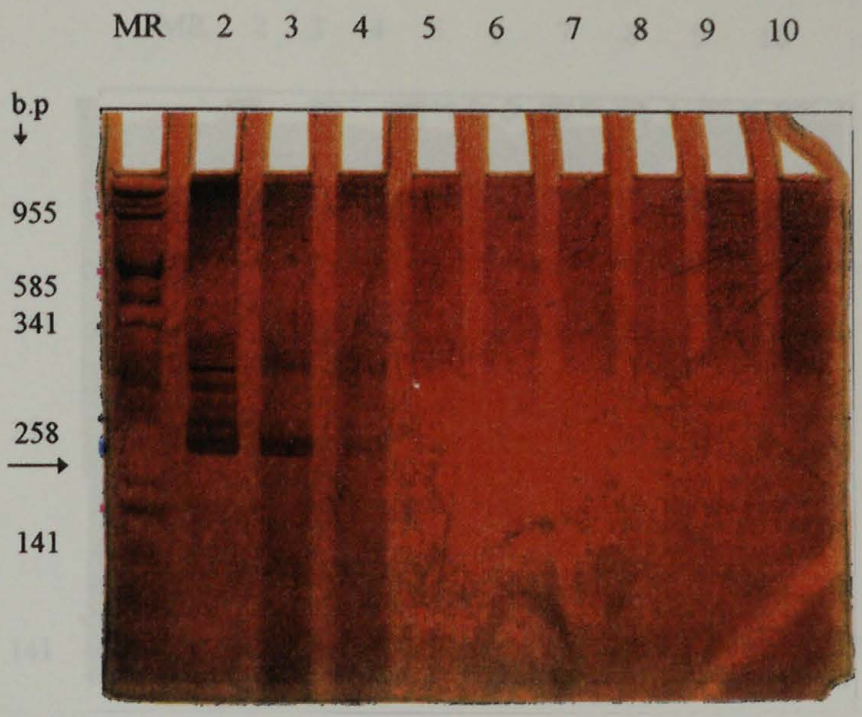


Figure 4.3a Photograph of silver stained PAGE of PCR products to test specificity assay using p67 *T. parva* primers.

Lane		Result
1	:pUC 19Sau DNA size marker	
2	:1.0 ng *T. parva* Muguga piroplasm DNA	+ve
3	:1.0 ng *T. parva* Muguga macroschizont DNA	+ve
4	:*T. annulata* macroschizont DNA	-ve
5	:1.0 ng Bovine DNA	-ve
6	:*B. bovis* (Lismore) piroplasm	-ve
7	:*B. bovis* (Kwanyanga) piroplasm	-ve
8	:*B. bovis* (Mexico) piroplasm	-ve
9	:*Anaplasma marginale* (Zimbabwean isolate)	-ve
10	Blank	-ve

Figure 4.3b Silver stained PAGE of PCR products from the amplification of *B. bovis* piroplasms: to show the presence of amplifiable *B. bovis* DNA after saponin lysis, proteinase K digestion of bovine blood.

Lane		Result
1	: pUC 19Sau DNA size marker	
2	:*B. bovis* (Mexico) 3.48%	+ve
3	:*B. bovis* (Lismore) 4.02%	+ve
4	:*B. bovis* (Kwanyanga) 1.93%	+ve
5	:*B. bovis* (Mexico) 5.23%	+ve
6	:*B. bovis* (Lismore) 3.88%	+ve
7	:*B. bovis* (Kwanyanga) 6.22%	+ve
8	Bovine DNA (1.0 ng)	-ve
9	Blank	-ve

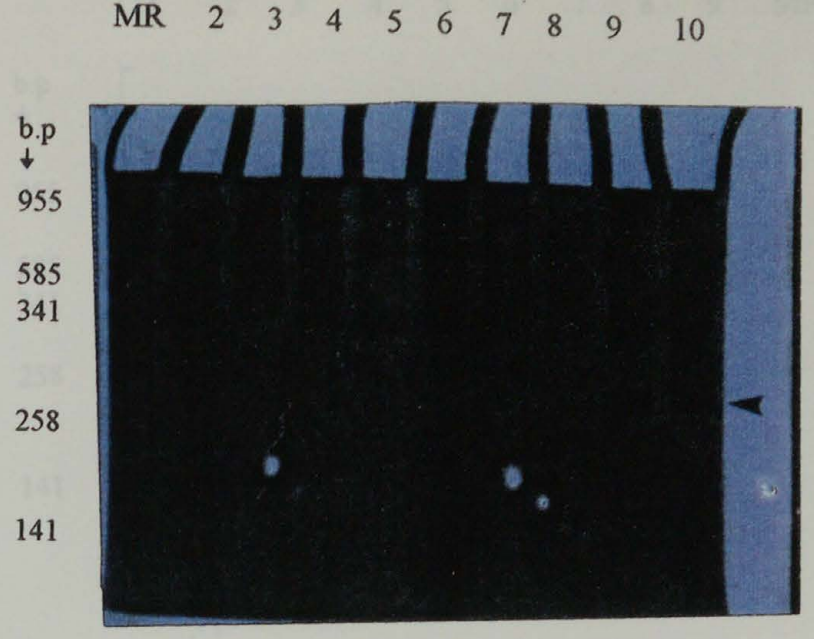


Figure 4.4 Photograph of silver stained PAGE of PCR products to test specificity assay using p67 *T. parva* primers

Lane		Result
1	: pUC 19Sau DNA size marker	
2	:1.0 ng Bovine DNA	-ve
3	:*T. buffeli* (Marula) piroplasm DNA	-ve
4	:*Trypanosoma congolense* DNA (c.c)	-ve
5	:*T. mutans* piroplasm DNA	-ve
6	:*T. taurotragi* macroschizont DNA (V953)(c.c.)	-ve
7	:*T. sergenti* (Shintoku) piroplasm DNA	-ve
8	:*T. annulata* macroschizont DNA (c.c.)	-ve
9	:*T. parva* Muguga macroschizont DNA (c.c.)	+ve
10	:*T. parva* Muguga piroplasm DNA	+e

Note: c.c. :DNA material prepared from cell culture material.

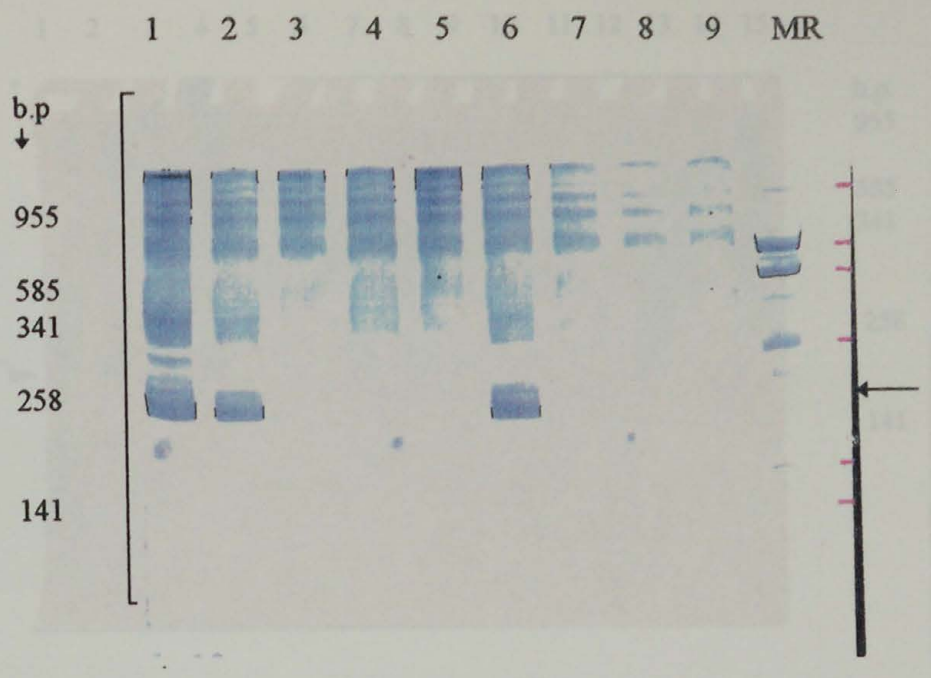


Figure 4.5 Photograph of silver stained PAGE of PCR products to test specificity assay using p67 *T. parva* primers with tempelate DNA of *Theileria* and *Babesia* species.

Lane		Result
1	:*T. parva* Muguga piroplasm DNA 1.0 ng	+ve
2	:*T. parva* Muguga macroschizont DNA 1.0 ng	+ve
3	:*T. annulata* macrochizont DNA	-ve
4	:*T. sergenti* piroplasm DNA	-ve
5	:*T. taurotragi* macroschizont DNA	-ve
6	:*T. parva* (Muguga) saponin lysed blood (0.0000021% piroplasm parasitaemia)	+ve
7	:*B. bovis* (Lismore) saponin lysed bovine blood extract (2% parasitaemia)	-ve
8	:*B. bovis* (Kwanyanga) saponin lysed bovine blood extract (2% parasitaemia)	-ve
9	:*B. bovis* (Mexico) saponin lysed bovine blood extract (2% parasitaemia)	-ve
10	:pUC 19Sau DNA size marker	

234 b.p. is the length of a positive amplification product.

MR DNA marker size

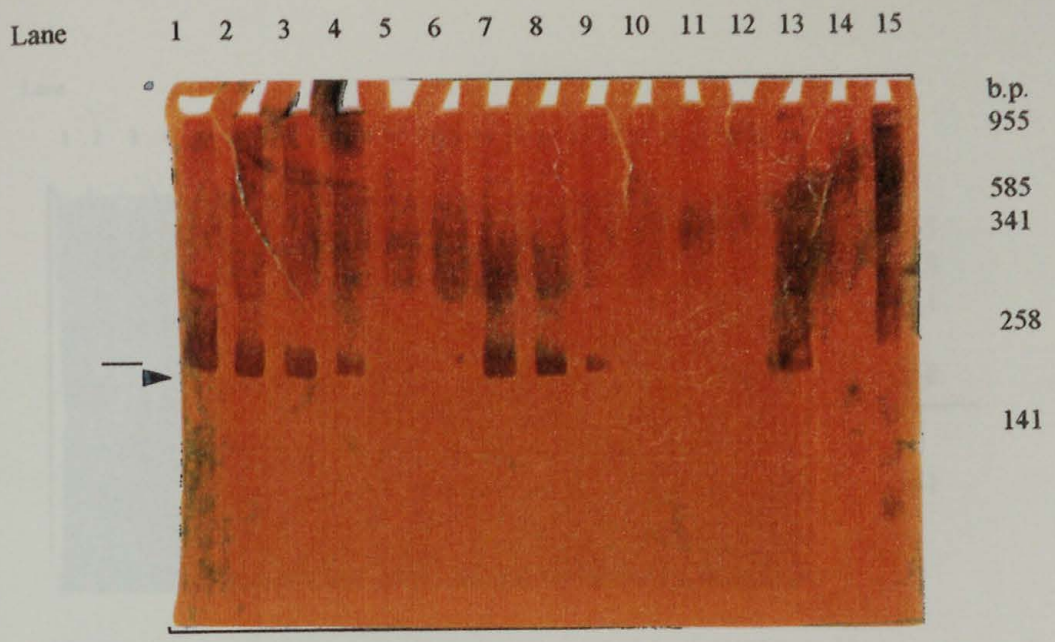


Figure 4.6 Silver stained PAGE of PCR products to determine sensitivity of assay. PCR amplification of *T. parva* Muguga piroplasm DNA (lane 1-6) and macroschizont DNA (lane 7-12). Samples were diluted in Tris-EDTA pH 7.6 and 2 µl amounts were added to each 25 µl PCR reaction.

Lane			Result
1	:*T. parva* Muguga (piroplasms DNA)	1 ng	+ve
2	:" " "	0.1 ng	+ve
3	:" " " "	0.01 ng	+ve
4	:" " "	0.001 ng	+ve
5	:" " "	0.0001 ng	+ve (weak)
6	:" " "	0.00001 ng	-ve
7	:*T. parva* Muguga (macroschizonts DNA)	1 ng	+ve
8	:" " "	0.1 ng	+ve
9	:" " "	0.01 ng	+ve
10	:" " "	0.001 ng	+ve (weak)
11	:" " "	0.0001 ng	-ve
12	:" " "	0.00001 ng	-ve
13	:Positive control		
14	:Negative control 1.0 ng Bovine DNA		
15	:pUC 19Sau DNA size marker		

+ve weak weak positive
234 b.p. is the length of a positive amplification product

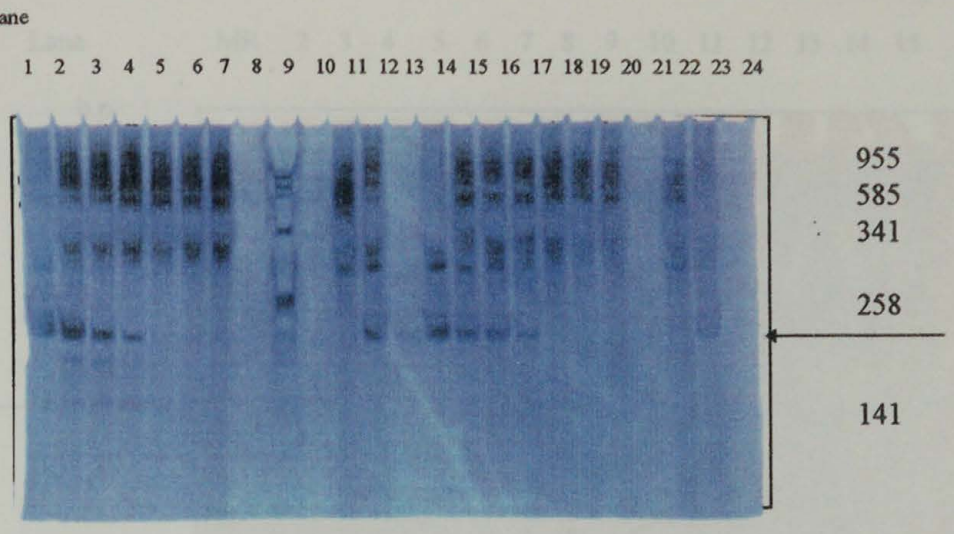


Figure 4.7 Photograph of silver stained PAGE of p67 PCR products of *T. parva* Muguga piroplasm saponin lysis extracts comparing the sensitivity between 30 and 40 cycle run .

30 cycles

Lane		Result
1	:*T. parva* Muguga 21% piroplasm	+ve
2	:*T. parva* Muguga 0.21% piroplasm	+ve
3	:*T. parva* Muguga 0.021% piroplasm	+ve
4	:*T. parva* Muguga 0.0021% piroplasm	+ve
5	:*T. parva* Muguga 0.00021% piroplasm	+ve
6	:*T. parva* Muguga 0.000021% piroplasm	+ve
7	:*T. parva* Muguga 0.0000021% piroplasm	+ve
8	:DNA size markers pUC 19 Sau 3A	
9	:DNA size markers pUC 19 Sau 3A	
10	:DNA size markers pUC 19 Sau 3A	
11	:Positive control *T. parva* Muguga	+ve
12	:Positive control *T. parva* Muguga	+ve
13	:Bovine DNA negative control	-ve
40 cycles		
14	:*T. parva* Muguga 21% piroplasm	+ve
15	:*T. parva* Muguga 0.21% piroplasm	+ve
16	:*T. parva* Muguga 0.021% piroplasm	+ve
17	:*T. parva* Muguga 0.0021% piroplasm	+ve
18	:*T. parva* Muguga 0.00021% piroplasm	+ve (weak)
19	:*T. parva* Muguga 0.000021% piroplasm	-ve
20	:*T. parva* Muguga 0.0000021% piroplasm	-ve
21	:Bovine DNA	-ve
22	:Positive control *T. parva* Muguga	+ve
23	:Positive control *T. parva* Muguga	+ve

234 b.p. is the length of a positive amplification product

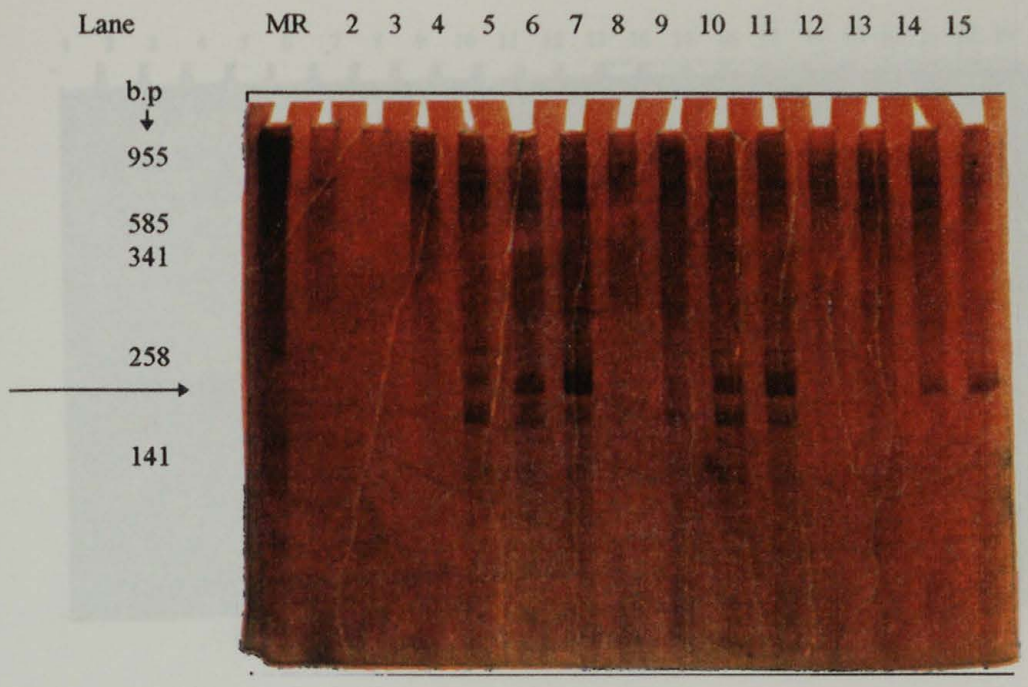


Figure 4.8 An investigation of the effect of increased dNTP concentration on the sensitivity of the p67 PCR assay. Ten-fold dilutions of known *T. parva* Muguga piroplasm positive blood was tested at 0.2 mM, 0.4 mM and 0.8 mM dNTPs.

Lane		Result
1	:pUC 19Sau DNA size marker	
2	:Negative control (Bovine DNA)	-ve
3	:Blank	-ve
	:0.8 mM dNTP	
4	:0.000021% piroplasm parasitaemia	-ve
5	:0.00021% piroplasm parasitaemia	+ve (extra bands)
6	:0.0021% piroplasm parasitaemia	+ve (extra bands)
7	:0.021% piroplasm parasitaemia	+ve
	:0.4 mM dNTP	
8	:0.021% piroplasm parasitaemia	+ve
9	:0.0021% piroplasm parasitaemia	+ve
10	:0.00021% piroplasm parasitaemia	+ve (extra bands)
11	:0.000021% piroplasm parasitaemia	-ve
	0.2 mM dNTP	
12	:0.021% piroplasm parasitaemia	+ve
13	:0.0021% piroplasm parasitaemia	+ve
14	:0.00021% piroplasm parasitaemia	+ve
15	:0.000021% piroplasm parasitaemia	-ve

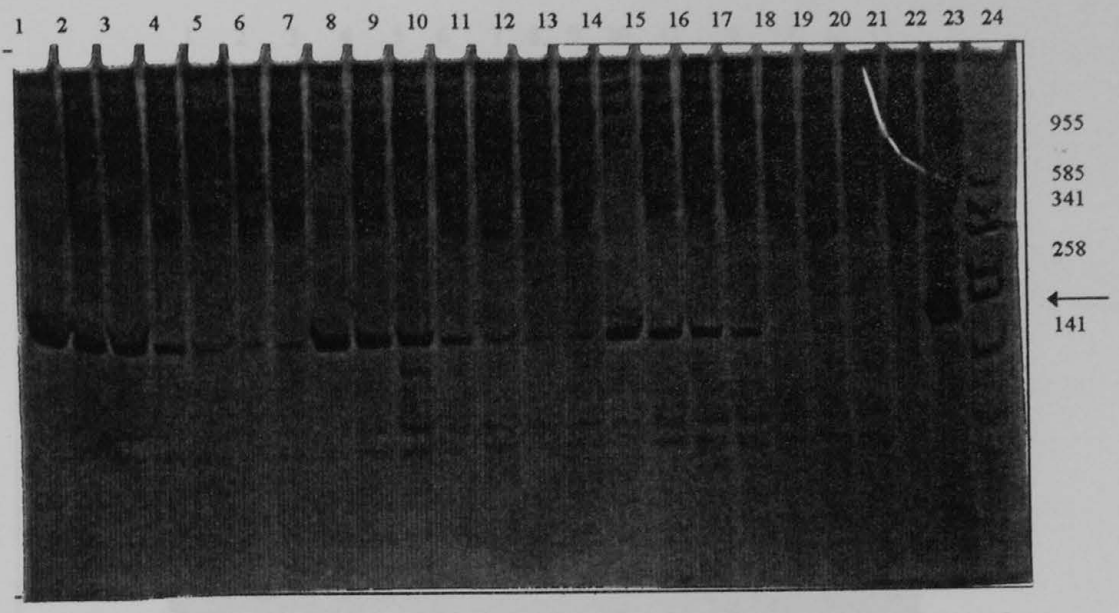


Figure 4.9 Photograph of silver stained PAGE of p67 PCR products comparing assay sensitivity to detect *T. parva* piroplasms in the presence of 2.5 mM, 5.0 mM and 10 mM $MgCl_2$.

Lane	Piroplasm parasitaemia	$MgCl_2$ concentration	Result
1	21%	2.5 mM	+ve
2	0.21%	2.5 mM	+ve
3	0.021%	2.5 mM	+ve
4	0.0021%	2.5 mM	+ve
5	0.00021%	2.5 mM	+ve
6	0.000021%	2.5 mM	+ve
7	0.0000021%	2.5 mM	+ve
8	21%	5.0 mM	+ve
9	0.21%	5.0 mM	+ve
10	0.021%	5.0 mM	+ve
11	0.0021%	5.0 mM	+ve
12	0.00021%	5.0 mM	+ve
13	0.000021%	5.0 mM	+ve weak
14	0.0000021%	5.0 mM	+ve weak
15	21%	10.0 mM	+ve
16	0.21%	10.0 mM	+ve
17	0.021%	10.0 mM	+ve
18	0.0021%	10.0 mM	+ve
19	0.00021%	10.0 mM	+ve
20	0.000021%	10.0 mM	+ve weak
21	0.0000021%	10.0 mM	+ve weak
22	0.00000021%	10.0 mM	+ve weak
23	*T. parva* Muguga positive control		
24	pUC 19Sau DNA size marker		

234 b.p. is the length of a positive amplification product

Figure 4.10 Silver stained PAGE of PCR products to test sensitivity of p67 PCR assay with different primer concentration

Lane		Result
	2.0 uM Primer concentration	
1	:0.00021% *T. parva* Muguga piroplasms	+ve
2	:0.000021% *T. parva* Muguga piroplasms	+ve
3	:0.0000021% *T. parva* Muguga piroplasms	+ve
4	:0.00000021% *T. parva* Muguga piroplasms	+ve
	4.0 uM Primer concentration	
5	:0.00021% *T. parva* Muguga piroplasms	+ve
6	:0.000021% *T. parva* Muguga piroplasms	+ve(bands)
7	:0.0000021% *T. parva* Muguga piroplasms	-ve
8	:0.00000021% *T. parva* Muguga piroplasms	-ve
	8.0 uM Primer concentration	
9	:0.00021% *T. parva* Muguga piroplasms	+ve(bands
10	:0.000021% *T. parva* Muguga piroplasms	+ve(bands)
11	:0.0000021% *T. parva* Muguga piroplasms	-ve
12	:0.00000021% *T. parva* Muguga piroplasms	-ve
13	:*T. parva* Muguga piroplasm 0.1ng DNA	+ve
14	:1 ng of bovine DNA	-ve
15	: pUC 19Sau DNA size marker	

Figure 4.11 Photograph of silver stained PAGE of PCR products : PCR amplification of saponin lysed EDTA blood samples from calf 818 infected with *T. parva* Marikebuni day 11 to 17 post infection. Two microlitres of sample were tested in PCR assay with p67 primers after saponin lysis and proteinase K digestion.

Lane		Result
1	:Day 11 p.i.	-ve
2	:Day 12 p.i.	-ve
3	:Day 13 p.i.	-ve
4	:Day 14 p.i.	+ve(weak)
5	:Day 15 p.i.	+ve
6	:Day 16 p.i.	+ve
7	:Day 17 p.i.	+ve

8 : Positive control *T. parva* Muguga piroplasms
9 :Negative control
10 :pUC 19Sau DNA size marker

p.i. :post infection
234 b.p. is the length of a positive amplification product

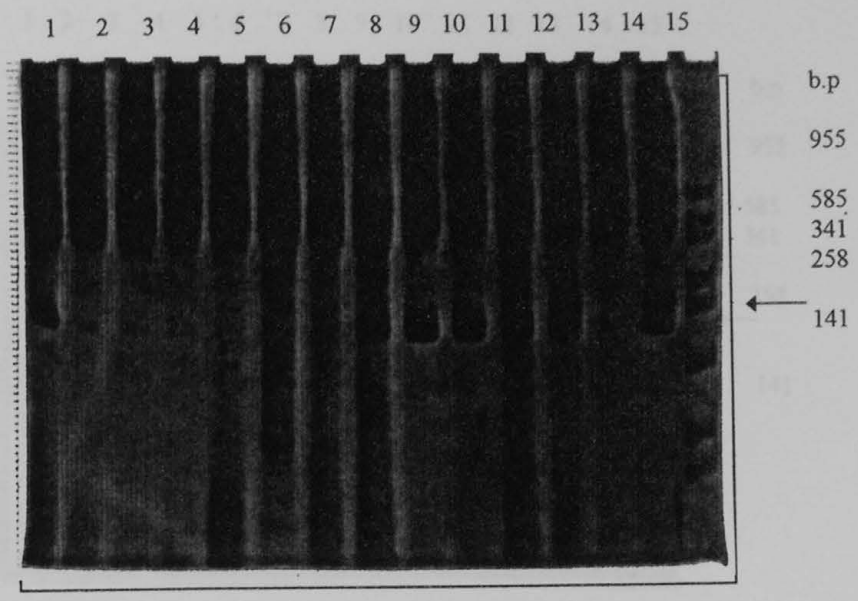


Figure 4.12 Photograph of silver stained PAGE of PCR products showing :
PCR amplification of saponin lysed EDTA blood, right prescapular gland (RPG) biopsy material,, left prescapular gland (LPG) biopsy material and PBM phenol chloroformed samples from calf 805 *T. parva* Boleni day 5 to 9 post infection. Two microlitres of sample were tested in PCR assay with p67 primers after saponin lysis and proteinase K digestion.

Lane		Result
1	:Positive control	+ve
2	:Day 5 p.i. RPG (p.c.)	w+ve
3	:Day 6 p.i. RPG (p.c.)	+ve
4	:Day 7 p.i. RPG (p.c.)	w+ve
5	:Day 8 p.i. RPG (p.c.)	-ve
6	:Day 8 p.i. EDTA (s.l.)	-ve
7	:Day 8 p.i. EDTA (s.l.)	-ve
8	:Day 8 p.i. PBM (p.c.)	+ve
9	:Day 8 p.i. LPG (p.c.)	+ve
10	:Day 9 p.i. LPG (p.c.)	+ve
11	:Day 9 p.i. PBM (p.c.)	+ve
12	:Day 9 p.i. RPG (p.c.)	+ve
13	:Day 9 p.i. EDTA (s.l.)	-ve
14	:*T. parva* Muguga macroschizont DNA	+ve
15	:pUC 19Sau DNA size marker	

234 b.p. is the length of a positive amplification product

-ve	:negative	s.l.	:saponin lysis extracted
+ve	:positive	w +ve	:weak positive

p.c. :phenol chloroform extraction after proteinase K digestion
p.i. :post infection
PBM :peripheral blood mononuclear cells

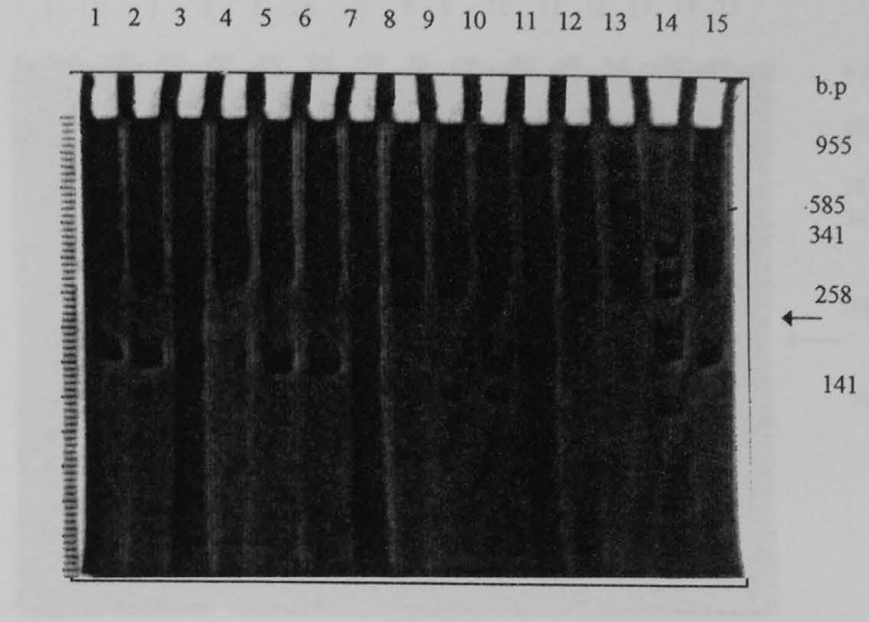


Figure 4.13 Photograph of silver stained PAGE of p67 PCR products showing : Samples from calf 805 *T. parva* Boleni for days 10, 11, 12 and 13 post infection. PCR amplification of extracts from

(a) right prescapular gland (RPG) biopsy material phenol/ chloroform extract,
(b) left prescapular gland (LPG) biopsy material phenol/ chloroform extract,
(c) PBM phenol/ chloroform extract
(d) saponin lysed EDTA samples .

Lane		**Result**
1	:Day 10 p.i. RPG biopsy material	+ve
2	:Day 10 p.i. LPG biopsy material	+ve
3	:Day 10 p.i. PBM	+ve
4	:Day 11 p.i. EDTA	-ve
5	:Day 11 p.i. RPG biopsy material	+ve
6	:Day 11 p.i. LPG biopsy material	+ve
7	:Day 11 p.i. PBM	+ve
8	:Day 12 p.i. EDTA	-ve
9	:Day 12 p.i. RPG biopsy material	+ve
10	:Day 12 p.i. LPG biopsy material	+ve
11	:Day 12 p.i. PBM	+ve
12	:Day 13 p.i. EDTA	-ve
13	:Negative control	
14	:pUC 19Sau DNA size marker	
15	:*T. parva* Muguga macroschizont DNA positive control (1.0 ng)	

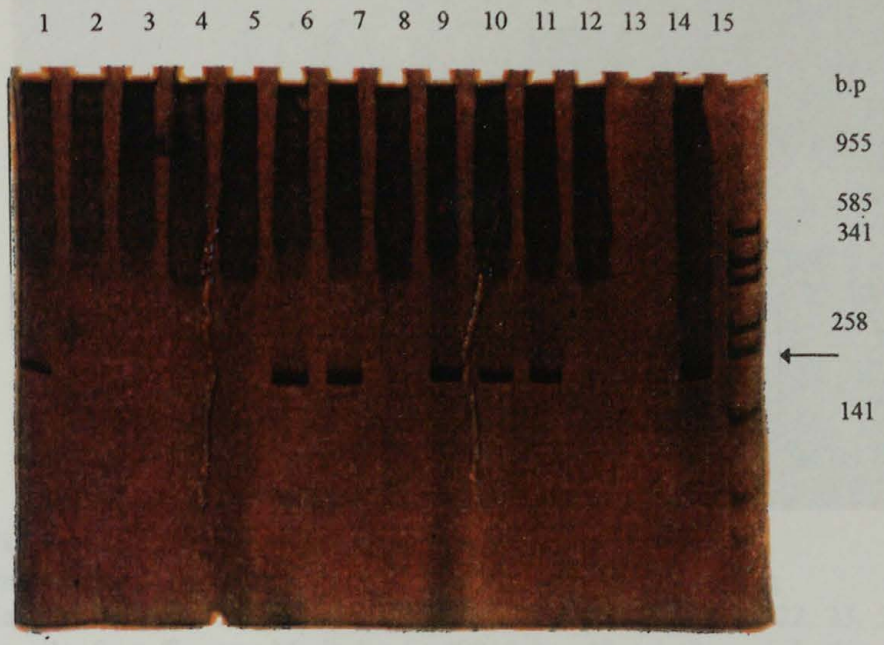


Figure 4.14 Photograph of silver stained PAGE of p67 PCR products showing : Samples from calf 805 *T. parva* Boleni for days 16, 17, 18 post infection.
PCR amplification of extracts from
(a) right prescapular gland (RPG) biopsy material phenol/ chloroform extract,
(b) left prescapular gland (LPG) biopsy material phenol/ chloroform extract,
(c) PBM phenol/ chloroform extract
(d) saponin lysed EDTA samples .

Lane		**Result**
1	:Day 16 p.i. PBM (p.c.)	+ve
2	:Day 16 p.i. LPG (p.c.)	-ve
3	:Day 16 p.i. RPG (p.c.)	-ve
4	:Day 16 p.i. EDTA blood (s.l.)	-ve
5	:Day 17 p.i. PBM (p.c.)	-ve
6	:Day 17 p.i. LPG (p.c.)	+ve
7	:Day 17 p.i. RPG (p.c.)	+ve
8	:Day 17 p.i. EDTA blood (s.l.)	-ve
9	:Day 18 p.i. PBM (p.c.)	+ve
10	:Day 18 p.i. LPG (p.c.)	+ve
11	:Day 18 p.i. RPG (p.c.)	+ve
12	:Negative control (1.0 ng Bovine DNA)	-ve
13	:Blank	-
14	:Positive control *T. parva* Muguga piroplasm DNA	-ve
15	:pUC 19Sau DNA size marker	

p.i. post infection
s.l. saponin lysis extraction
p.c. phenol chloroform extraction

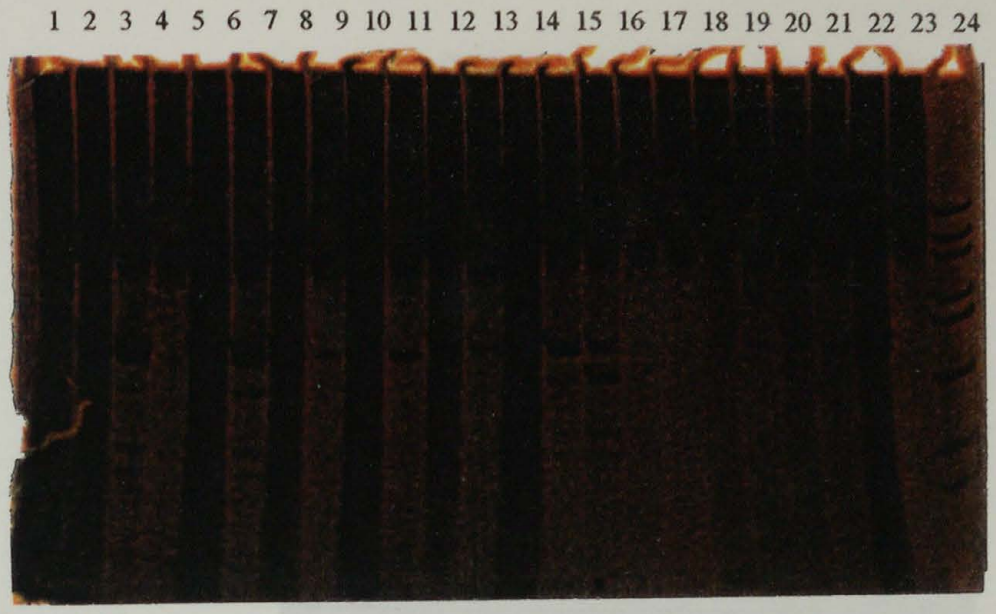


Figure 4.15

Photograph of silver stained PAGE of p67 PCR products showing :
Samples from calf 805 *T. parva* Boleni for days 19, 20, 21, 22, 23, 24 25 and EDTA samples from *T. parva* Marikebuni calf 818 days 47 and 48 post infection.
PCR amplification of extracts from
(a) right prescapular gland (RPG) biopsy material phenol / chloroform extract,
(b) left prescapular gland (LPG) biopsy material phenol / chloroform extract,
(c) PBM phenol/ chloroform extract
(d) saponin lysed EDTA samples .

Lane		Result	Lane		Result
Calf 805					
1	:Day 19 p.i. PBM (p.c.)	-ve	11	:Day 21 p.i. RPG (p.c.)	+ve
2	:Day 19 p.i. LPG (p.c.)	+ve	12	:Day 21 EDTA (s.l.)	+ve
3	:Day 19 p.i. RPG (p.c.)	+ve	13	:Day 22 p.i. PBM (p.c.)	+ve
4	:Day 19 EDTA	+ve (w)	14	:Day 22 p.i. LPG (p.c.)	+ve
5	:Day 20 p.i. PBM (p.c.)	+ve	15	:Day 22 p.i. RPG (p.c.)	+ve
6	:Day 20 p.i. LPG (p.c.)	+ve	16	:Day 22 EDTA (s.l.)	+ve
7	:Day 20 p.i. RPG (p.c.)	+ve	17	:Day 23 p.i. PBM (p.c.)	+ve (w)
8	:Day 20 p.i.EDTA (s.l.)	+ve (w)	18	:Day 24 p.i. LPG (p.c.)	+ve (w)
9	:Day 21 p.i. PBM (p.c.)	-ve	19	:Day 25 p.i. RPG (p.c.)	+ve (w)
10	:Day 21 p.i. LPG (p.c.)	+ve			
Calf 818					
20	:Day 47 p.i. calf 818 EDTA (s.l.)				+ve (w)
21	:Day 48 p.i. calf 818 EDTA (s.l.)				+ve
22	:*T. parva* Muguga piroplasm positive control				+ve
23	:Bovine DNA 1.0 ng Negative control				
24	:pUC 19Sau DNA size marker				

p.i. post infection
s.l. saponin lysis extraction
p.c. phenol chloroform extraction
+ve (w) weak positive

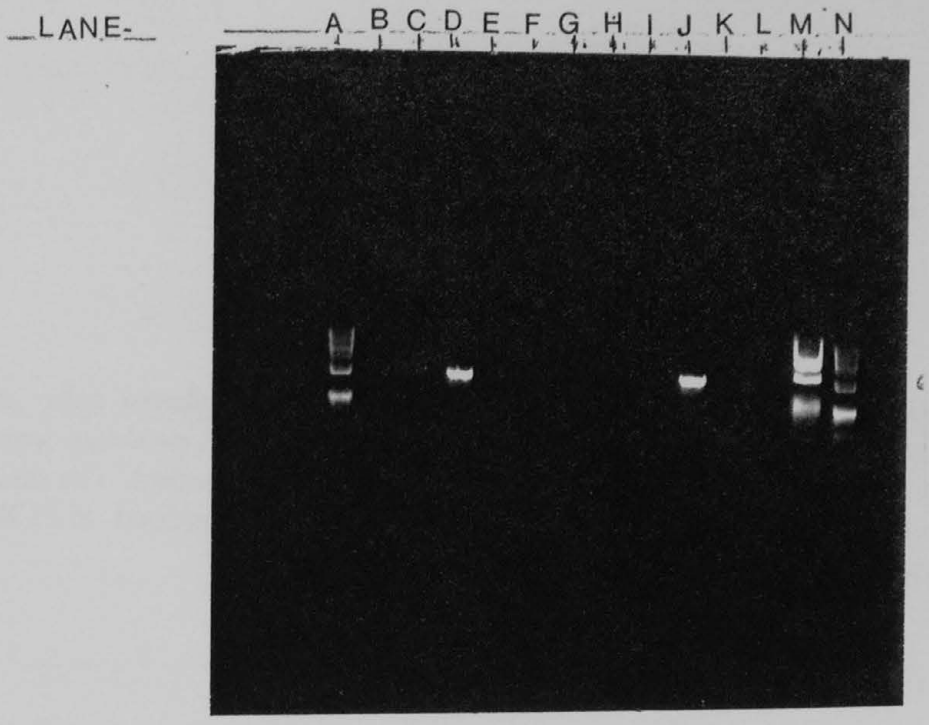


Figure 4.16 Agarose gel electrophoresis of PCR products for p104 PCR specificity

Lane		Result
A	:Ø X174 Hae III dig DNA size marker	
B	:Bovine DNA 1.0 ng	-ve
C	:Blank	-ve
D	:T. parva Muguga piroplasm DNA 1.0 ng	+ve
E	:*T. mutans* (1.0 ng piroplasm DNA tested in PCR assay)	-ve
F	:*T. taurotragi* (1.0 ng macroschizont DNA added to PCR reaction)	-ve
G	:*Trypanosoma congolense* 1.0 ng DNA added to PCR reaction)	-ve
H	:*T. buffeli* (Marula) (1.0 ng piroplasm DNA added to PCR reaction)	-ve
I	:*T. sergenti* (Shintoku) (1.0 ng piroplasm DNA added to PCR reaction)	-ve
J	:*T. parva* Boleni (1.0 ng macroschizont DNA added to PCR reaction)	+ve
K	:*B. bovis* DNA	-ve
L	:Blank	-ve
M	:Ø X174 Hae III dig DNA size marker	
N	:Ø X174 Hae III dig DNA size marker	

Figure 4.17 DNA prepared from various parasite infected cells, and piroplasms were run on a PCR assay using p104 primers. PCR products were slot-blotted, probed with *T. parva* Muguga random labelled PCR product. The result of dot blot hybridisation to confirm the PCR product of 497 bp hybridised to PCR amplification product from *T. parva* only:

A5 :*T. parva* Muguga PCR product (10 µl)

A11 :*T. parva* Muguga PCR product (30 µl)

C1 :*T. mutans* (1.0 ng piroplasm DNA tested in PCR assay)

C3 :*T. taurotragi* (1.0 ng macroschizont DNA added to PCR reaction)

C5 :Blank

C7 :Bovine DNA (1.0 ng added in PCR reaction tube)

E1 :*Trypanosoma congolense* (1.0 ng DNA added to PCR reaction)

E3 :*T. buffeli* (Marula) (1.0 ng piroplasm DNA added to PCR reaction)

E7 :*T. sergenti* (Shintoku) (1.0 ng piroplasm DNA added to PCR reaction)

F5 :*T. parva* Boleni (1.0 ng macroschizont DNA added to PCR reaction)

F11 :*T. parva* Boleni (1.0 ng macroschizont DNA added to PCR reaction)


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

Table 4.1 PCR results for extracts prepared from 3 types of sample for *T. parva* Marikebuni infected calf No: 818 using p67 primers from the first 30 days post infection (p.i.)

Day p.i	PCR PBM	PCR LPG	PCR EDTA blood	IFAT titre
0	-ve	-ve	-ve	<1:10
1	-ve	-ve	-ve	
2	-ve	-ve	-ve	
3	-ve	-ve	-ve	
4	-ve	-ve	-ve	
5	-ve	-ve	-ve	
6	-ve	-ve	-ve	
7	-ve	-ve	-ve	1:10
8	-ve	-ve	-ve	
9	-ve	-ve	-ve	
10	+ve	-ve	-ve	
11	+ve	-ve	-ve	
12	-ve	-ve	-ve	
13	-ve	+ve	-ve	
14	+ve	-ve	-ve	1:160
15	-ve	-ve	+ve	
16	-ve	-ve	+ve	
17	-ve	-ve	+ve	
18	-ve	+ve	+ve	
19	-ve	-ve	+ve	
20	-ve	w +ve	+ve	
21	-ve	w +ve	+ve	1:640
22	-ve	-ve	+ve	
23	-ve	-ve	+ve	
24	-ve	-ve	ns	
25	-ve	-ve	+ve	
26	-ve	ns	ns	
27	-ve	ns	ns	1:2560
28	-ve	ns	-ve	
29		ns	ns	
30		ns	ns	
+ve proportion	3/28	4/25	10/25	

-ve :negative LPG :left prescapular gland
+ve :positive
ns :not sampled
234 b.p. product is the length of a positive amplification

Table 4.2 PCR results for extracts prepared from 4 types of sample for *T. parva* Boleni infected calf No: 805 using the p67 primers from the first 30 days post infection (p.i.)

Day p.i	PCR PBM	PCR RPG	PCR LPG	PCR EDTA blood	IFAT titre
0	-ve	-ve	ns	-ve	<1:160
1	-ve	-ve	ns	ns	
2	-ve	-ve	ns	ns	
3	-ve	-ve	ns	ns	
4	-ve	± ?	ns	ns	
5	-ve	w +ve	ns	-ve	
6	-ve	w +ve	ns	-ve	
7	-ve	w +ve	ns	-ve	1:160
8	+ve	-ve	ns	-ve	
9	+ve	+ve	+ve	-ve	
10	+ve	+ve	+ve	-ve	
11	+ve	+ve	+ve	-ve	
12	+ve	+ve	-ve	-ve	
13	+ve	-ve	+ve	-ve	
14	-ve	+ve	+ve	-ve	1:160
15	+ve	+ve	+ve	-ve	
16	-ve	+ve	+ve	-ve	
17	-ve	+ve	+ve	-ve	
18	+ve	+ve	+ve	+ve	
19	-ve	+ve	+ve	w +ve	
20	+ve	+ve	+ve	+ve	
21	-ve	+ve	+ve	+ve	1:640
22	+ve	+ve	+ve	+ve	
23	+ve	-ve	-ve	+ve	
24	-ve	+ve	-ve	+ve	
25	-ve	-ve	-ve	+ve	
26	-ve	ns	-ve	-ve	
27	-ve	ns	-ve	-ve	
28	-ve	ns	-ve	-ve	1:640
29	ns	ns	ns	-ve	
30	ns	ns	ns	-ve	
+ve proportion	11/28	18/25	13/20	8/29	

+ve :positive
-ve :negative
ns :not sampled
LPG :left prescapular gland
RPG :right prescapular gland
w +ve :weak positive

CHAPTER FIVE

Detection of *Theileria* parasites in infected / recovered cattle by tick application

5.1 Introduction

The three host, brown ear tick *Rhipicephalus appendiculatus* is the principle field vector of *T. parva*. The nymphal tick has also been known to transmit the disease (Koch *et al* ., 1993; Koch, 1990; Ochanda, 1994). Experiments by Ochanda (1994) have shown that *R. appendiculatus* nymphae infected as larvae developed lower prevalence and intensity of infection compared to adults infected as nymphae. Transmission is always transtadial and transovarian transmission has not been demonstrated.

In this chapter clean, *R. appendiculatus* nymphs from a laboratory colony were used to pick up the infection from the infected / carrier recovered cattle (discussed in chapter 3) in order to determine the infectivity of the *Theileria* infections to ticks in the laboratory. The nymphal ticks were applied on the calves post recovery in order to demonstrate that infections could be passed onto ticks when the animals were not showing any clinical reactions. Four tick feeds were performed on the *Theileria* infected calves at approximately 3 months intervals. One tick feed was done on the calves which survived on the Hunyani estates second exposure in order to confirm the occurrence of a mixed infection and the presence of a virulent *Theileria* parasite on the farm. At the same time as tick feeds, PCR, serology and blood smear examinations were performed. The nymphs were allowed to moult to the next instar and the salivary glands of the adult ticks were dissected out and stained to determine the prevalence of *T. parva* infection. The Feulgen technique to

identify *Theileria* infection in adult ticks has been demonstrated to be rapid and simple method (Blewett and Branagan, 1973; Walker *et al.*,1979). The Schiff's reagent used in the Feulgen stain is stable at 4°C when prepared, easy to use and is commercially available. Several staining techniques for the detection of *Theileria* in whole salivary glands have been compared and the Feulgen stain supplemented by the superimposition of Giemsa stain was superior to other methods (Norval, *et al.*, 1992). A comparison of conventional Feulgen staining with DNA probes found 90-100% correlation in *Theileria* infected tick salivary glands (Conrad, et al., 1989a), however this depends on the parasite stock used. There still remains problems with quantification of *Theileria* parasites in salivary glands of field ticks because more than one theilerial species may occur in individual ticks, [eg. *T. parva* and *T. taurotragi* in *R. appendiculatus; T. mutans* and *T. velifera* in *Amblyomma variegatum*] (Norval, *et al.*, 1992), thus the development nucleic acid techniques is necessary.

The following terms are used to describe levels of infection and conform to those given by Buscher and Otim (1986);

Prevalence :Number of infected ticks per number of ticks examined.

Abundance :Number of infected acini per infected plus uninfected tick examined.

Mean intensity :Number of infected acini per *infected* tick.

A batch of ticks was defined as a group of ticks of the same stock that fed to repletion on an animal and were removed on a single occasion. Since piroplasms are believed to be the stage infective for the feeding tick (Gonder, 1911; Mehlhorn and

Schein, 1984), their presence or absence in the circulation determines whether an animal is a carrier at a given time. In this study nymphal *R. appendiculatus* ticks were applied onto the recovered animals different occasions over a period of 19 months and dissected at the next instar to determine if the Zimbabwean *T. parva* parasites can effectively cause animals to be persistent or sporadic carriers. Quantitative studies on *T. parva* salivary glands of *R. appendiculatus* adults have shown that the age and the feeding site of nymphs on an infected calf and its parasitaemia had no detectable influence on the tick infections and higher infections were found in female ticks from heavier nymphs, in females versus male (Buscher and Tangus, 1986).

5.2 Materials and Methods

5.2.1 Rabbits and Ticks

Pure bred New Zealand white rabbits, weighing at least 2 kilograms were used for tick feeding and were obtained from the Tick unit at the Veterinary Research Laboratories-Harare.

Theileria free *R. appendiculatus* nymphs (McIlwaine colony) were used for tick feeds. These ticks were originally obtained from the Lake Chivero region 30 kilometres south of Harare and have been maintained in a closed colony by feeding stages of the ticks on New Zealand White rabbits at the Veterinary Research Laboratories, Harare since 1976 (Short & Norval, 1981).

5.2.2 Application of ticks to cattle to pick up *Theileria* infections

The experimental animals had their backs washed, shaved and zipped cotton cloth (57 cm x 24 cm) were attached with glue (Gemchem, Harare). Uninfected *R. appendiculatus* nymphs were applied in batches of 1000 at a time per animal.

Nymphal tick applications and feeds on the carrier / recovered animals were performed on the days shown on Table 5.1 below.

The nymphs were left to feed to repletion and when engorged transferred into incubators at 28°C and relative humidity 85% for 28 days to complete their moult. Adult ticks were left for another two weeks to harden before processing for assessment of infection.

5.2.3 Assessment of ticks for *Theileria* infections using Feulgen stain

Feulgen technique was used to detect *Theileria*-infected acini in the adult tick salivary glands (Young and Leitch, 1982). In order to examine tick batches for infections approximately 300 adult ticks from each batch were allowed to feed on New Zealand White rabbit's ears for 4 days so that the *T. parva* sporoblasts could mature to sporozoites. Approximately 75 males and 75 female adult ticks were applied and placed in each ear bag.

Adult ticks were placed in cotton bags attached onto rabbit's ears using elastoplast (Zinc Oxide Elastic Adhesive Bandage-Smith and Nephew Ltd Pinetown S.A.). Any adult ticks which had not fed by the first day were removed and destroyed, and those continuing to feed were left until day 3 after application (day of application counted as day zero). Adult ticks which had successfully fed were removed on the fourth day from the rabbit's ears, counted and were partially embedded in paraffin wax

(Histoplast-Shandon Scientific Ltd. Runcorn Cheshire, England) with their dorsal sides uppermost and submerged in normal saline. Salivary glands were dissected out after having cut out the cuticle of the tick using fine pointed surgical blades (Size No:11) and fine pair of forceps.

Five pairs of salivary glands were spread individually on each glass slide and were allowed to dry. These were fixed in methanol for 5 minutes dried, and then hydrolysed in 5N HCL for one hour. The excess acid was drained off and the glands stained in Schiff's reagent stain for one hour (Buscher & Otim 1986). After the Feulgen staining the stained glands were washed thoroughly under running tap-water, air dried at room temperature and then mounted with 50mm x 22mm coverslips with DPX mountant. The salivary glands were examined under a magnification of X200 to assess the number of acini infected with *Theileria* sporozoites per individual tick. The number of ticks dissected and examined varied from batch to batch and animal to animal as shown in the Tables 5.2 to 5.7. One of the reasons for this variation is that it was found that nymphal ticks fed less successfully with repeat applications due to dermal hypersensitivity induced in the host. The degree of the reactions varied from animal to animal and feed to feed. In order to improve the number of nymphae feeding successfully the nymphs were applied on the ears of the calf or the numbers applied were reduced to 200 at a time so they could feed to repletion. It has been shown that the site of tick feeding does not influence the level of infection developing in subsequent instars.

Table 5.1

Days of application of *R. appendiculatus* nymphs to pick up *Theileria* infections

Group	Parasite	Calf No:	Batch I	Batch II	Batch III	Batch IV	Batch V
I	Boleni	W70	95 p.i.	218 p.i.	305 p.i.	456 p.i.	nd
I	Boleni	W73	95 p.i.	218 p.i.	305 p.i.	456 p.i.	nd
I	Boleni	W74	95 p.i.	218 p.i.	305 p.i.	nd	nd
I	Boleni	W75	95 p.i.	218 p.i.	305 p.i.	nd	nd
II	Avery	1442	95 p.i.	218 p.i.	305 p.i.	456 p.i.	nd
II	Avery	1454	95 p.i.	218 p.i.	305 p.i.	456 p.i.	nd
III	Bally Vaughan	1447	95 p.i.	218 p.i.	305 p.i.	456 p.i.	nd
III	Bally Vaughan	1459	95 p.i.	218 p.i.	305 p.i.	456 p.i.	nd
IVa	Ayrshire	1456	nd	264 p.t.a.	348	499	nd
IVb	Chikeya	1437	138 p.t.a.	264 p.t.a.	nd	nd	nd
Va	Hunyani Estates	1372	140 post exposure	266 post exposure	350 post exposure	501 post exposure	nd
Vb	Hunyani Estates	E1					137 post exposure
Vb	Hunyani Estates	E4					137 post exposure
Vb	Hunyani Estates	E5					137 post exposure

nd :not done

p.t.a. :post tick application

p.i. :post infection

5.3 Results

Table 5.2 shows the numbers ticks which fed successfully for four days as adults on the rabbits and were dissected. After the first batch the numbers decreased as the animals reacted to the attachment of the nymphs resulting in the death of a large numbers of adult ticks on feeding on rabbits before the dissections.

The prevalence of *Theileria* infections in adult *R. appendiculatus* ticks that fed as nymphae on the *Theileria* recovered cattle are shown in Tables 5.3 to 5.7.

For day 95 tick applications, 3 out of 4 batches of the ticks fed on *T. parva* Boleni calves W70, W73, and W75 were positive with infection prevalence of 0.4%, 5.96% and 7.14 % respectively. For day 218 applications 3 out of 4 batches were positive with animal number W74 having transmitted the infection but was negative for the first batch of ticks. *Theileria* parasites were not detected in ticks applied to the group I animals on days 305 and 456 post infection. No *Theileria* parasites were detected in the Boleni infected calves on days 305 and 456 post infection.

Bally Vaughan infections: The tick batches which fed on group II and III calves had no parasites seen in the adult tick salivary glands in any of the four batches.

Ayrshire ticks application: Uninfected *R. appendiculatus* nymphs were applied on days 264, 348 and 499 post adult tick application and the subsequent adult ticks had prevalence of infection of 11.6%, 10.24% and 11.6% respectively.

Chikeya ticks application: A single acini was *Theileria* infected in the two batches fed on 138 and 268 days post adult ticks application on calf 1437.

Hunyani estates infections;

A sample of 28 adult *R. appendiculatus* ticks from the paddock at Hunyani estates had an infection rate of 10.71%, seven acini were infected in the 3 ticks that were infected. A 68.03% prevalence of infection was shown in the ticks which fed on the recovered animal 1372 on days 140 post exposure(Table 5.3). The prevalence of infection in the subsequent ticks were high (Tables 5.4, 5.5 & 5.6) with 60.6%, 27.2% and 37.82 % prevalence for applications on days 266, 350 and 501 respectively. The second group of animals exposed (E1, E4 & E5) had uninfected nymphs applied 137 days post exposure and the prevalence of infection in the subsequent adult ticks were 18.88, 12.39 and 90.44% respectively. The highest range of number of infected acini per tick was 1-79 in the day 266 days post exposure adult ticks.

Table 5.2

Tick numbers successfully dissected after feeding on rabbits

Group	Animal tag no:	Batch I	Batch II	Batch III	Batch IV	Batch V
I	W70	235	193	200	134	
	W73	250	213	144	124	
	W74	218	190	145	* nd	
	W75	224	191	190	* nd	
II	1442	131	186	137	23	
	1454	152	244	244	147	
III	1447	143	204	500	228	
	1459	148	24	76	231	
IV	1456	nd	199	127	185	
	1437	260	201	* nd	* nd	
V	1372	122	221	353	220	
VI	E1					233
	E4					218
	E5					136

* ticks dead

nd not done

5.4 Discussion

In this study Boleni infected / recovered animals were shown to be infective carriers of *T. parva* to the vector ticks for a period of 218 days post infection. Three of the tick applications on this date on 4 Boleni infected animals were positive. Thus transmission of *T. parva* Boleni to ticks from immunised animals was shown. As transmission studies were not performed in this study, to prove that the infected tick batches could transmit disease it cannot be stated that onward transmission of the Boleni parasite would occur. Koch *et al.*, (1992) demonstrated transmission of disease 7-9 months after *T. parva* Boleni immunisation by application of adult ticks onto susceptible cattle from nymphs fed on Boleni immunised animals.

Group II *T. parva* Avery and group III buffalo derived *T. parva* Bally Vaughan infected and recovered calves were not capable of transmitting infection to ticks. Maritim *et al* (1989) in 5 out of 11 infected animals infected with buffalo derived *T. parva*. In this study 1054 adult *R. appendiculatus* ticks fed as nymphae on *T. parva* Bally Vaughan infected / recovered calves were negative. Much higher infection rates (10 to 90%) have been obtained from *R. appendiculatus* adults fed as nymphs on African buffalo than obtained from *T. parva* or buffalo derived *T. parva* carrier cattle (Young and Grootenhuis., 1984). Previous workers have demonstrated that buffalo derived parasites are difficult to transmit to ticks because of their low levels of parasites and low numbers of piroplasms.

R. appendiculatus adult ticks collected in field from vegetation transmitted *T. parva* infection to susceptible calves (Group IV) resulting in a moderate *Theileria* reaction. The two properties, Ayrshire and Chikeya had cattle immunised in the

previous years and these could have added the parasite population in the ticks and the farmers were not experiencing any outbreaks of disease.

Both experiments at Hunyani Estates were complicated by the possibility of infection with *T. taurotragi*. High tick infection rates following application of ticks to recovered calves suggest a biologically different *Theileria* from the other laboratory stocks. The severity of the reactions exposed at this property meant that the farmer could not graze his stock in the particular paddock used in this study. It is possible that the high tick infection rates which were detectable after 501 days post exposure at Hunyani estates, 499 days post tick application of field ticks were mixed infections of *T. parva* and *T. taurotragi* in the ticks. However in this chapter it has been shown that an infection prevalence of 90.44% in adult ticks fed as nymphae 137 days post exposure at Hunyani Estates (Hunyani 2nd exposure), and in Chapter three a *T. taurotragi*-like monoclonal antibody profile appeared yet the parasite was pathogenic contrary to work by Grootenhuis. *et al.*, (1979) who found that a tick derived stabilate of *T. taurotragi* was uniformly infective to cattle and mild reactions developed and the survivors after treatment were immune to *T. parva* Avery challenge. The Hunyani exposed calves were positive for *T. parva* (chapter 4) in this study by PCR using p104 primer sequences designed for microneme / rhoptry antigens of *T. parva*.

Young *et al.*, (1983) demonstated that higher *T. parva* infections (96%) developed in salivary glands of ticks exposed in the field than those incubated at 28 or 23°C. In this study an infection rate of 90.44% was achieved in adults fed as

nymphae 137 days post exposure at Hunyani estates, higher than the infection rate of 10.71% in grass-top ticks from the same paddock.

From these results it can be concluded that field parasites cause higher levels of carrier status capable of causing tick infections higher than those caused by the Boleni immunizing stock. Another explanation could be the degree of infectivity of the ground up tick stabilates is low because of liquid nitrogen storage.

It was difficult to achieve a minimum of 200 feeding adult ticks per batch for each animal as the animals reacted to the ticks resulting in the variability of the number of ticks successfully feeding on an animal. The large variation in the infection rate in the ticks fed on Hunyani exposed calves and the Boleni infected can be a pointer in distinguishing field parasites from the vaccine stock, as the two properties Hunyani and Ayrshire have had cattle immunised. Further characterisation of the Hunyani parasite is required because the cattle were infected with *T. taurotragi* or a very atypical strain of *T. parva.* Variations within the same group of animals can occur as it is possible that at the time of nymphal application the animals did not have piroplasms in the peripheral blood vessels.

Results: Table 5.3

Prevalence of infections in adult *R. appendiculatus* ticks that fed as nymphae on *Theileria*-infected cattle.

Batch I:

Animal No: Parasite	Day p.i.	Number of ticks dissected	Number of ticks infected	Mean No. infected acini per tick	Prevalence of infection %	Range of Number of infected acini per tick (min-max)
W70 Boleni	95 p.i.	235	14	1.43	5.96	1 - 4
W73 Boleni	95 p.i.	250	1	1	0.4	1
W74 Boleni	95 p.i.	218	0	0	0	0
W75 Boleni	95 p.i.	224	16	1.44	7.14	1 - 3
1442 Avery	95 p.i.	131	0	0	0	0
1454 Avery	95 p.i.	152	0	0	0	0
1447 Bally Vaughan	95 p.i.	143	0	0	0	0
1459 Bally Vaughan	95 p.i.	148	0	0	0	0
1437 Chikeya.	138 p.t.a	260	1	1	0.38	1
1372 Hunyani Estates	140 p.e.	122	83	5.66	68.03	1 - 29

p.i. :post infection
p.t.a. :post tick application
p.e. :post exposure

Table 5.4 Prevalence of infections in adult *R. appendiculatus* ticks that fed as nymphae on *Theileria*-infected cattle.

Batch II:

Calf No. Parasite	Day p.i.	Number of ticks dissected	Number of ticks infected	Mean no. of infected acini per tick	Prevalence of Infection %	Range of Number of infected acini per tick (min-max)
W70 Boleni	218 pi	193	0	0	0	0
W73 Boleni	218 pi	213	7	1	3.29	1
W74 Boleni	218 pi	190	2	1	2.22	1
W75 Boleni	218 pi	191	2	1.5	1.05	1 - 2
1442 Avery	218 pi	186	0	0	0	0
1454 Avery	218 pi	244	0	0	0	0
1447 B V	218 pi	204	0	0	0	0
1459 BV	218 pi	24	0	0	0	0
1456 Ayrshire	264 p.t.a	199	23	2.52	11.6	1 - 8
1437 Chikeya	264 p.t.a.	201	1	1	0.50	1
1372 Hunyani Estates	266 p.e.	221	134	13.30	60.60	1 - 79

p.i. :post infection
p.t.a. :post tick application
p.e. :post exposure
B.V. :Bally Vaughan

Table 5.5
Prevalence of infections in adult *R. appendiculatus* ticks that fed as nymphae on *Theileria*-infected cattle.
Batch III.

Calf No: Parasite	Day p.i.	Number of ticks dissected	Number of ticks infected	Mean No. of infected acini per tick	Prevalence of Infection %	Range of Number of infected acini per tick (min-max)
W70 Boleni	305 p.i.	200	0	0	0	0
W73 Boleni	305 p.i.	144	0	0	0	0
W74Boleni	305 p.i.	145	0	0	0	0
W75Boleni	305 p.i.	190	0	0	0	0
1442 Avery	305 p.i.	137	0	0	0	0
1454 Avery	305p.i.	137	0	0	0	0
1459 BV	305 p.i.	76	0	0	0	0
1447 B V	305 p.i.	All ticks dead nd	0	0	0	0
1456 Ayrshire Farm	348 p.t.a.	127	13	2.08	10.24	1 - 8
1372 Hunyani Estates	350 p.e.	353	96	4.74	27.20	1 - 33

nd :not done
p.i. :post infection
p.t.a. :post tick application
p.e. :post exposure
B.V. :Bally Vaughan

Table 5.6
Prevalence of infections in adult *R. appendiculatus* ticks that fed as nymphae on *Theileria*-infected cattle.
Batch IV:

Calf No. Parasite	Day p.i.	Number of ticks dissected	Number of Ticks infected	Mean no. of infected acini per tick	Prevalence of infection %	Range of Number of infected acini per tick (min-max
W70 Boleni	456	134	0	0	0	0
W73 Boleni	456	124	0	0	0	0
W74 Boleni	Died 11/12/95					0
W75 Boleni	Died 26/09/95					0
1442 Avery	456	23	0		0	0
1459 B. V.	456	231	0	0	0	0
1447 B V.	456	228	0	0	0	0
1456 Ayrshire Farm	499 p.t.a.	185	23	2.52	11.6	1 - 2
1372 Hunyani Estates exposure	501 post exposure	220	81	4.32	37.82	1 - 34

B.V. :Bally Vaughan
nd :not done
p.i. :post infection
p.t.a. :post tick application

Table 5.7
Prevalence of infections in adult *R. appendiculatus* ticks that fed as nymphae on *Theileria*-infected cattle.
Hunyani Estates 2nd exposure
Batch V:

Calf No.	Day post exposure	Number of ticks dissected	Number of ticks infected	Mean no: of infected acini per tick	Prevalence of Infection %	Range of Number of infected acini per tick (min-max)
E1	137	233	44	3.48	18.88	1 - 22
E4	137	218	27	1.78	12.39	1 - 4
E5	137	136	123	8.12	90.44	1 - 43

CHAPTER SIX

IFA titres of anti-schizont antibodies in relation to *T. parva* carrier state

6.1 Introduction

Serological investigations using *T. parva* macroschizont antigens can be used to detect previous exposure to *Theileria* parasites, but are not considered to provide an accurate indicator of carrier state. The IFAT can be used to monitor experimental infections even though with a few drawbacks. A Theileriosis serological survey in 1986 revealed that between 20 to 70% of adult cattle in the commercial farms and 50% of calves in the communal areas under 4 months of age were seropositive using the IFAT (Koch *et al.*, 1986), showing the presence of reactors in areas where no outbreaks have been reported thus indicating the limitations of the test specificity and sensitivity. The causes of this were not understood, although it was suggested that enzootic stability may be widespread and that mild strains causing inapparent infections may occur. The movement of carrier recovered animals from theileriosis outbreaks at cattle sales to areas infested with the tick vector has also been suggested as an explanation for the high anti-schizont antibody titres in areas which have not reported outbreaks. The diagnostic method used to detect the *Theileria* parasites during the acute phase of infections by microscopic examination is not reliable post recovery since the piroplasms appear intermittently and schizonts are not detectable as described in previous chapters. With the current wide scale use of Bolvac® vaccine there is a need to validate the IFAT and relatively new tests such as the ELISA which are currently being developed for *Theileria* parasites (Wright *et al.*, 1993). This may assist in the detection of mild *Theileria* species which cause

seroconversions similar to *T. parva*. The IFAT can be used for the detection of antibodies indicating previous exposure to infection. In Zimbabwe the interpretation of the IFAT has always been found wanting in its specificity in the detection of *T. parva* parasite carriers when exporting animals or in the selection of properties for immunisation against theileriosis. The test is also used routinely for selecting animals for experimental purposes and for export to countries which do not accept animals with evidence of antibodies for *Theileria*.

In the previous chapters the reactions of experimental calves to *T. parva* infection and challenge and their carrier state were discussed. In this chapter, the serological response of the same calves pre- and post-infection and post-challenge will be described using IFAT with macroschizont antigen of the Zimbabwean, Berea stock of *T. parva*. The preference of schizont antigen over the piroplasm antigen in the IFA test results from the work of Burridge and Kimber (1973). In their studies a significant antibody titre to *T. parva* cell culture schizont antigen was demonstrated for between 12 and 73 weeks after infection in the sera of 35 cattle recovered from experimental East Coast Fever.

The aim of this chapter is to determine the distribution of titres of calves from a *T. parva* free property and to determine the optimum cut-off titre for use in the IFAT for the identification of carrier animals post-recovery from *T. parva* infection.

6.2 Materials and Methods

The indirect fluorescent antibody test was used to determine levels of antibodies against macroschizont antigen in sera of calves before and after infection with *Theileria* parasites.

6.2.1 *T. parva* Berea macroschizont antigen preparation

A vial of 1.8 ml *T. parva* Berea (Passage 52) lymhoblastoid cells stored in liquid nitrogen was thawed in a water bath at 37°C and the cell suspension was resuspended in 10 ml of L15 Leibovitz medium without foetal calf serum and centrifuged at 200 xg at room temperature. After the second wash the lymphoblastoid cell pellet was resuspended in sterile Gibco -L15 with L-glutamine medium with 10% foetal calf serum (Gibco) at a pH of 7.2 in a 25 cm^2 culture flasks (Flow Laboratories) The medium also contained 100 iu/ml of penicillin and 100µg/ml of streptomycin (Sigma) added under aseptic conditions (this medium requires no atmospheric carbon dioxide since it has a bicarbonate-free buffering system). The culture was maintained at 37°C incubator and was subcultured every 48 hours until there was at least 500 ml containing 10 x 10^6 cells per ml on harvesting. For specific counts of viable cells, an aliquot of cell suspension was diluted in 0.2% Trypan blue prepared in PBS and counted using a haemocytometer. An aliquot of cells was centrifuged in a Shandon cytospin and Giemsa stained to establish the number of infected cells. The antigen was prepared from cultures exhibiting a cell viability greater than 85% and an infection rate of at least 85% as detailed in the next section.

6.2.2 Preparation of formalin-fixed *Theileria* macroschizont antigen slides

T. parva Berea infected lymphoblastoid cells were used when the culture was in log phase of cell growth. The cell suspension was centrifuged at 200 xg for 10 minutes at 4°C and resuspended in chilled sterile PBS pH 7.2. Sterile tubes were used to separate the cell suspension (viable from dead) through a Ficoll-Paque (Pharmacia) gradient solution spinning at 400 xg for 30 minutes at 4°C with the ratio of 4.5 ml

PBS gave no loss in titre of the positive control sera and simultaneously no specific fluorescence of the negative control serum at 1 in 40. The conjugate was stored as per manufacturer's recommendations.

6.2.5 IFAT procedure

The IFAT using *T. parva* Berea schizont antigen, performed as described by Burridge and Kimber (1972) with a few modifications was used to test all sera in the experiment. The bovine sera was diluted in PBS pH 7.2 to the following dilutions 1/80, 1/160, 1/320, 1/640, 1/1280, 1/2560, 1/5120 and 1/10240 using a multichannel pipettor on a U-bottomed 96-well microtitre plate including the positive and negative control sera. The tissue culture schizont antigen coated slides (section 5.2.1) with 10 wells were removed from -80°C freezer, unwrapped immediately, and placed on trays and left at room temperature for 30 minutes to dry. Using a single-channel pipette, 20 µl of the 1/10240 serum dilution was added onto test slide well H as shown in test slide plan below (figure 5.1), and onto well G 20 µl of the 1/5120 serum dilution. The same volume was dropped onto the rest of the wells F, then E, D, C and B, until 1/80 was dropped in well A.

Figure 6.1

Drawing of antigen-coated slide with reference number X and wells A to H for serum dilutions 1/80 to 1/10240.

A	B	C	D	E	
					X
+ve	-ve	H	G	F	

After having proceeded in the same way with the rest of the antigen slides so that each serum was placed on one slide, known positive and negative control sera (that were serially diluted) were also placed on two slides with 20 µl in each well. The negative and positive control sera were added last on the test slides at 1:80 dilutions, in wells "-ve" and "+ve" at as indicated (figure 6.1) in 20 µl volumes, followed by incubation in a wet chamber at room temperature for 1 hour. After incubation the cold PBS was rinsed off each slide well using a wash-bottle and placed in a staining jar with cold PBS pH 7.2 and washed twice for 15 minutes with gentle agitation. Fifty microlitres of conjugate at a dilution of 1 in 60 in PBS containing Evans Blue (Sigma) at a final concentration of 0.1% (v/v) as a counterstain was added after draining excess PBS ensuring that each well was completely covered and incubated in a moist wet chamber for 30 minutes. The slides were washed twice again as in the above in cold PBS, excess PBS drained off and mounted in 50% glycerol with a 22 x 40 mm coverslip. Slides were examined for fluorescence using a x50 oil immersion objective using an ultraviolet incident light fluorescent microscope (Leitz). The degree of fluorescence was recorded as follows:

+ strong fluorescence (positive reaction)

+/- weak positive

- no fluorescence negative reaction)

This routine method is used at the Veterinary Research laboratories Harare - Protozoology section and has used on the FAO Theileriosis project studies on the Control of Tick and Tick-borne diseases.

6.3 RESULTS

6.3.1 Pre-infection titres

Pre-infection titres of less than 1:320 (Figure 6.2) were observed in *T. parva* negative animals pre-infection (day 0) from Lanark farms and the negative control had no fluorescence at 1 in 40. Out of the forty eight calves sampled at Lanark farms, eight had titres of 1 in 320, eighteen had titres of 1 in 160, forty had titres of 1 in 80 and one was negative at 1 in 40. Figure 6.2 shows the distribution of Lanark farms bovine sera samples tested on IFAT.

Figure 6.2 IFAT results of bovine sera samples tested from Lanark farms

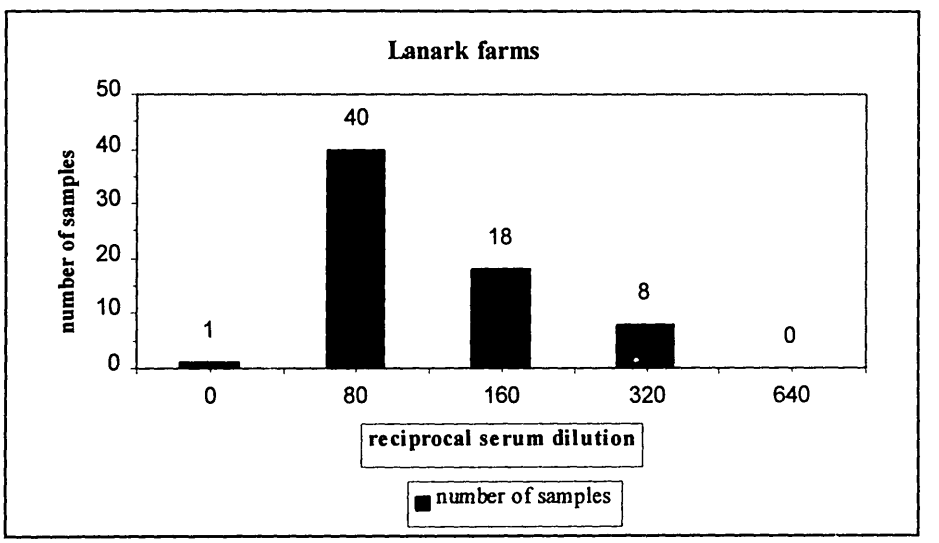
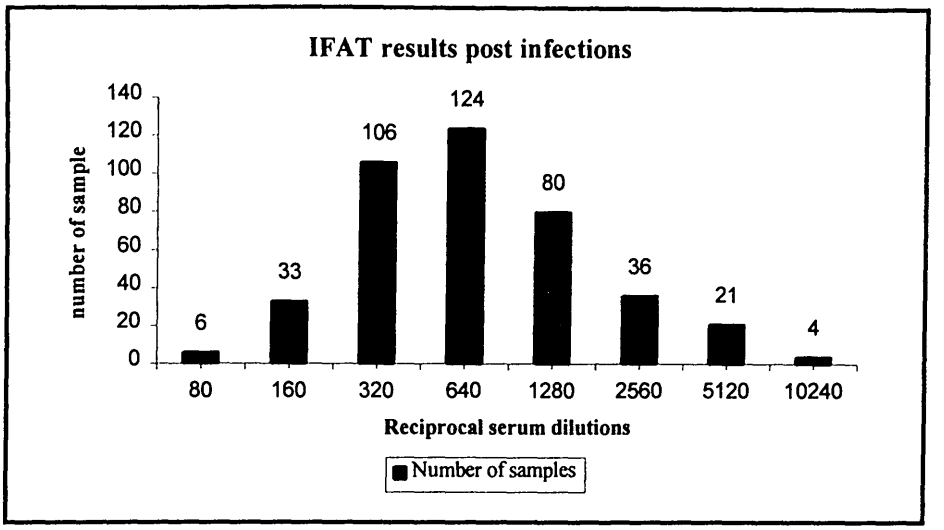


Figure 6.3 IFAT results for samples tested post infection



6.3.2 Post infection titres

During the 19 months post infection the antibody titres were not maintainedand were not constant as shown in Figure 6.6, 6.7 and 6.8. Figure 6.3 shows the distribution of ani-schizont antibody titres in the experomental animals post infection. Table 6.1 shows the sensitivity and specificity of the IFAT test of the post infection results in the experimental animals. Of the 410 occasions when the 15 animals were tested, 6 were positive at 1:80, 53 positive at 1:160, 125 positive at 1:320, and 307 had titres equal to or greater than 1:640. The serological titres post infection are summarised in Figure 6.3, and the sensitivity and specificity of 64.63% and 100% respectively was achieved at a cut-off titre of 1:640 (Table 6.1). Details of indirect fluorescent test results are shown in Appendix V.

The sensitivity of a diagnostic method is the proportion of true positives that are detected by the method; and the specificity of the method is the proportion of true negatives that are detected (Thrusfield, 1995).

Using the IFAT results pre- and post-infection with *T. parva* parasites (Figure 6.2 and 6.3) the sensitivity and specificity of the IFAT were calculated as shown in Table 6.1 (Thrusfield, 1995).

Table 6.1 Sensitivity and specificity of Indirect fluorescent antibody test for *T. parva* macroschizont antibodies to different =/- cut-off titres.

Cut-off titre (+ve)	SENSITIVITY (%)	SPECIFICITY (%)
1:80	100	61.19
1:160	98.54	88.06
1:320	90.49	100
1:640	64.63	100
1:1280	34.39	100
1:2560	14.88	100
1:5120	6.10	100
1:10240	0.98	100

6.3.3 Serological reactions to initial *T. parva* infection

An animal with an antibody titre of ≤ 1:320 was considered to be serologically negative to *T. parva* prior to infection. On day 40 after infection, tick application or field tick exposure all the infected calves were serologically positive.

During the period of 19 months post infection the calves were monitored under tick-proof conditions, the group I calves maintained anti-schizont antibody levels of between 1:160 and 1:5120 as shown in Figure 6.4. The reciprocal serum dilutions for the bovine sera are detailed in Appendix V.

Group II calves infected with *T. parva* Avery maintained anti-schizont antibody titre of between 1:160 and 1:10240 during the 19 months post infection. However, one of the calves (calf 1442) which did not show a *Theileria* clinical reaction had a titre of 1:10240 on day 88 post infection only, and for the rest of the

experiment had a maximum of 1: 640 until challenged. Antibody titres to the schizont antigen for group II calves are shown in Figure 6.5 and details for the reciprocal antibody titres are detailed in Appendix V.

The group III calves had a four-fold increase in antibody titres 40 days post infection and through the 19 months ranged from 1:160 and 1:2560, and calf 1447 had a antibody titre of 5120 on 249 post infection. The antibody titres for the calves are shown in Figure 6.6 and the reciprocal serum dilutions which were positive in the IFAT are detailed in Appendix V.

Calves 1456 and 1437 which had infected ticks applied had antibody levels ranging from 1:80 and 10240 during the period before challenge. Calf 1437 which had a *Theileria* infection from Chikeya farm ticks had a four-fold rise in antibody titre 40 days post infection (Figure 6.7).

The calf (1372) exposed at Hunyani maintained a range of 1:320 to 1:10240 through the 22 months post infection. A four-fold rise in antibodies to *T. parva* macroschizont antigen at 40 days post exposure was noted, and the titres were below the cut-off point on some occasions tested (Figure 6.8) The second group of calves exposed at Hunyani estates which also had buparvaquone treatment had titres ranging between 1:320 and 1:2560. The serological response is shown in Figure 6.9 and details of the reciprocal serum dilutions are in Appendix V.

6.3.4 Serological reaction to *T. parva* Avery challenge

Seven animals with pre-challenge antibody titres to *T. parva* Berea schizont antigen of 1:320 and less showed no clinical reaction to *T. parva* Avery challenge although microscopic examination of blood smears showed (Table 3.8) piroplasms of less than

0.1% at least 21 days post challenge. The piroplasms appeared for periods ranging from 1 to 7 consecutive days from day 21 post infection.

All the experimental animals had antibody titres ranging from 1:640 to 1:1280 day 40 post challenge. However one of the group II animals (calf 1442) did not have a theileriosis clinical reaction and the anti-schizont antibody titre remained low despite showing a titre of 1:10240 on day 88 post infection. Also, this animal had a piroplasm parasitaemia of less than 0.1% on day 178 and 193 in the Giemsa stained blood smear which was confirmed by other laboratory personnel. The antibody titre went up to 1:1280 forty days post *T. parva* challenge despite absence of reaction to *T. parva* Avery challenge.

6.3.5 Control calves results

The control calves underwent severe *Theileria* reactions. Calf 4855 was infected with the same volume of *T. parva* Avery stabilate at a later date not on the same day as calf 4853. Calf 4853 had ten days of febrile reaction and 15 days of macroschizont parasitosis, a 4-fold rise in antibodies to macroschizont antigen and only four days of piroplasm parasitaemia from day 25 post challenge.

6.3.6 Relationship of IFAT titre to infectivity to ticks

The differences in serological titres in relation to the ability to infect ticks are shown in Figure 6.10.

There was no correlation (Pearson's correlation) between the antibody titre and the ability of the animal to infect ticks except that the animals which underwent a severe *Theileria* reaction in the Hunyani estates field exposure produced high tick infection prevalences in the adult ticks fed as nymphae on the recovered calves (Figure6.10).

6.4 Discussion

The experimental animals seroconverted by day 40 post infection and the antibody titres were between 1:640 and 1:2560 except for one calf (1442) which only had a titre of 1:320 but was immune on *T. parva* Avery challenge. Antibody titres started to decrease 100 days post infection for most of the animals and even went below the positive cut-off titre of 1:640 in the Boleni infected calves. The duration of antibody responses to *Theileria* varies considerably and is dependent on numerous factors such as variations in individual animals, and the carrier status (low levels of schizont parasitosis). In the absence of re-challenge, schizont IFA antibodies have been known to decline by six months after infection (Burridge and Kimber, 1973). However, development of the carrier state may cause antibody levels to be maintained for longer.

The examination of fluorescent stained cells in the IFAT make it a very subjective test and in some cases very difficult to interpret. However in this controlled experiment it was easy to do several repeats in order to confirm a sudden lowering of titres and to confirm non-specific fluorescence. In this study antibodies to macroschizonts started to wane at about 4 months post infection in all the groups of experimental animals but the animals were still immune to challenge.

Calf W70 although exhibiting a negative antibody titre (1:320) was immune to *T. parva* Avery at the time of challenge. Thus from the validation results, a 100% sensitivity and 90.49% specificity of the IFAT at a cut-off titre of 1:320 would detect this serum sample as from an animal previously exposed to *T. parva*.

The anti-macroschizont antibody titres which were being exhibited at the time of tick application do not have a positive correlation with the infection rate detected in the adult ticks. However the Hunyani (Figure 6.7 and 6.8) exposed animals which underwent a severe *Theileria* clinical reaction achieved higher infection rates which could have been a result of the *T. taurotagi* mixed infection with the *T. parva* group. It has been reported that *T. parva* antisera cross-react strongly in the IFAT with *T. taurotragi,* with the latter antisera having low titres to *T. parva* schizont antigens (De Vos and Roos, 1981; Uilenberg *et al.*, 1982), and in this study the IFAT titres were equal to or greater than 1:640 supporting the presence of *T. parva* infection. The use of a cut-off titre of 1:640 generally used at VRL-Harare has difficulties in the interpretation of results. The optimum cut-off for both sensitivity and specificity was at 1:320, where sensitivity and specificity of 90 and 100% respectively were found. At 1:640, the sensitivity dropped to 64.63%, but the specificity rose to 100%. Baseline titres for *T. parva* schizont antigen are commonly taken at 1:40, in our study the baseline for the IFA test was 1 in 320 and titres of 1 in 640 or more were considered to be positive. While this increases test specificity [minimises false positive results], it decreases test sensitivity [increases the proportion of low antibody titres] (Norval *et al.*, 1992). Thus for live animal exports in Zimbabwe a titre of 1:160 would increase the sensitivity to 100% and as for epidemiological surveys for the determination of presence of infection, a cut-off of 1:640 would be adequate because of the high specificity, and therefore false positives would be rare. But a high number of false negatives should not be expected from a *T. parva* endemic areas.

In this case the severity of the initial reaction to *T. parva* infection was not reflected in the anti-schizont antibody levels as demonstrated with the Hunyani second exposure calves. The lack of correlation between the antibody titres and the ability to infect ticks shows that the IFAT cannot be used as a tool for the identification of *T. parva* carriers. Antibody titres have been known to fall to insignificant levels in animals capable of transmitting infection to ticks (Mackenzie and Lawrence, 1979).

The fluctuating antibody titres to schizont antigen have been reported before (Mackenzie and Lawrence, 1979), and have been linked with the presence of piroplasms as these are a result merozoites from schizont infected cells.

Direct and indirect fluorescent antibody techniques can also be used to detect piroplasms and schizonts of *Theileria* in blood smears and tissue sections (Morrison *et al.*, 1981). It is likely that a new generation of tests for both antibody and antigen detection will soon be available for the *theilerial* diseases. Katende *et al.*, (1990) describes an ELISA test for *T.mutans*. Recent work in collaboration with ILRI workers has shown the IFA test using the *T. parva* Berea schizont antigen to be as sensitive as the ELISA test for *T. parva* when testing experimental animals in Zimbabwe preinfection and 40 days post infection only (Katende, J. personal communication).

Figure 6.4 *T. parva* anti-schizont antibody response to *T. parva* Boleni infection

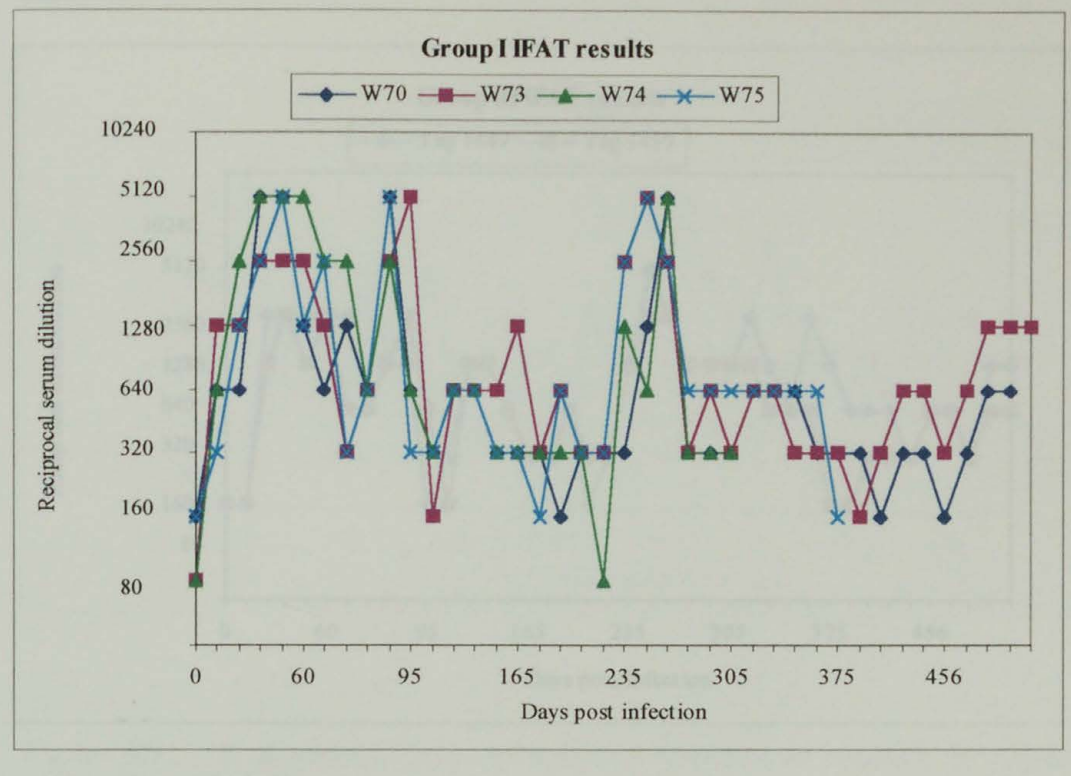


Figure 6.5 *T. parva* anti-schizont antibody response to *T. parva* Avery infection

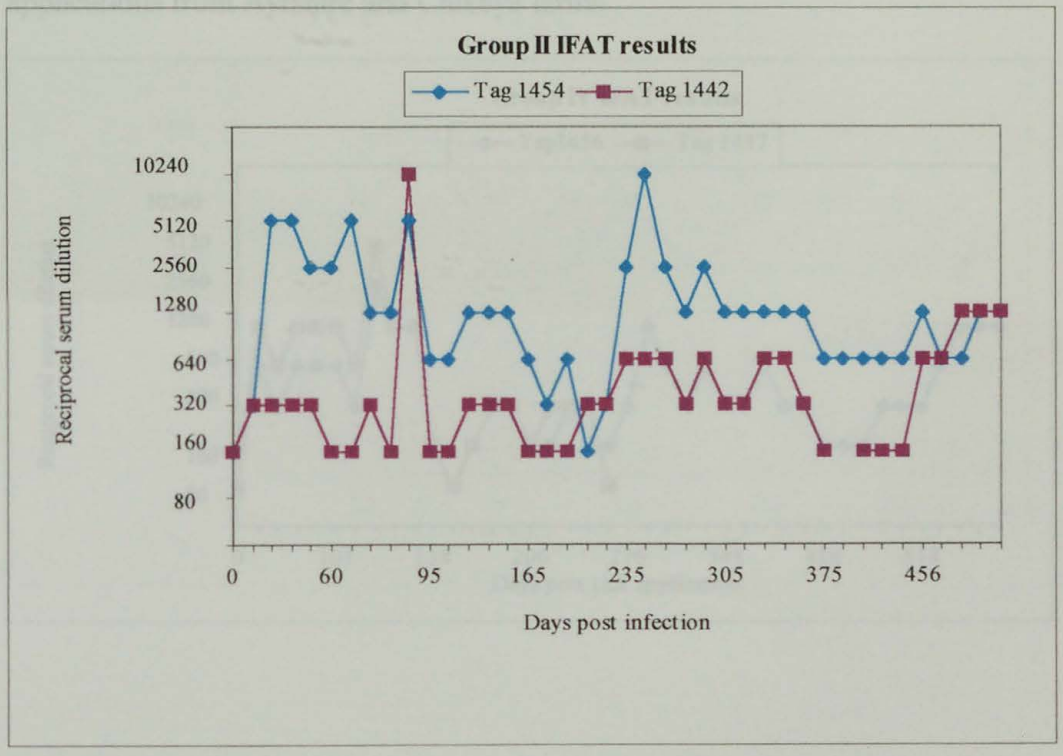


Figure 6.6 *T. parva* anti-schizont antibody response to *T. parva* Bally Vaughan infection

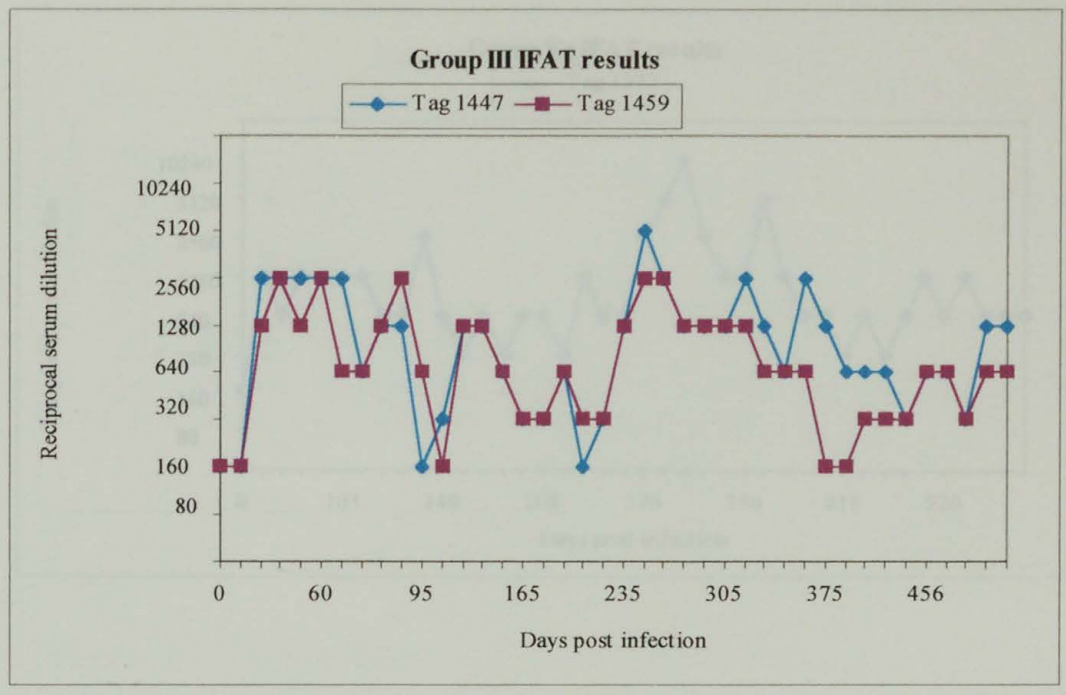


Figure 6.7 *T. parva* anti-schizont antibody response to infection by infected tick applications from Ayrshire and Chikeya farms

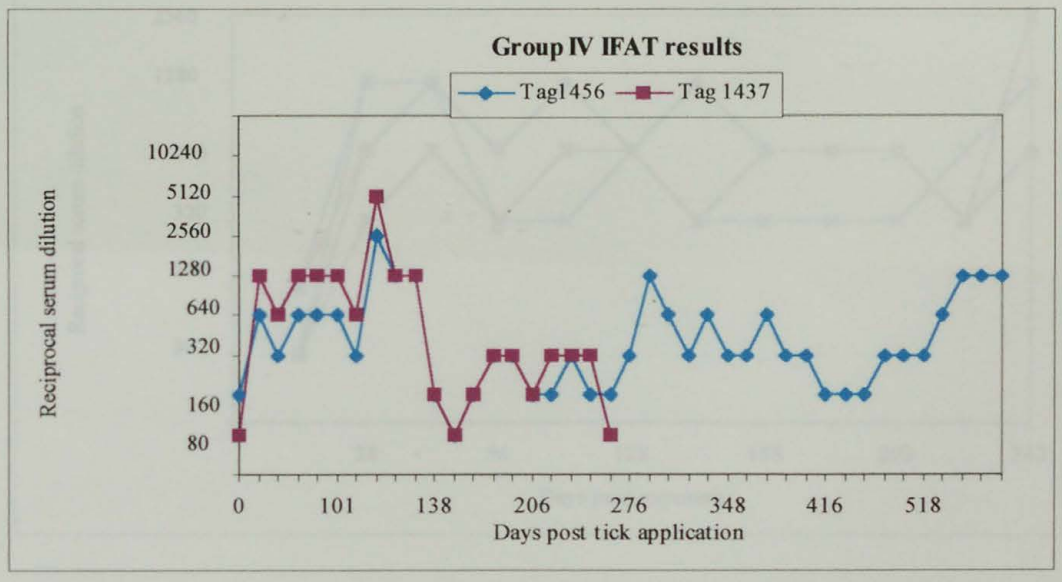


Figure 6.8 *T. parva* anti-shizont antibody response to infection by exposure at Hunyani Estates. (1st)

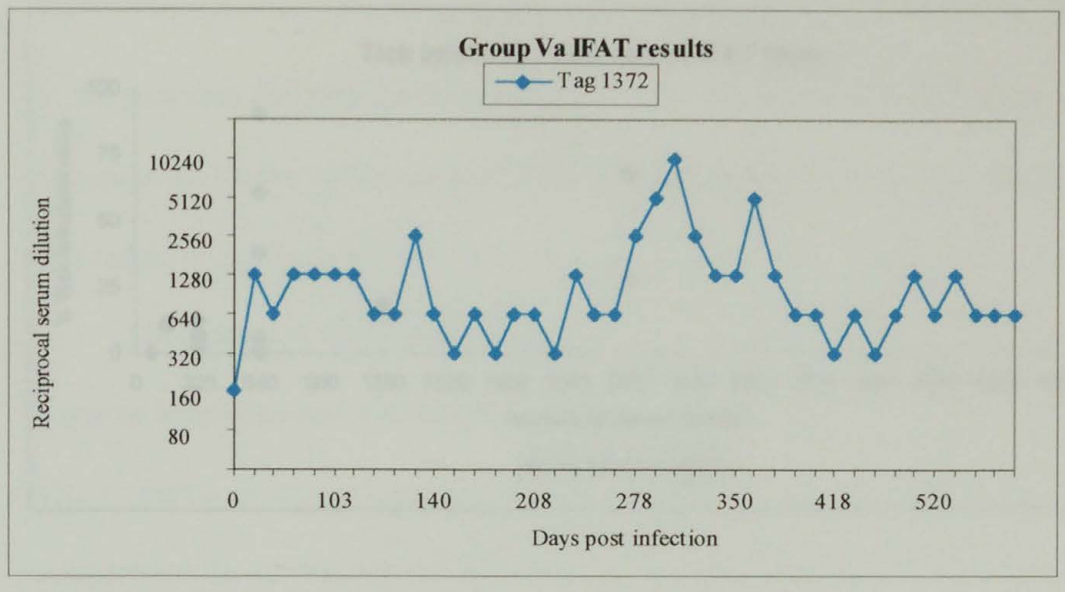


Figure 6.9 *T. parva* anti-schizont antibody response to infection by exposure at Hunyani Estates. (2nd)

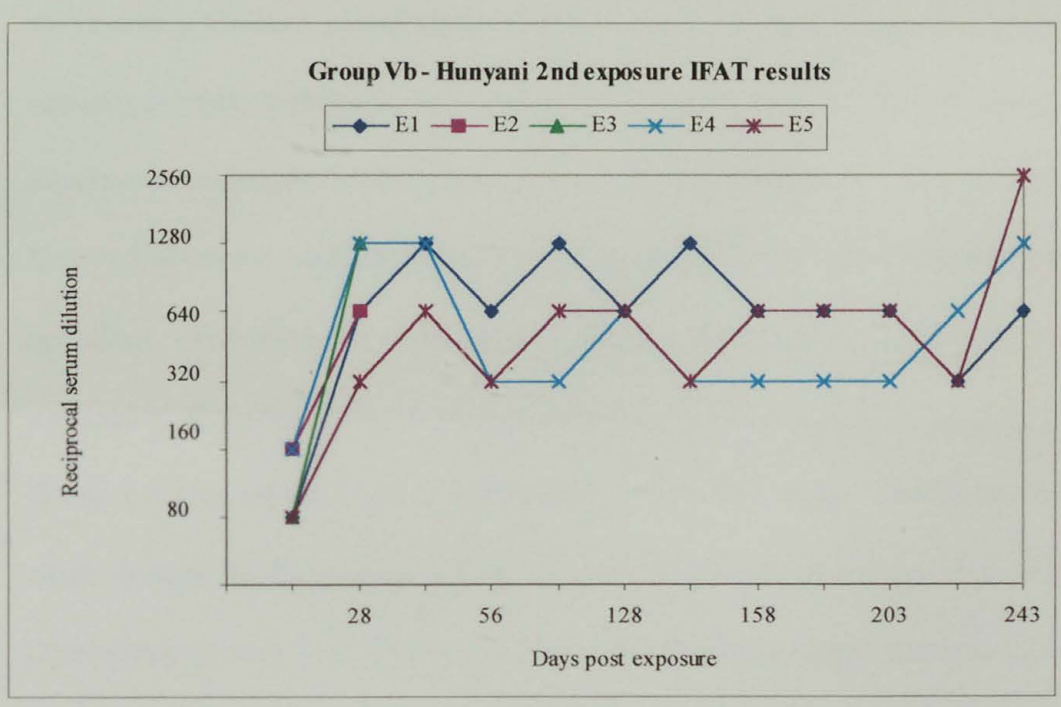
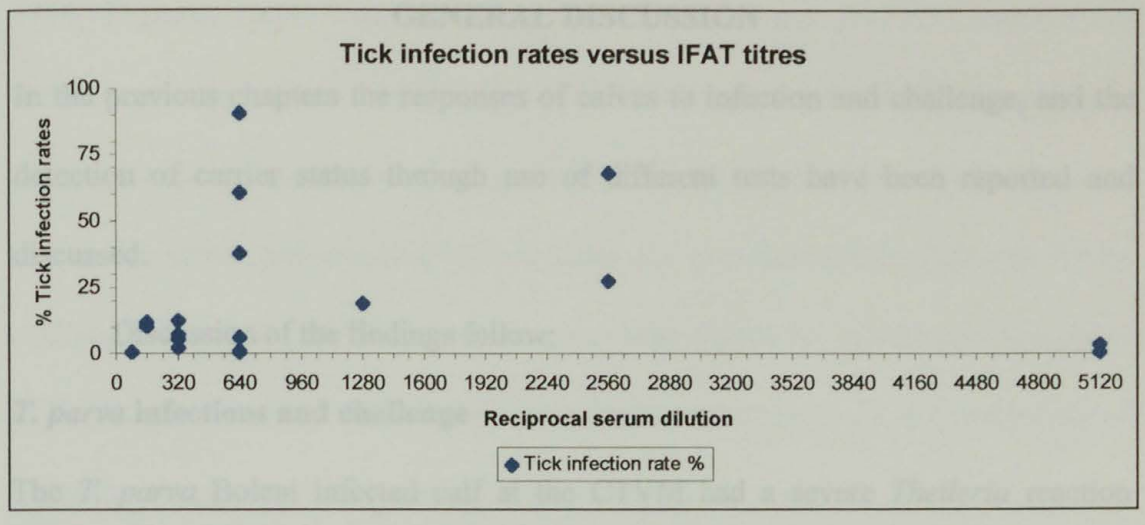


Figure 6.10: Relationship between tick infection rates and *T. parva* anti-schizont antibody titres



CHAPTER SEVEN

GENERAL DISCUSSION

In the previous chapters the responses of calves to infection and challenge, and the detection of carrier status through use of different tests have been reported and discussed.

Discussion of the findings follow:

T. parva infections and challenge

The *T. parva* Boleni infected calf at the CTVM had a severe *Theileria* reaction compared to the calves infected with the same parasite stock at VRL-Harare and was immune to *T. parva* Muguga (Kenya) sporozoite challenge. A mild clinical reaction ensued (Table 3.6), accompanied by a prolonged low level schizont parasitosis and a maximum piroplasm parasitaemia of 0.6%. The animals in groups infected with ground up tick stabilates, tick applications from Ayrshire and Chikeya farms developed moderate *Theileria* reactions, while calves exposed to ticks at Hunyani Estates had severe reactions and five animals were lost due to theileriosis on the two occasions. However the experimental animals (groups I-V; Table 3.8), were all immune to *T. parva* Avery challenge, irrespective of the initial severity of disease. Group I calves infected with *T. parva* Boleni showed a marked difference from the other groups in the number of days when piroplasms were detected under the microscope at very low levels of less than 0.1% continuously between 22 and 35 days from day 13 post primary infection.

Although one calf (1442-group II) which was inoculated with *T. parva* Avery stabilate did not show parasites during primary infection, it was *T. parva* positive

usisng the p104 PCR assay during primary infection and post infection. Also calf 1459, *T. parva* Bally Vaughan infected did not show any *Theileria* reaction but evidence of infection was confirmed by p104 PCR during primary infection and later. Although these calves did not show any *Theileria* reaction, calf 1456 had a four-fold rise in the serum anti-schizont antibody titre and was immune to *T. parva* Avery challenge. Since the use of live parasites has been successful in immunising against ECF, the establishment of infection is a basic requirement for the induction of protective responses (McKeever and Morrison, 1990) and their studies have also revealed that protection is mediated by parasite specific cytotoxic T cells which are restricted by class I major histocompatibility complex (MHC) products (Morrison *et al.*, 1995).

Following challenge with *T. parva* Avery the six groups were solidly immune 522 days post infection, 566 days post tick application, 563 and 182 days post exposure following the second Hunyani exposure. The *T. parva* Boleni infected calf at the CTVM was immune to challenge with Muguga challenge 63 days post infection, and two *T. parva* (Boleni) recovered animals at VRL-Harare were immune to heterologous challenge 522 days post infection, irrespective of the presence of initial macroschizont infection. However, considerable piroplasm parasitaemias were evident in the animals after challenge with *T. parva* Avery, an indication that the challenge parasites were not completely blocked and there was a variation between individual animals.

Although the Hunyani exposure calves underwent a severe clinical reaction two of the calves (E4 and E5) developed macroschizonts on challenge with *T. parva*

Avery and this was not evident in the other groups infected with laboratory ground-up-tick stabilates. It is considered that animals which recover spontaneously from the disease are solidly immune to homologous challenge, and it has been shown that this protection lasts for up to 3.5 years in the absence of challenge (Burridge *et al.*, 1972). In our study each group of animals irrespective of immunising stock, was protected against *T. parva* Avery, and the reactions to challenge were not related to the severity of initial reactions to infection. This finding is in agreement with Barnett (1957) and Burridge *et al* (1972). The severity of *T. parva* Avery challenge was demonstrated in the control calves 4853 and 4855, in which the later died 30 days post infection. The Avery stabilate has been known to be pathogenic even when used at low doses (0.01 ml equivalent to 0.1 tick), (Koch, 1990).

The carrier state of these animals was assessed using Giemsa-stained blood smears to detect the presence of piroplasms; PCR and Dot-blot hybridisation to detect the presence of *Theileria parva* parasite DNA in any form (macroschizonts, microschizonts or piroplasm); and the feeding of *R. appendiculatus* nymphs to pick up infection. The establishment of infection in the experimental animals was confirmed by the schizonts seen in the lymph node biopsy smears post infection. Piroplasms were detected intermittently in all the calves during the period when they showed clinical reactions and post recovery in Giemsa-stained blood smears.

Detection of carrier state by PCR

The sensitivity and specificity of the PCR was found to be high post-recovery in comparison with the detection of piroplasms in Giemsa-stained blood smears. Of the samples tested by PCR post infection 42.42% and 62.66% were positive in the PBM

phenol / chloroformed and EDTA blood saponin lysed extracts respectively; in comparison 32.39% of blood smears were positive for *Theileria* piroplasms and 53% of serum samples were considered to be positive at a titre of 1:640 (summary of results shown in Appendix IV). In the samples from group I calves there was moderate agreement (P = 0.0001) using the Kappa statistic between the PCR results on the phenol chloroformed PBM and EDTA saponin lysed extracts. However, the PCR results on EDTA saponin lysed extracts were not in agreement with the blood smear microscopic examinations for the piroplasms as shown in summary of results in Appendix IV. In comparisons of PCR on EDTA saponin lysed extracts and blood smears, 82% were not in agreement with more positive results in the PCR assays.

The mode of action of buparvaquone as described by McHardy (1988) on *T. parva* schizonts in cultured lymphoid cells demonstrated progressive vacuolation of the cytoplasm as the principal lesion. This would explain why the development of piroplasms in the treated calves undergoing a theileriosis reaction was not affected, and it was evident in this study that the piroplasm parasitaemias were persistent in the butalex treated calves.. Also studies by Dolan (1986a, 1986b) demonstrated a high prevalence of carriers in cattle recovered after treatment with parvaquone (Clexon, Pittman-Moore). The PCR assay was able to demonstrate persistent carrier state after infection and recovery in all the different modes of infections even in calves which did not show any *Theileria* clinical reactions.

However, because of the expense and technical expertise involved with the PCR assays, the use of PCR can be used to confirm the presence of *T. parva* infection after the examination of Giemsa stained blood smears and the IFAT as

these remain less expensive tests. As for cases involving import and exports of live animals the PCR could be applied in order to avoid the spread of pathogenic strains. There must be a definite risk associated with the introduction of cattle infected / recovered from theileriosis strains such as were present on the Hunyani estate, as these could transmit a lethal infection. This kind of situation has been suggested if dipping is relaxed since ticks infected with the parasites from carrier animals can transmit lethal infection (Maritim *et al.*, 1989). The PCR assay could be used in such circumstances to identify pathogenic strains.

Comparison of methods for carrier status detection

No significant difference was found between the preparation of samples positive by PCR (EDTA blood) and positive microscopy. Statistical analysis comparing the PCR positive EDTA bloods and positive microscopy results during the 19 months post-infection showed no significant differences (McNemar test for correlated proportions for individuals sampled twice). However, analysis of the pooled results showed a significant difference ($P = 0.01$) at one degree, of freedom with the PCR being more sensitive. A comparison between PCR results on phenol / chloroformed peripheral blood mononuclear cell extracts and Giemsa-stained blood smears showed no statistically significant difference (calves infected with ground-up-tick stabilates).

Although the IFAT used in our study only detects anti-schizont antibodies, it can be used as an indicator of previous infection. When IFAT positive antibody titres (cut-off 1:640) are compared with PCR positive results there was no significant difference between the proportion of positive results with the two tests during the period of 19 months post-infection. However, with the pooled data (for all

infections) there was significant difference (p = 0.05) [McNemar test] with the PCR having a higher proportion of positives. There was no significant difference between PCR and IFAT in the proportion of tests giving a positive result, when IFAT was interpreted with a cut-off of 1:320.

Thus the current cut-off titre of 1:640 for *T. parva* schizont antibodies used at the VRL is recommended in combination with a PCR assay for samples from theileriosis areas in order to ascertain absence of previous infection. For areas which practice strict tick control and known to be free of theileriosis a cut off of 1:320 would be ideal in screening animals for previous infection using the IFAT.

These results have demonstrated that the conventional methods of *Theileria* parasite detection can be used to determine previous exposure to the parasite in combination with the specific PCR. The latter is important in distinguishing *T. parva* from *T. taurotragi* infections. The Indirect Fluorescent Antibody test for anti-schizont antibodies can be used to determine previous exposure to *T. parva* and a comparison with the ELISA test (J. Katende- ILRI, Nairobi, personal communications) has shown a high degree of correlation between the two. The sensitivity and specificity of the p67 and p104 PCR assays was found to be high post recovery compared to the detection of piroplasms in Giemsa-stained blood smears. However the problem arises when there are mixed infections with *T. taurotragi* in field situations.

Detection of carrier state by tick application

In this study 2 *T. parva* (Avery) and 2 buffalo-derived *T. parva* (Bally Vaughan) infected calves failed to transmit *T. parva* to ticks although parasites were detected in

these cattle by PCR amplification. This could be due to failure of the ticks to either pick up the parasite; or alternatively, the parasites may not have been available for pick up at the time of tick application, as intermittent transmission has been demonstrated (Dolan, 1986). However, the Hunyani Estates calf which survived the first exposure was able to infect ticks applied on days 140, 266, 350 and 501 post-exposure. It was also possible to achieve tick infections with Chikeya farm parasite infected calves on days 138, and 264 post tick application and 264, 348 and 499 days for the calf infected with ticks from Ayrshire farms. Out of a total of 42 tick applications (including the group II and III animals) on all the experimental animals 43% were positive post recovery from infection. All the animals infected with field parasites, that is Hunyani Estates, Chikeya and Ayrshire parasites infected the *R. appendiculatus* ticks (12 tick applications) and the Boleni had 6 out of 14 tick applications positive, also post recovery from infection.

To compare PCR assay with the detection of the parasite in the ticks would be difficult because repeated tick feeds in the recovered animals were not very successful due to poor feeding as the cattle became sensitised to *R. appendiculatus*. Acquired resistance to ixodid ticks has been recognised as a possible biological control method (Treger, 1939). The acquired immunity is expressed as a reduction in the number of ticks which attach to the host, a reduced engorgement weight, a reduction of viability and capacity to moult and a reduced egg and larval production, resulting in a significantly reduced tick population (Willadsen, 1980). Ideally to determine if the Boleni-infected cattle were infective to ticks for a long time, they must be left naive to ticks for a longer time after infection with a stabilate before

applying clean nymphs to be dissected at the next instar. As demonstrated on the third and fourth tick feeds efforts to feed nymphal ticks on the animals were not successful hence the negative results in the adult tick dissections of the ticks which fed on the two *T. parva* Boleni infected cattle. In contrast, *Theileria* infections were detected in the calf which recovered from the Ayrshire tick application and the Hunyani-exposed survivor.

Ochanda (1994) considered that there was an overriding factor in "days post-infection" that determines the prevalence of infection in ticks irrespective of the individual bovine host. The possibilities were advanced that the age of the piroplasm could be an important factor in determining infection levels developing in the ticks, or that piroplasms themselves were not infective agent to the tick. However, we noted that carriers following field exposure and infection were infective to the tick 499 days post application of ticks, and 501 days post exposure to field ticks although the latter was considered to be a mixed infection. The *T. parva* Boleni infected animals were to the contrary not infective on / after day 305 post infection. The concern of Zimbabwean farmers that Boleni immunised cattle are carriers was supported, but long term carriage (> 12 months) was not found to occur in the two calves (W70 and W73) and this could be due to the hypersensitive reactions induced by the tick feeds. Although Boleni immunised animals may not have long term infectivity to ticks the carrier status with field *Theileria* parasites could pose a problem if the *Theileria* recovered animals are moved to other properties where the tick vector is present. Parasite survival was explained by Neitz, (1957); transovarial transmission does not occur in *T. parva* and adult ticks cleanse themselves while

feeding, thus it is the recovered animals which will maintain the parasite for onward transmission to the tick vector. It has been demonstrated that *T. parva* can survive for nearly two years under field conditions in Kenya, virtually the same survival period as that of the tick itself (Newson *et al.*, 1984; Young *et al*, 1983), but this period is likely to be shorter at lower, warmer altitudes like in Zimbabwe which has a lower altitude and further emphasises the important role played by ticks in the survival of the parasite. However further studies on the behaviour of *T. parva* Boleni-carrier status are necessary to determine the period when the host is infective to the tick as this plays a major role in maintaining enzootic stability when nymphs feed in the subsequent dry season (Koch *et al.*, 1992). Although there was no correlation between the tick infections and anti-schizont antibody titre, high tick infection rates occurred when ticks were fed on the calves with titres of <1:640, currently considered sero-negative for *T. parva.*

Serology for detection of carrier status

Antibody responses of the studied animals to infection and challenge were monitored by the IFAT using macroschizont antigen as a conventional test. Although the test is considered to be unreliable for the detection of carrier animals, (Mackenzie and Lawrence, 1979) its reliability was enhanced when used in combination with the PCR. The IFAT revealed that all the experimental animals might not maintain the antibody titres achieved at 40 days post infection; in some cases the titres were negative during the period between recovery and challenge. Due to the development of a carrier status the anti-schizont antibody titres were expected to be maintained for longer, however in this study one *T. parva* Boleni recovered and a Hunyani

recovered animal did not show titres indicative of previous exposure to theileriosis and yet were immune to *T. parva* Avery challenge. In 3 of the Boleni immunised animals, *T. parva* Avery, and Bally Vaughan infected animals significant schizont antibody titres were maintained up to approximately 14 weeks. The results on the duration of significant antibody titres in this study are similar to reports by Burridge and Kimber (1972) who found that significant schizont antibody titres in sera from 5 ECF recovered animals were maintained for 14 weeks, and in 3 animals after 26 weeks using the IFAT.

Calf 1442 (group II) did not have a *Theileria* reaction but was solidly immune on challenge possibly due to very low levels of parasites which were not detectable by microscopy. The macroschizont IFAT results showed a relatively similar pattern in all calves except for calf 1442, in Group II infected with *T.parva* Avery.

The severity of the clinical reactions did not match the antibody titres in the different groups although all calves sero-converted after primary infection, exhibiting anti-macroschizont antibodies.

We demonstrated that the application of infected *R. appendiculatus* ticks from Chikeya and Ayrshire farms, and the exposure of susceptible calves to field ticks at Hunyani Estates produced a higher level of infection than the injection of ground-up tick stabilates. Comparison of the theileriosis reactions experienced during the infection, and exposure to tick infections and field exposure, revealed a great variation in the degree of virulence of the various *Theileria* infections studied. One complication of the study was tick toxicosis observed on Hunyani Estates due to the high tick burdens seen subsequently on calves on the second exposure

experiment. The occurrence of *Theileria* parasites which are highly infective to the vector-tick population is a major factor in the spread of pathogenic theileriosis in Zimbabwe as demonstrated by the 5 out of 9 calves lost on exposure at Hunyani Estates. On these farms, the mixed infection of *T. parva* and *T.taurotragi* was highly infective to the laboratory tick colony, unfortunately it was not possible in this study to fully characterise these parasites. Theileriosis cases that differed from the usual ECF in a variety of ways have been encountered before the eradication of East Coast Fever (ECF) from Zimbabwe in 1954. Among the differences noted were the scarcity of schizonts and rarity of intraerythrocytic piroplasms. Outbreaks of theilerosis seen at present in Zimbabwe are caused by *Theileria parva* which is either cattle derived or buffalo derived, and in contrast to ECF cause less severe clinical reactions. *T. taurotragi* has been isolated in Zimbabwe (Lawrence and Mackenzie, 1980; Uilenberg, *et al.*, 1982) and is known to show some cross reaction with the *T. parva* group on the IFAT (de Vos and Roos., 1981; Uilenberg *et al.*, 1982;. Barnet, 1957). On characterisation of *T. parva* isolates from Zimbabwe Koch (1990), three batches of adult ticks had mean numbers of infected acini of 5.3, 24.4 and 5.4 per tick for the batches fed as nymphs on sick animals exposed for two weeks at Willsbridge Farm. In our study mean numbers of infected acini of 5.66, 13.3, 4.7 and 4.32 per tick were observed day 140, 266, 350 and 501 days post exposure at Hunyani Estates when the animal was not undergoing a clinical reaction. Furthermore, high tick infection rates were demonstrated in 3 calves exposed at Hunyani Estates a year later after nymphs had been applied on the recovered calves 137 days post exposure. Koch (1990) attributed the high infection rate of the *R.*

appendiculatus ticks to mixed infection with *T. taurotragi*, a common species in Zimbabwe (Lawrence and Mackenzie, 1980; Uilenberg *et al.*, 1982), which is capable of causing high infection rates in ticks (Young *et al.*, 1980).

In our study the Bally Vaughan tick stabilate produced a moderate *Theileria* reaction. *T. parva* derived from buffalo is highly infective to cattle causing a severe disease with high mortality characterised by low numbers of schizonts infected cells and low piroplasm parasitaemias (Neitz., 1955; 1957; Barnett and Brocklesby ., 1966; Young *et al.*, 1973). In Kenya and Tanzania cattle which were infected with buffalo-derived *T. parva* and which survived and became carriers provided a source of *T. parva* to ticks for subsequent transmission to cattle (Maritim *et al.*, 1989a, b). In contrast, in our study the two calves infected with *T. parva* Bally Vaughan were not able to transmit infection to nymphs of *R. appendiculatus* on all four occasions post recovery.

Although the application of *R. appendiculatus* nymphae to pick up *Theileria* infections and adult tick dissections is very laborious, currently there are no other methods of determining infection-rates in the tick. It is the ideal method for confirming the carrier animals are transmitting viable piroplasm to complete the life cycle of *T. parva* in the ticks, and it also provides a quantitative estimate of infection rate. The polymerase chain reaction is now used for finding the infection prevalence of vectors with pathogens of livestock [Anaplasmosis] and human diseases [Chaga's disease](Stiller, 1990; Steindel, *et al.*, 1993). The Boleni-infected animals appeared to have a relatively low risk of transmitting the disease to ticks due to the very low parasitaemias which they exhibited, the inability to infect ticks 305 days post

infection and the very low tick infection rates achieved on the two occasions the ticks were *Theileria* infected after having fed as nymphs on the recovered animals. Although tick numbers were low due to poor tick feeding and low piroplasms parasitaemias were not observed by microscopic examination of Giemsa-stained blood smears; these factors could be a disadvantage for the Bolvac® vaccine against theileriosis as the presence of carriers is considered to maintain tick infection, which acts to boost vaccinal immunity when the cattle are challenged (Norval *et al.*, 1992; Koch, 1990). However, field parasites which produce severe theileriosis could pose a problem for the cattle farmers and to the Veterinary Department in the event of *Theileria* outbreaks if there is a breakdown in dipping or disease breakthroughs. Thus there is a need to constantly monitor theileriosis outbreaks and more important develop techniques to differentiate between *T. parva* Boleni (Bolvac®) and field infections as available monoclonal antibodies do not distinguish Zimbabwean *T. parva* isolates (Koch *et al.*, 1988).

Bovine *T. taurotragi* has always been described as a mild strain in Southern Africa (Uilenberg *et al.*, 1982). *T. taurotragi* may be to some extent related to *T. parva* considering their partial serological relationship, their transmission by the same vector and the morphological similarity of the macroschizonts. Their differences are immunological (absence of cross-immunity), serological (partial), differences in the infection rate in the vector and in the developmental cycle in the tick (Young *et al.*, 1980), morphological (more rod- and comma-shaped piroplasms in *T parva*) as well as differences in the infectivity for the eland. However, in our study the *T. taurotragi* / ?*T. parva* mixed infection was pathogenic to cattle and

could be maintained in cattle producing high tick infection rates in adult *R. appendiculatus* ticks. This could pose a dangerous situation to farmers who might purchase animals from a property like Hunyani Estates and may not practice strict tick control. The infected acini though were not morphologically different from *T. parva* as described by Young *et al* (1980), were similar to the results of Fawcett *et al.* (1985) in the estimations of the number of sporozoites suggesting *T. parva* infection in the ticks. Further characterisation of the *Theileria* parasite at Hunyani Estates is of interest in order to investigate the possibility that cattle-pathogenic *T. taurotragi,* parasites capable of onward transmission in cattle exist on this Estate.

It was not possible to conclusively show that the Hunyani theileriosis was *T. taurotragi* or *T. parva* on parasitological, clinical and serological grounds. Although the monoclonal antibody profile showed a *T. taurotragi* profile, the recovered animals were positive on the *T. parva* PCR assay which is specific for *T parva* and does not amplify *T. taurotragi.* A tick stabilate prepared from ticks fed on animals recovered from the second Hunyani exposure was negative on the PCR. Since *T. parva* and *T. taurotragi* are transmitted by the same vector and presence of wild ungulates at Hunyani, a mixed infection cannot be discounted. *R. appendiculatus* adults fed as nymphs on eland (*Taurotragus oryx*) can achieve 100% infection rates (Young *et al.*, 1980), and in tick batches fed on *T. parva* carrier buffalo can vary from 10% to 70% (Grootenhuis *et al.*, (1987). In this study high infection rates (12.39% to 90.44%) were observed 137 days post exposure at Hunyani Estates.

The possibility of detection and subsequent removal, or restriction of movement, of carrier animals in the hope of eradicating theileriosis in Zimbabwe, as originally

suggested by Mackenzie and Lawrence (1979), has proved to be an impossible goal to achieve, thus the Zimbabwe Veterinary Department has taken up immunisation using the live Bolvac® vaccine in order to control theileriosis on endemic properties.

Conclusions

1. The indirect fluorescent antibody test should be retained, in combination with PCR; at a titre of 1:640 the IFAT is 100% specific, but with a sensitivity of 64.63%. Therefore the specific PCR should be used on IFAT negative animals from endemic areas.

2. Animals exposed to *T. parva* infection remained carriers for duration of the experiment, irrespective of initial severity.

3. Cattle immunised with the Boleni strain were shown to carry infection to 493 days post infection by PCR assay using p104 primers, but *Theileria* transmission to ticks was at extremely low frequency. The study suggests Bolvac® immunised calves can be considered to carry infection transmissible to vector ticks up to 218 post infection in the absence of challenge.

4. The carrier status for pathogenic *T. parva,* in Bolvac® immunised animals after virulent challenge was not determined, and therefore the risk of transmission by immunised, field exposed cattle must be considered.

5. A highly pathogenic *T. parva* infection was present at one property, which resulted in high transmission rate to ticks. *R. appendiculatus* nymphs fed on recovered animal kept under tick-free conditions were infected at 500 days after the bovine host had been infected and recovered and had high tick infection rates when

dissected as adults. Such a parasite stock has a high potential to spread to susceptible animals and presents a considerable risk to Zimbabwean livestock industry.

6. Animals were protected against virulent challenge after 522 days, despite the absence of re-infection from ticks and irrespective of whether a primary "reaction" occurred to infection. This indicates that of post vaccinal reactions are not reliable evidence of vaccine infection failure.

7. Current monitoring of post-vaccinal responses by IFAT 40 days post immunisation should be maintained as was observed in the *T. parva* Boleni infected calves. From the results of this study, 100% sensitivity in the detection of infection in Boleni immunised animals occurred when an IFAT was used at a cut-off titre of 1:640, between day 40 and 67 post infection.

8. The sensitivity and specificity of the p67 and p104 PCR assays was found to be high post recovery compared to the detection of piroplasms in Giemsa-stained blood smears.

Summary of features of the methods of *T. parva*-carrier detection

METHOD	TIME	LABOUR	EXPENSE	SPECIFICITY	SENSITIVITY
Blood smear	1 hour	-	-	-	-
Tissue culture	6 weeks	+	+	+(MAbs)	-
Tick transmission	8 weeks	++	++	(+)*	+
PCR	24 hours	+/-	+	+	+

* would require to follow up with tissue culture and Mab phenotyping from cattle infected by ticks.

The above table partly shows that currently there is no ideal method for the detection of carrier state in *T. parva* infected animals and thus more research is required in this area.

The order of the day for the farmers in the beef industry in Zimbabwe was summarised by Matson, (1967) as "NEVER BUY BREEDING STOCK FROM A THEILERIOSIS PROPERTY", in order to control the spread of pathogenic strains of theileriosis, because of the variations noted in the pathogenesis of the disease.

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APPENDIX I

SOLUTIONS AND BUFFERS FOR PCR AND BLOT HYBRIDISATION

1. Tris-Acetate-EDTA (TAE) buffer kept as a concentrate stock

   242 g Tris base

   57.1 ml Glacial acetic acid

   100 ml 0.5 M EDTA (pH 8.0)

   Distilled water to 1 litre

2. 10X DNA Loading Buffer:

   20 mM EDTA (EDTA)

   25% Ficoll (type 400) (Pharmacia)

   0.2% Bromophenol blue (Sigma)

3. Ethidium bromide (EtBr) stock 5 mg/ml in water. Care: EtBr is a powerful mutagen. Handle with gloves and take care in disposal of contaminated solutions.

4. 5XTBE:

   54 g Tris base (Sigma)

   27.5 g Boric acid

   20 ml 50mM EDTA (pH 8.0) per litre

5. 20X SSC (Standard Saline Citrate)

   174 g / l NaCl (Sigma)

   88.2 g / l sodium citrate; pH 7.0

6. Saponin Lysis Buffer

   0.22% Sodium chloride (Sigma)

   0.015% Saponin (Sigma)

   Distilled water up to 100 ml

7. Tris EDTA pH 7.6 (TE)

   10 mM Tris-HCL

   1.0 mM EDTA

8. Dot Blot Hybridisation

a) Alkali Denaturing Solution — per 2 litres

1.5 M	Sodium Chloride (Sigma)	175.3 g
0.5 M	Sodium Hydroxide (Merck)	40.0 g

Neutralising Solution (pH 8.0 — per 2 litres

1 M	Tris Base	242.2 g
1.5 M	Sodium chloride	175.3 g

Solution made up to final volume after adjusting to pH with concentrated HCL.

20 X SSC pH 7.0 — per litre

	Sodium chloride	175.2 g
	Sodium citrate (Sigma)	88.2 g

pH adjusted to 7.0 with 10 N sodium hydroxide before solution made up to final volume

b) Hybridisation

Hybridisation buffer

5xSSC	
5%	Blocking reagent (Amersham)
0.1%	N-lauroylsarcosine (Sigma)
0.02%	SDS
50%	Formamide (Pharmacia)

Formamide added after the solution has been dissolved at 50°C. The buffer is stored at -20°C.

APPENDIX II

Figure (a): Febrile reaction to *T. parva* Boleni primary infection

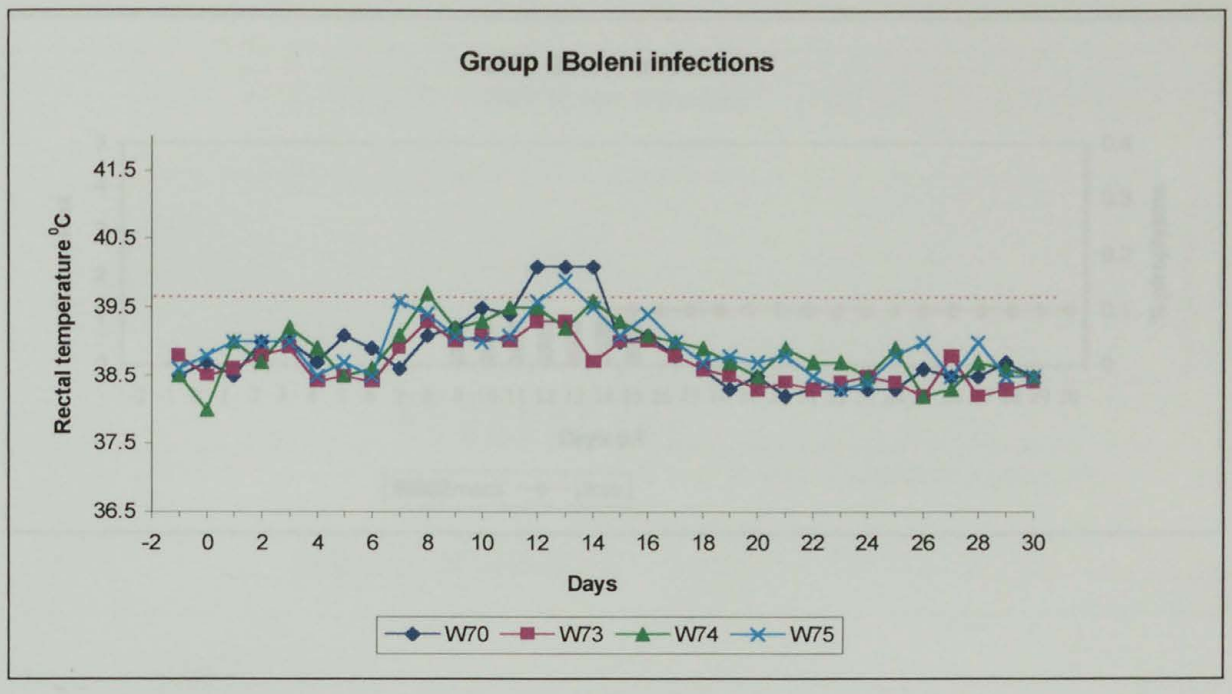
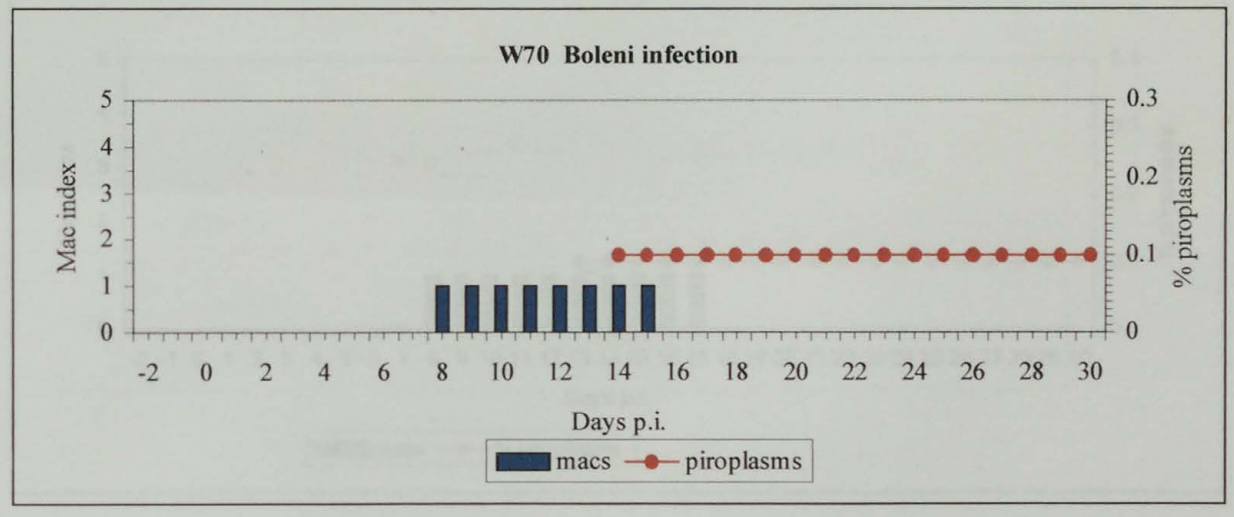


Figure (b): Parasitic reaction to *T. parva* Boleni primary infection



APPENDIX II

Figure (c): Parasitic reaction to *T. parva* Boleni primary infection

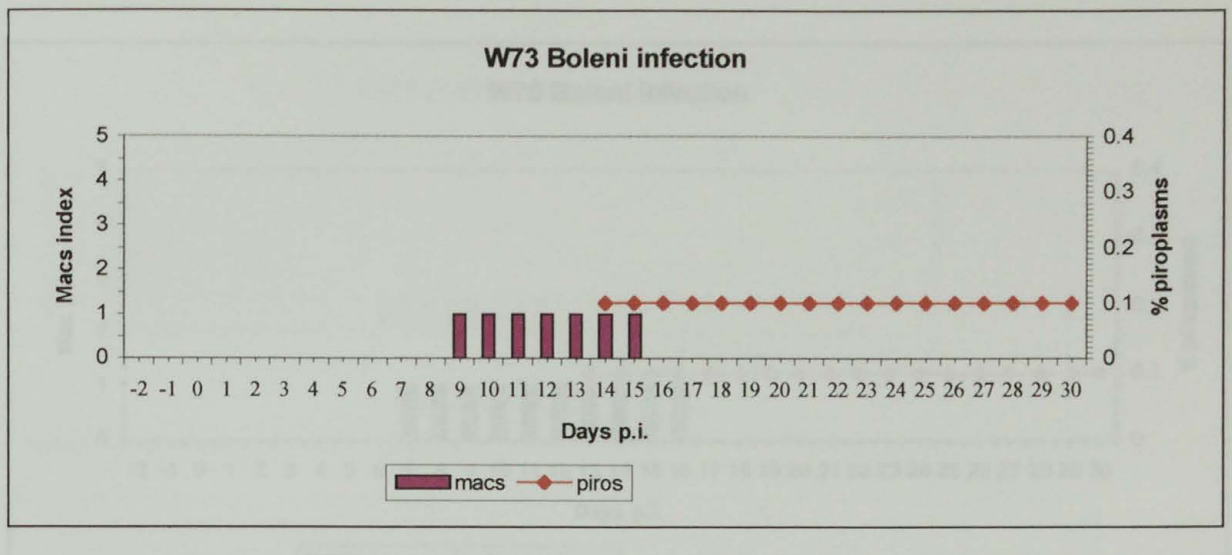
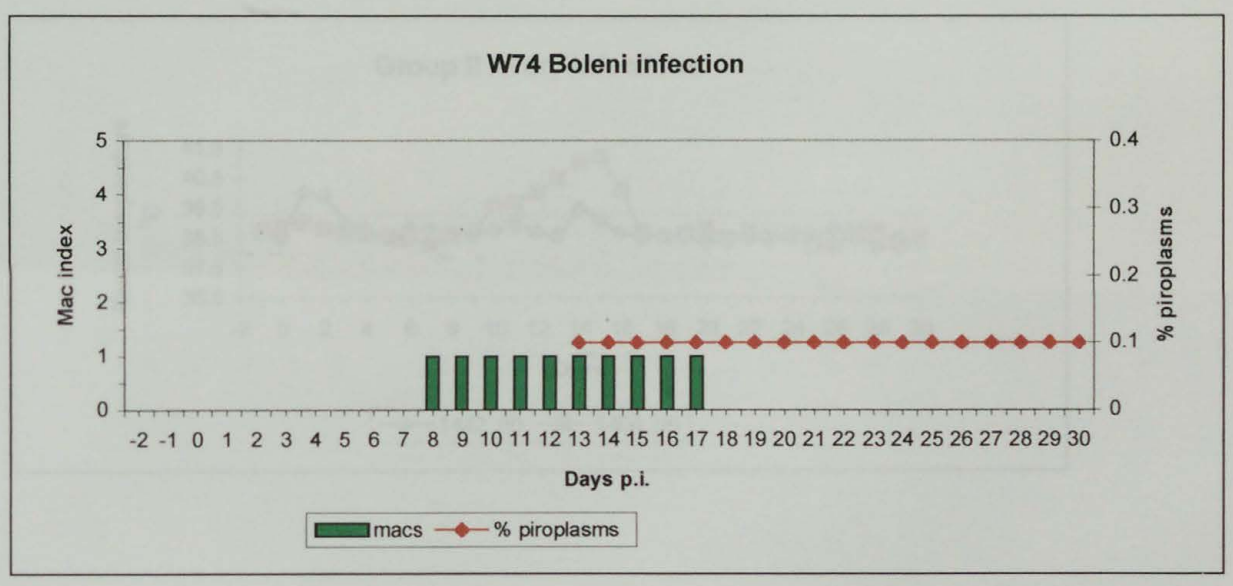


Figure (d): Parasitic reaction to *T. parva* Boleni primary infection



APPENDIX II

Figure (e): Parasitic reaction to *T. Parva* Boleni primary infection

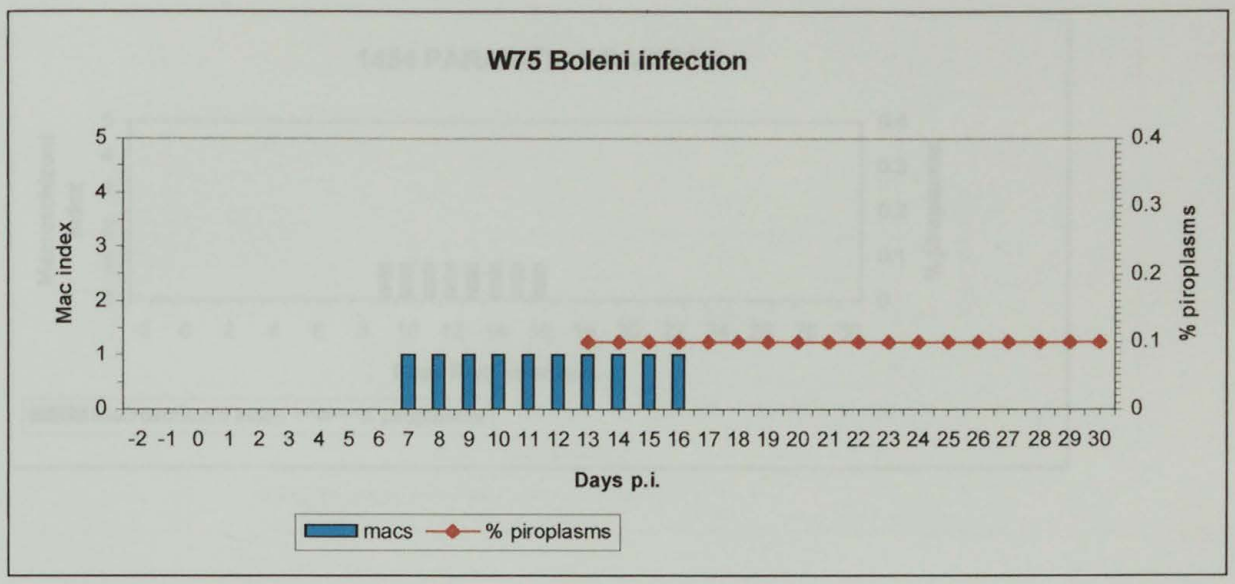
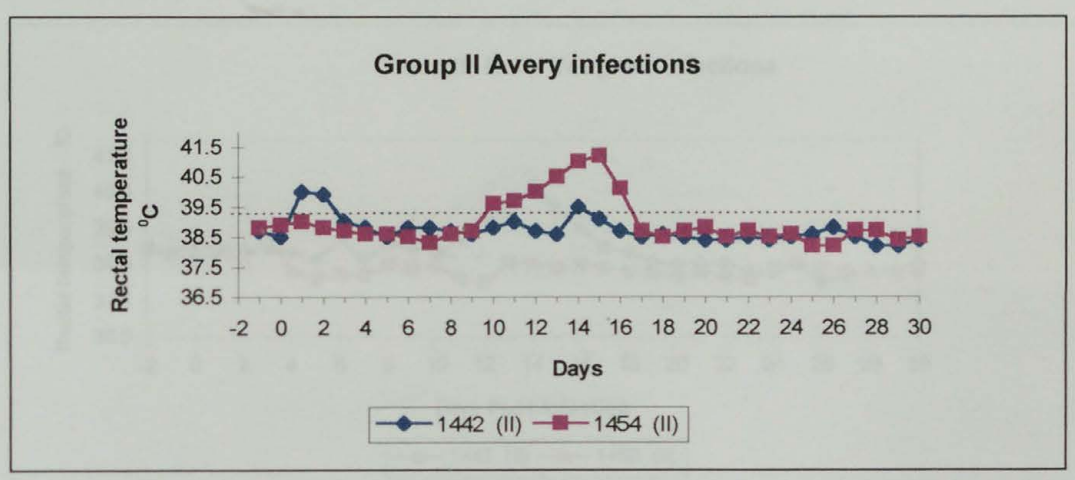


Figure (f): Group II Febrile reaction to *T. parva* Avery primary infection



APPENDIX II

Figure (g): Parasitic reaction to *T. parva* Avery primary infection

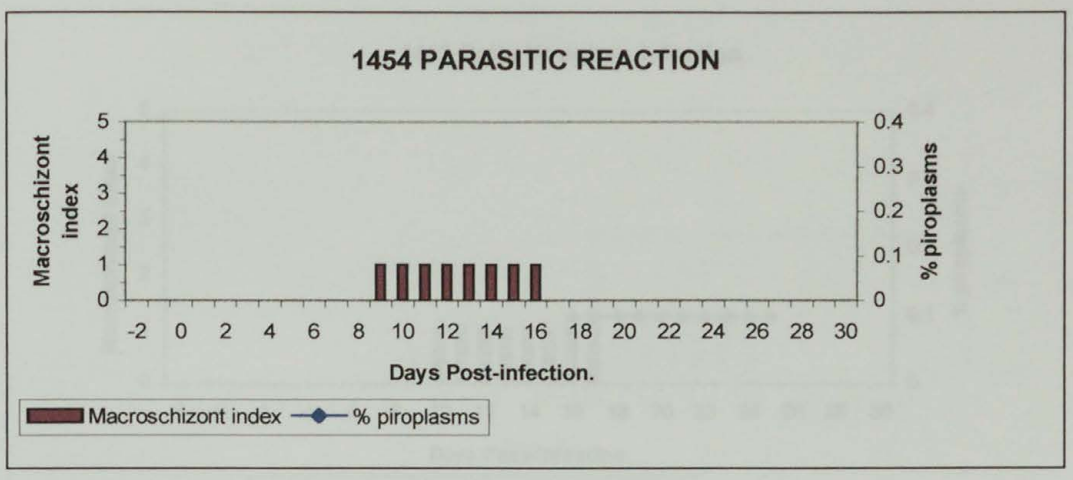
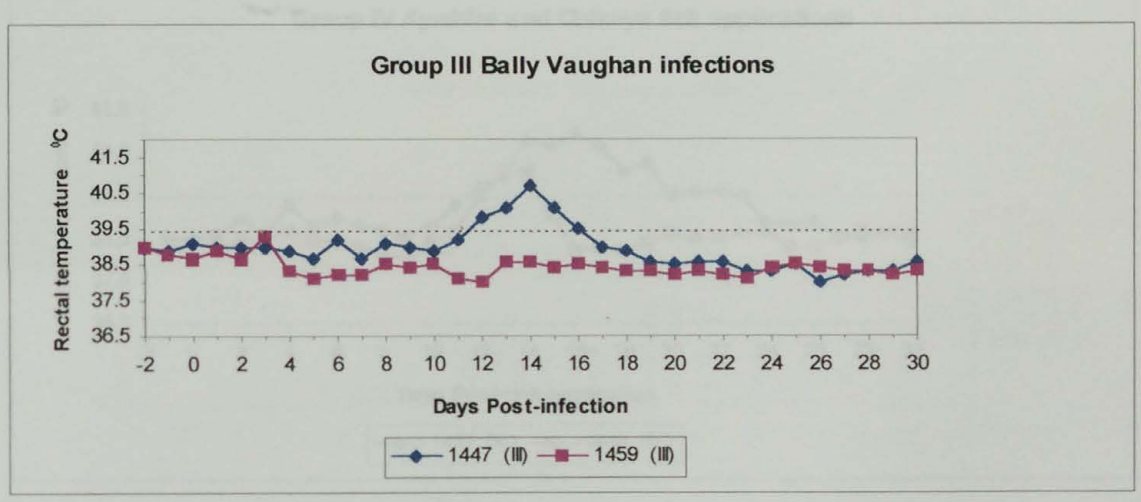


Figure (h): Group III Febrile reaction to *T. parva* primary infection



APPENDIX II

Figure (i): Parasitic reaction to *T. parva* primary infection

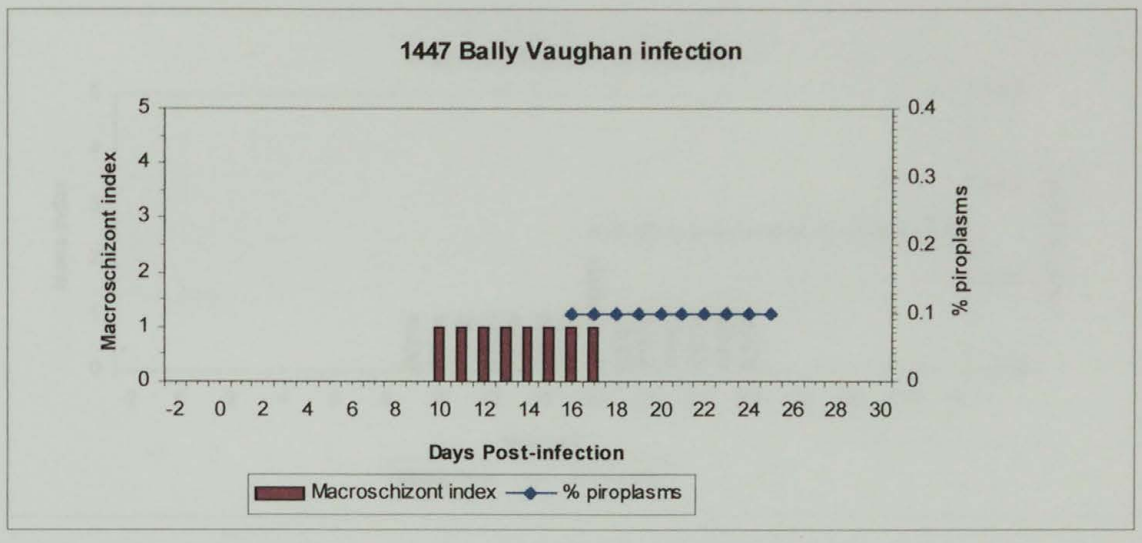
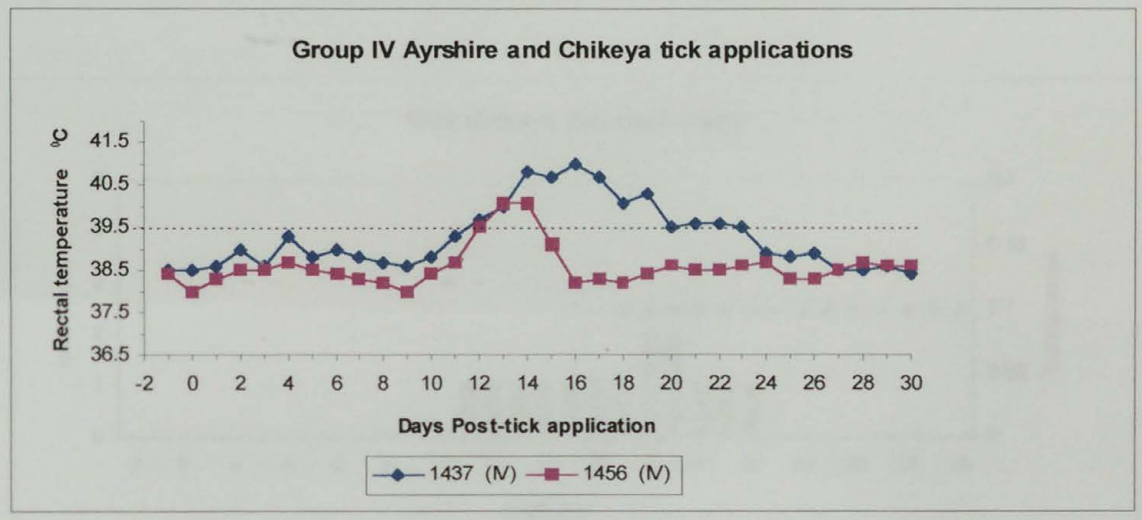


Figure (j): Group IV Febrile reaction to *Theileria* infected ticks application



APPENDIX II

Figure (k): Group IV Parasitic reaction to infection by ticks

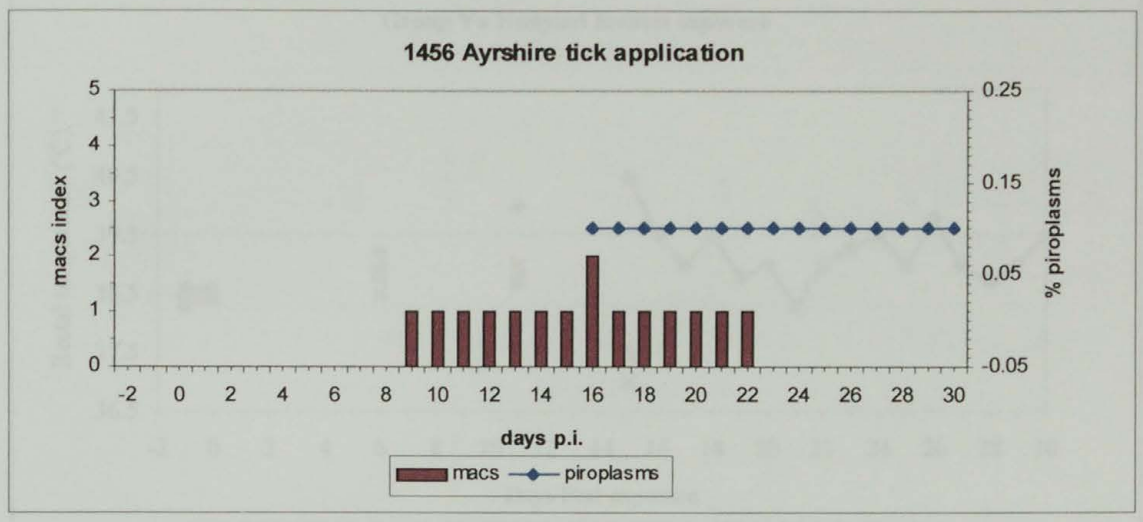
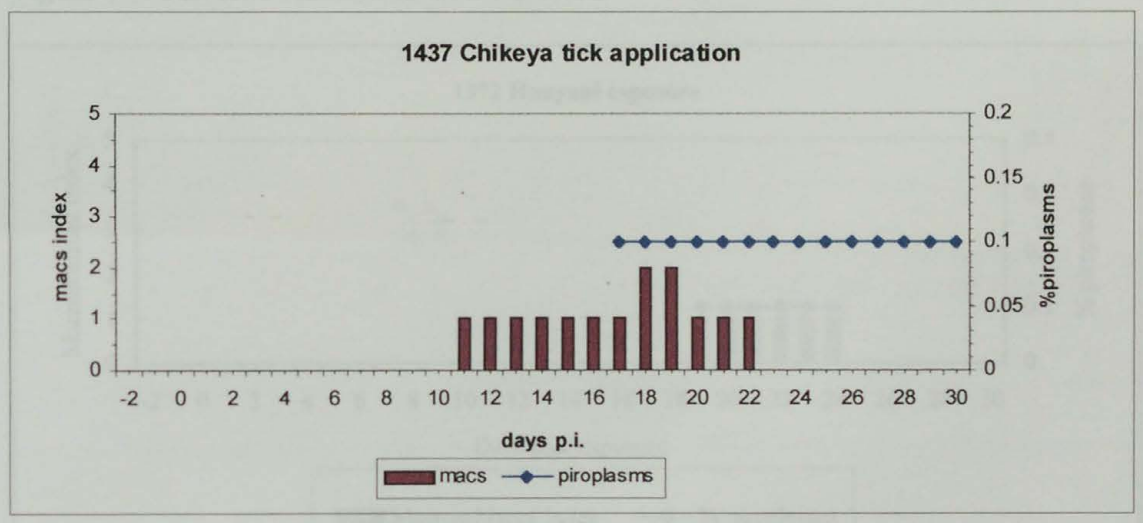


Figure (l): Group IV Parasitic reaction to infection by ticks



APPENDIX II

Figure (m): Febrile reaction to tick exposure

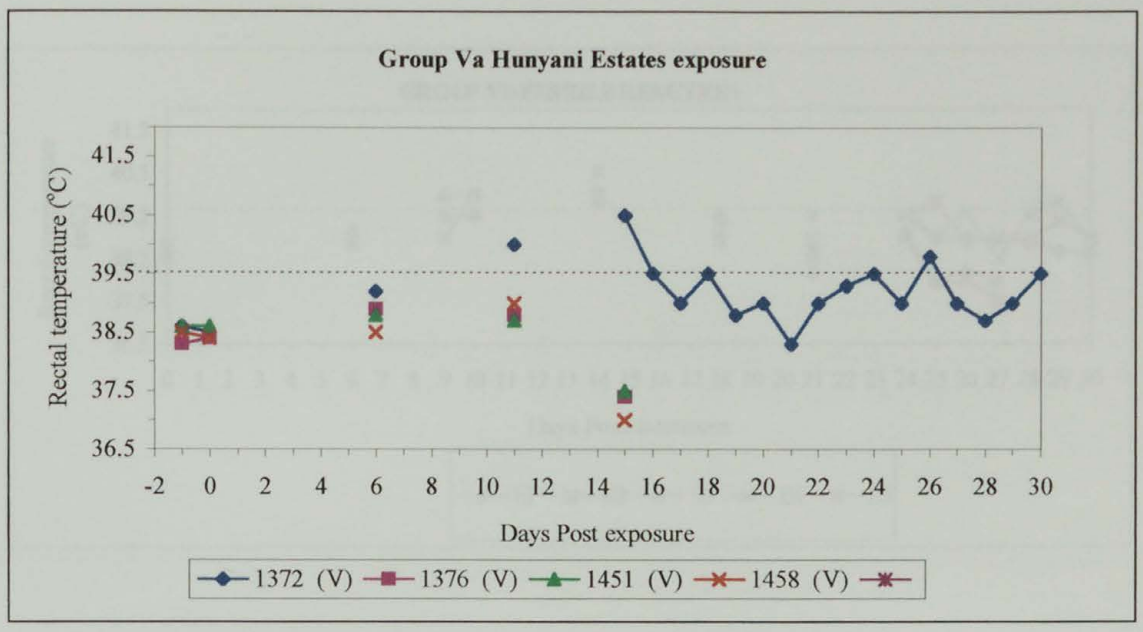
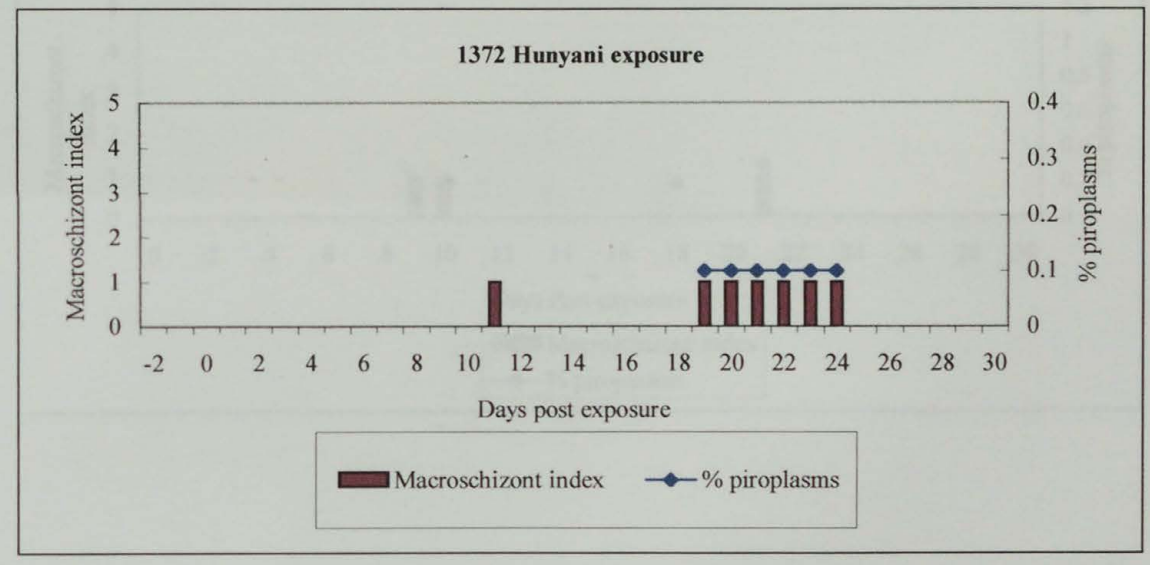


Figure (n): Parasitic reaction to tick exposure



APPENDIX II

Figure (o): Febrile reaction to tick exposure (2^{nd})

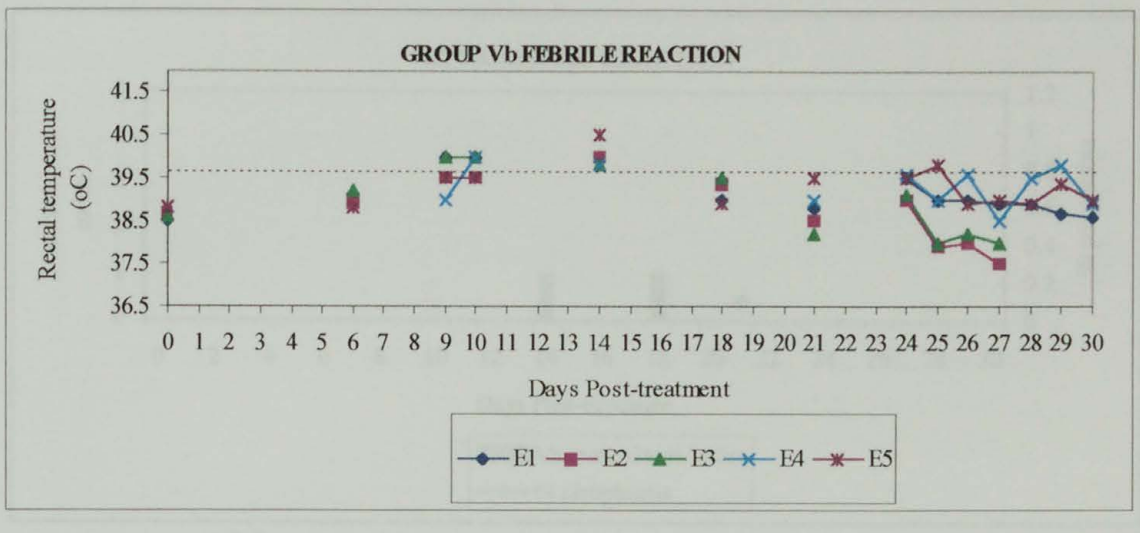
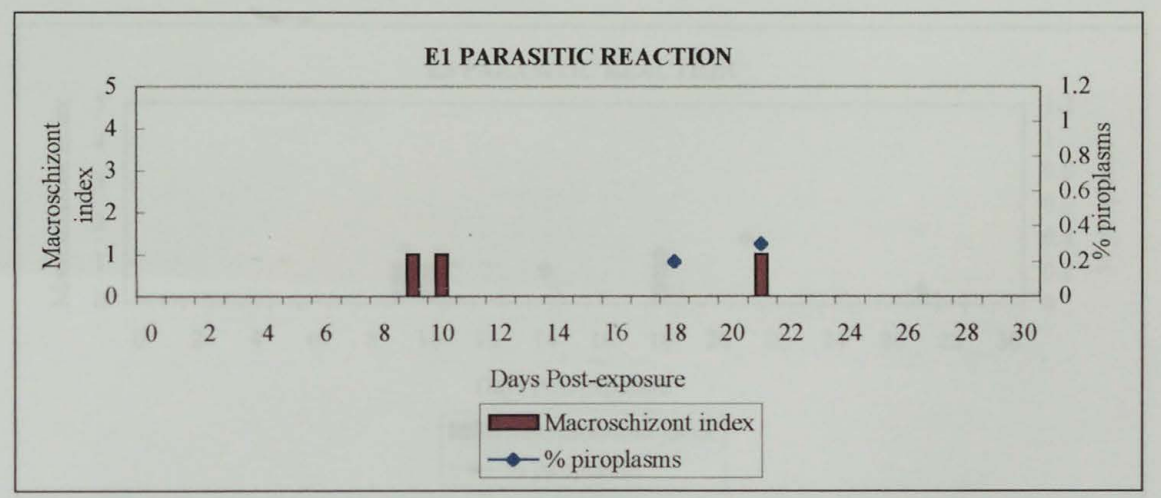


Figure (p): Parasitic reaction to tick exposure (2^{nd})



APPENDIX II

Figure (q): Parasitic reaction to tick exposure (2nd)

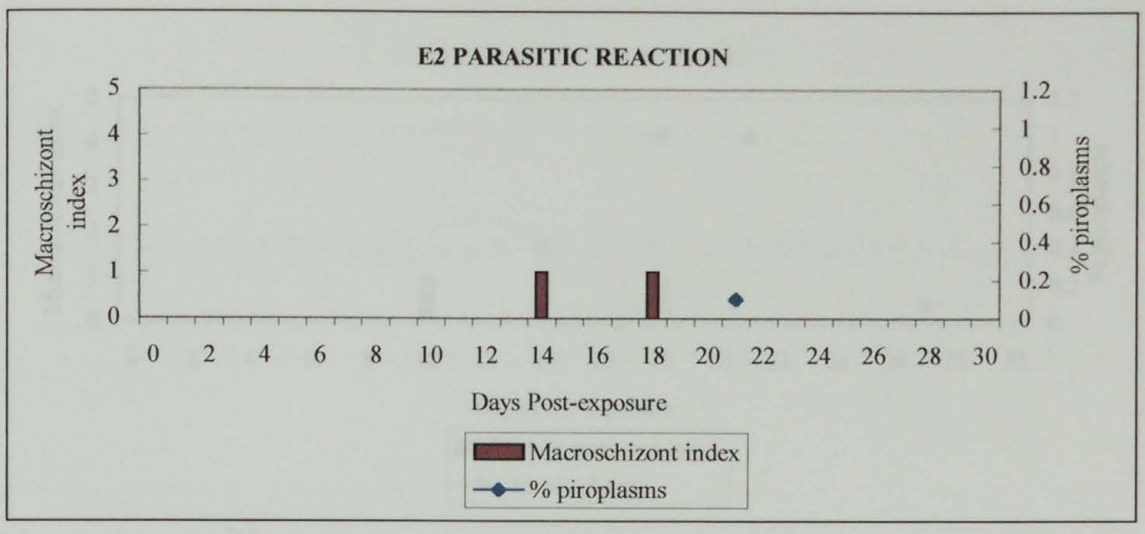
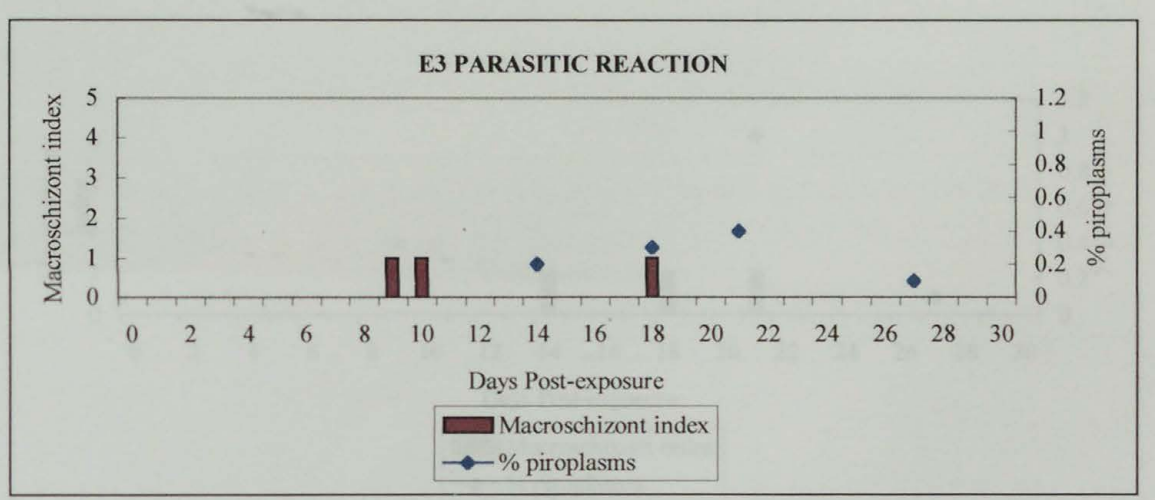


Figure (r): Parasitic reaction to tick exposure (2nd)



APPENDIX II

Figure (s): Parasitic reaction to tick exposure (2nd)

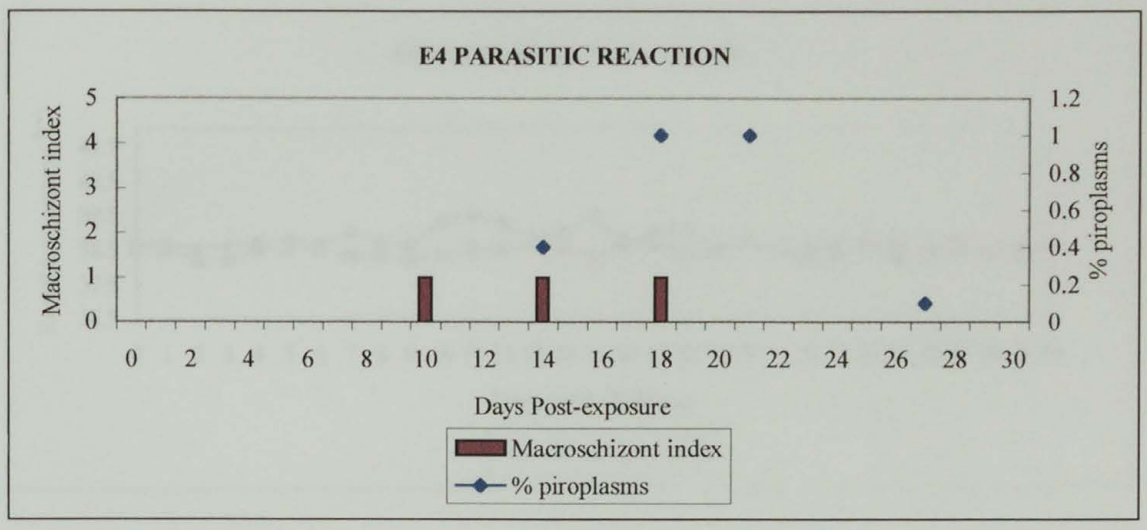
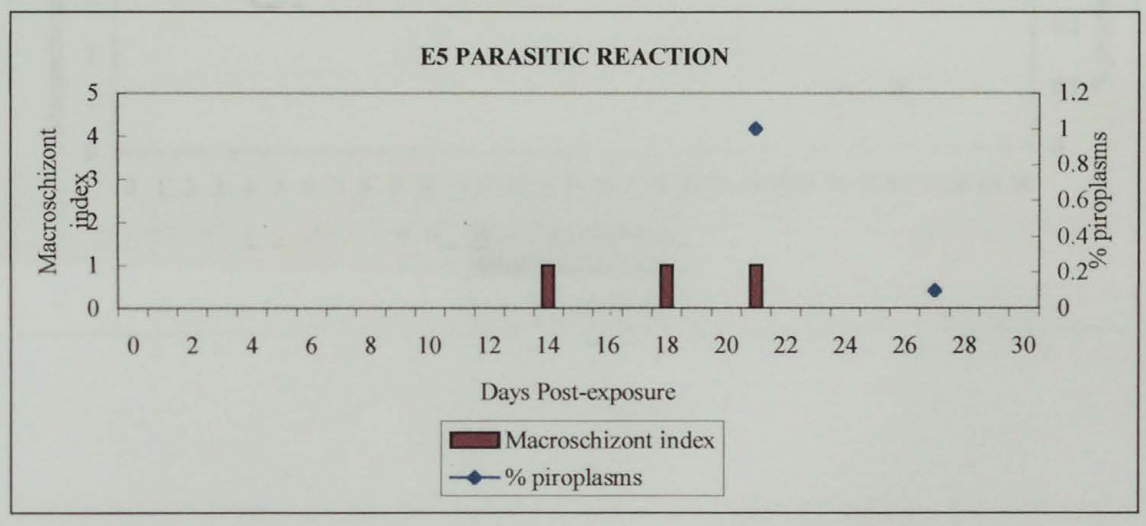


Figure (t): Parasitic reaction to tick exposure (2nd)



APPENDIX III

Figure 3.7(a): Febrile reaction to *T.parva* Avery challenge

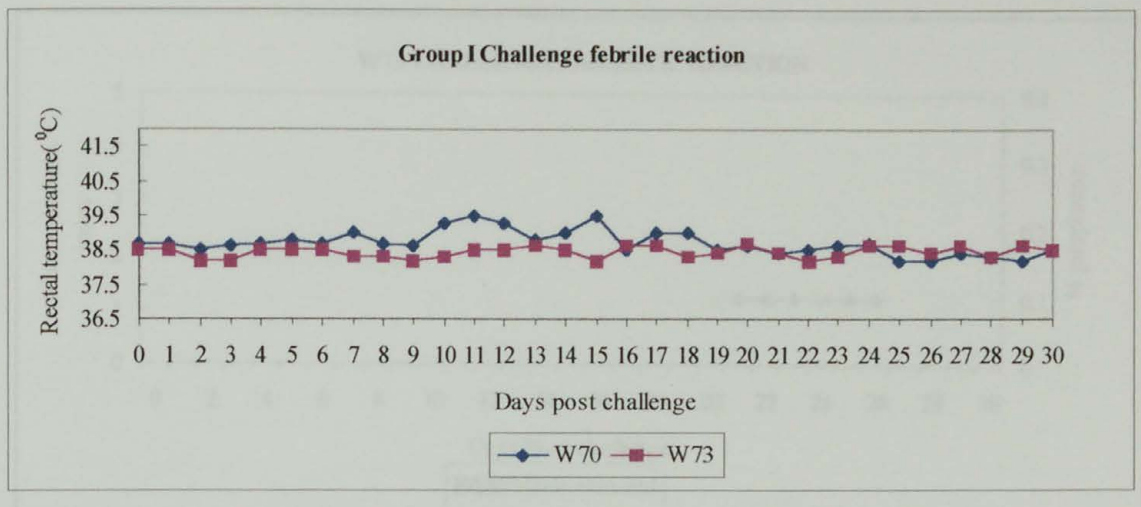
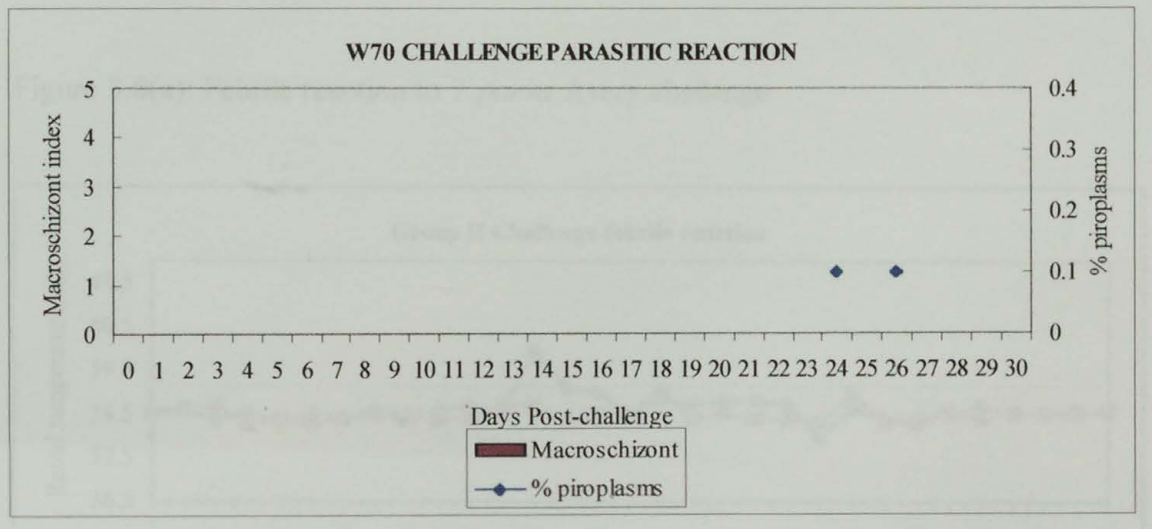


Figure 3.7(b): Parasitic reaction to *T.parva* Avery challenge



APPENDIX III

Figure 3.7(c): Parasitic reaction to *T.parva* Avery challenge

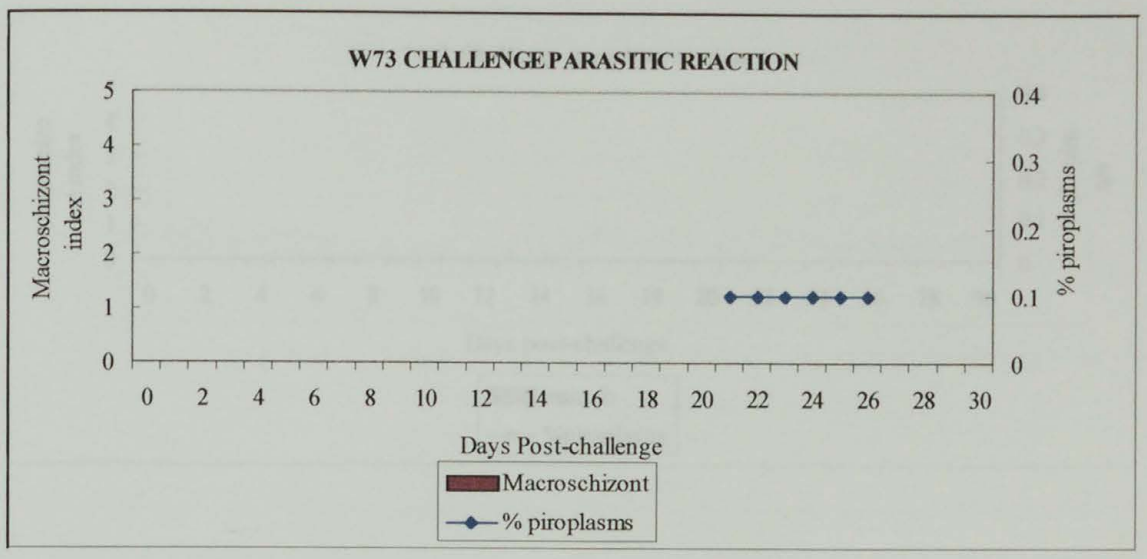
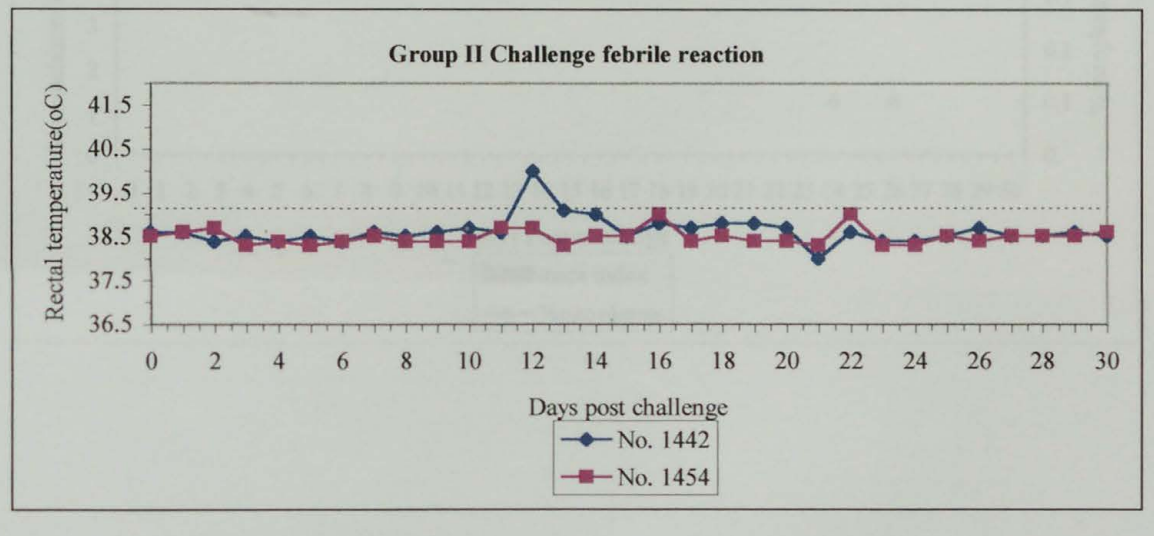


Figure 3.8(a): Febrile reaction to *T.parva* Avery challenge



APPENDIX III

Figure 3.8(b): Parasitic reaction to *T.parva* Avery challenge

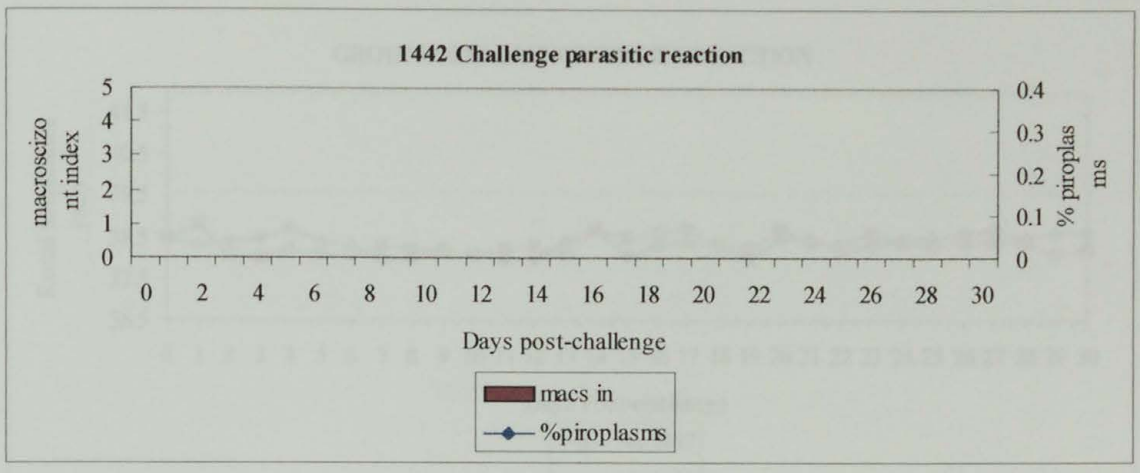
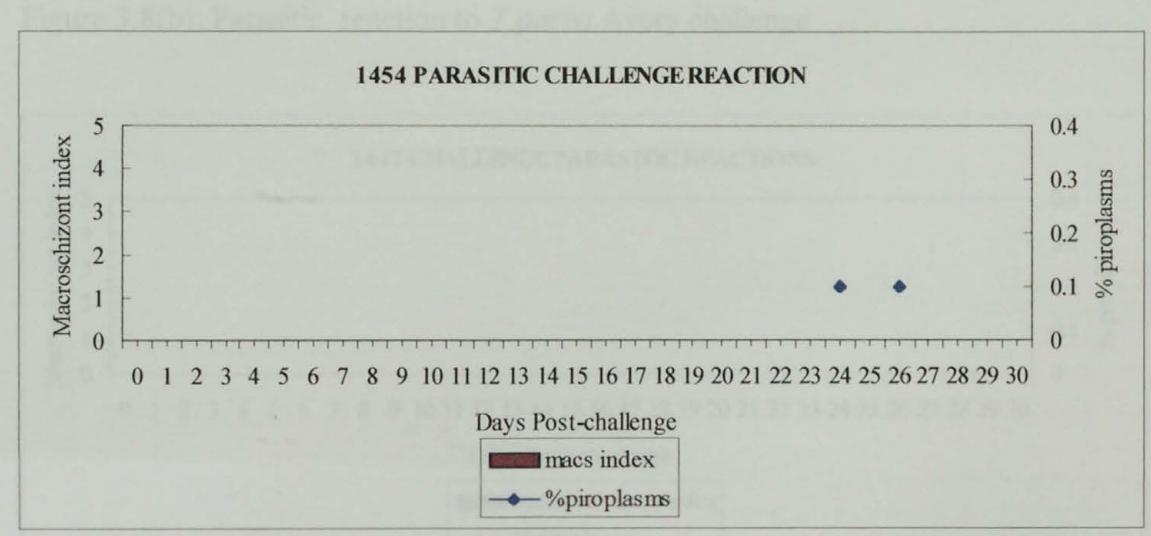


Figure 3.8(c): Parasitic reaction to *T.parva* Avery challenge



APPENDIX III

Figure 3.9(a): Febrile reaction to *T.parva* Avery challenge

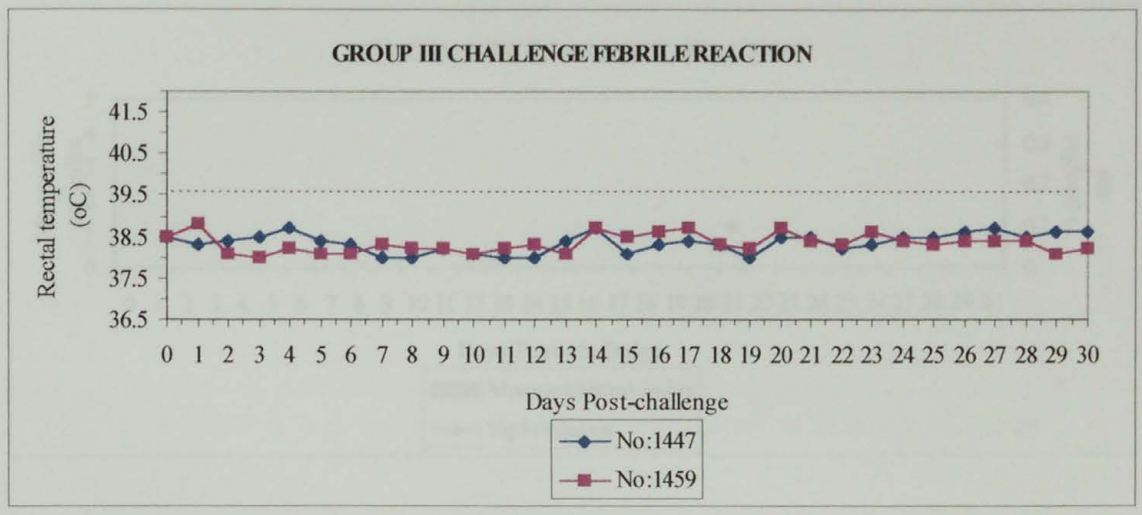
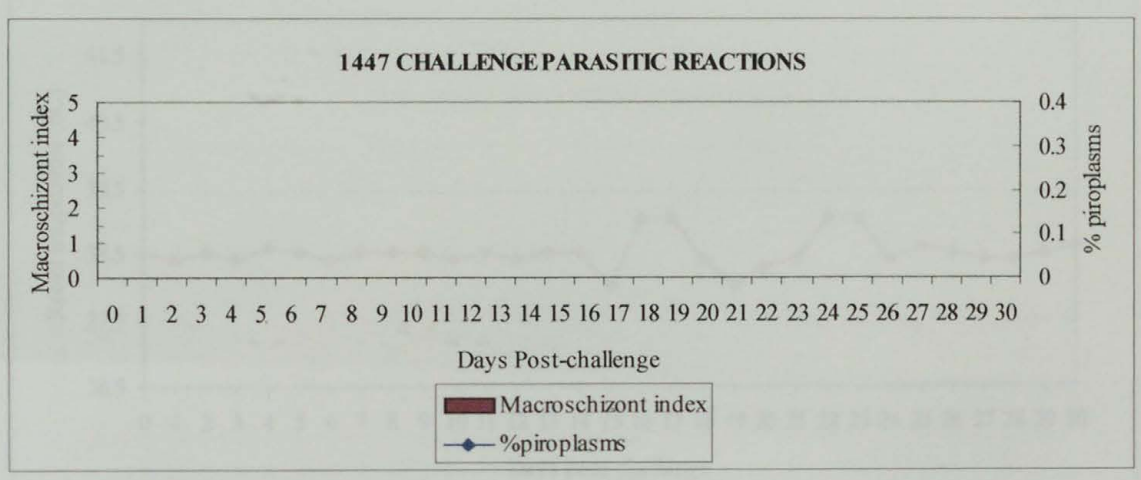


Figure 3.8(b): Parasitic reaction to *T.parva* Avery challenge



APPENDIX III

Figure 3.8(c): Parasitic reaction to *T.parva* Avery challenge

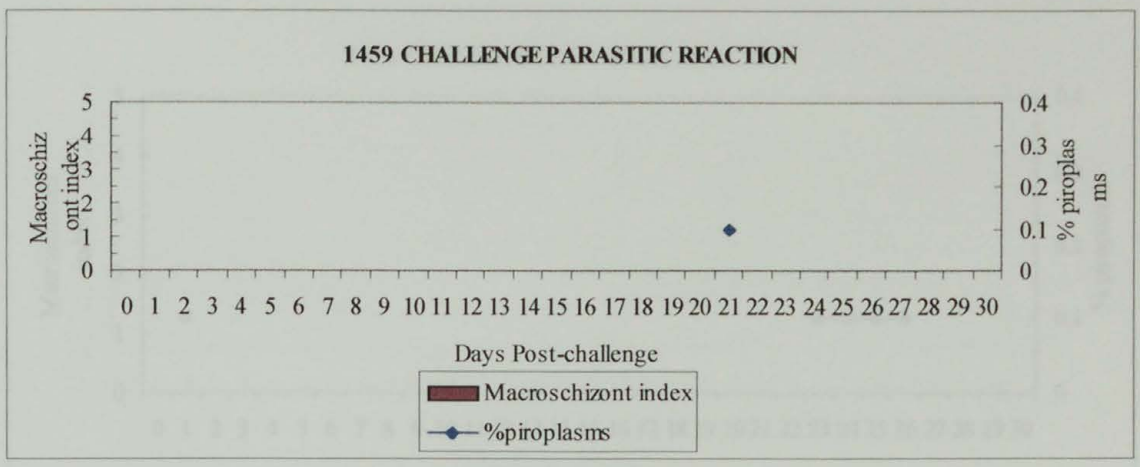
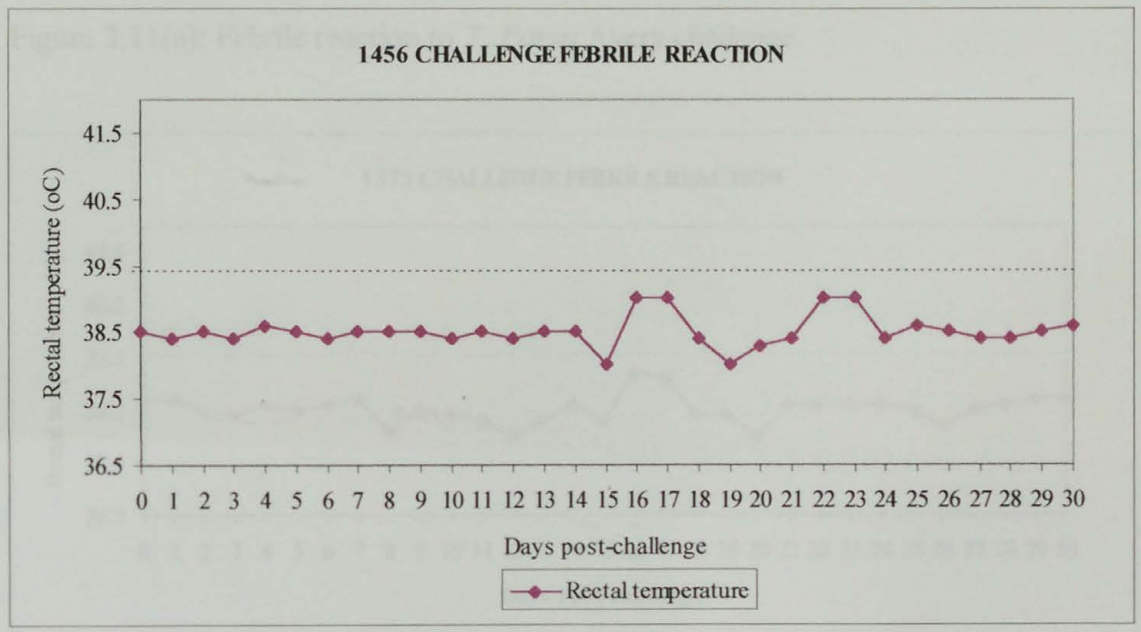


Figure 3.10(a): Febrile reaction to *T.parva* Avery challenge



APPENDIX III

Figure 3.10(b): Parasitic reaction to *T.parva* Avery challenge

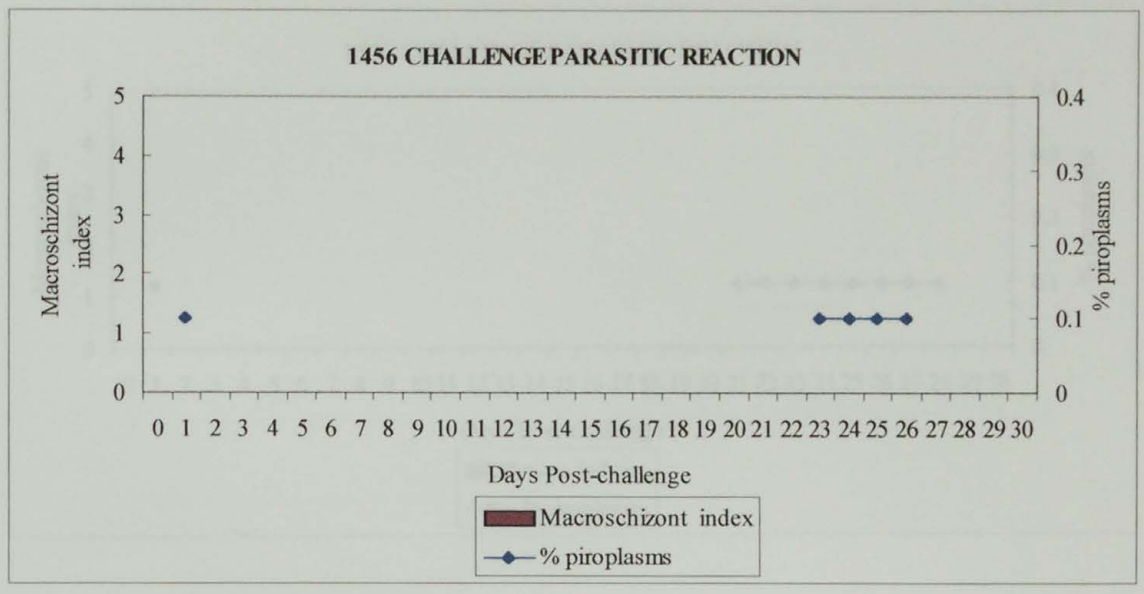
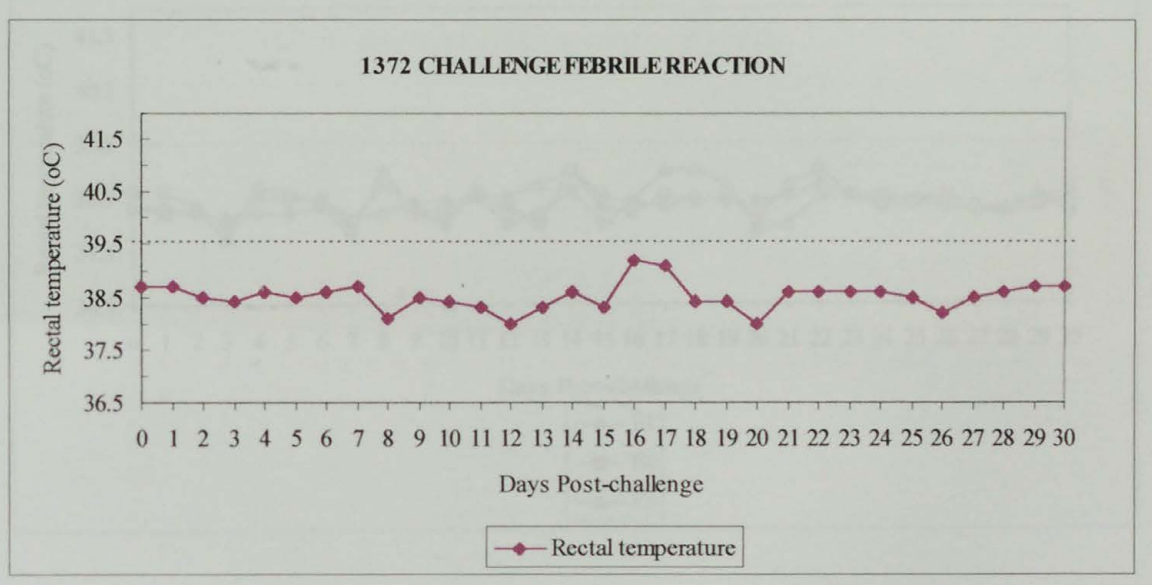


Figure 3.11(a): Febrile reaction to *T. Parva* Avery challenge



APPENDIX III

Figure 3.11(b): Parasitic reaction to *T. Parva* Avery challenge

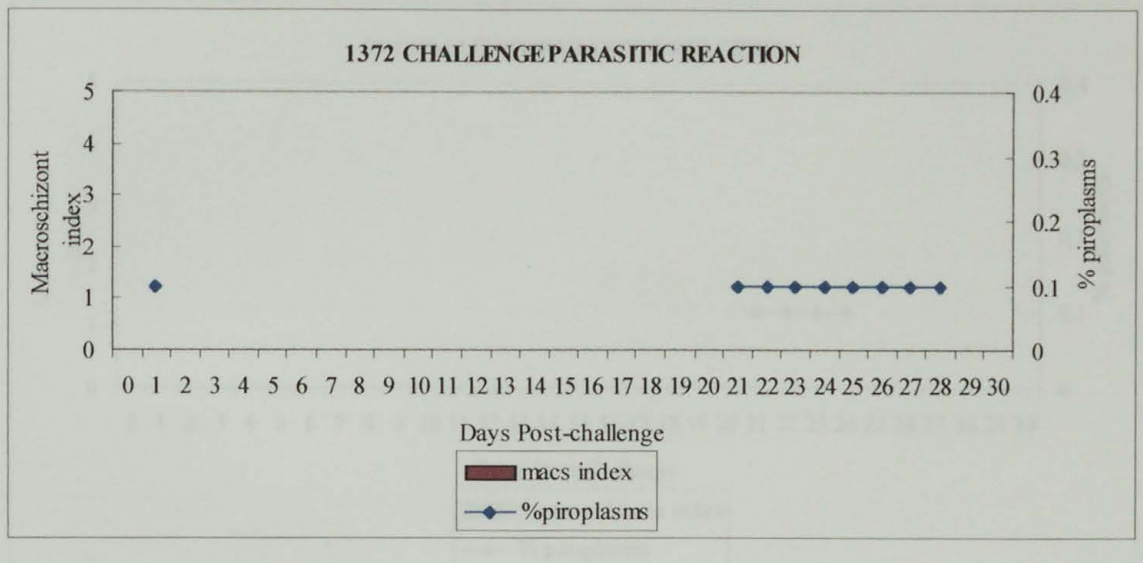
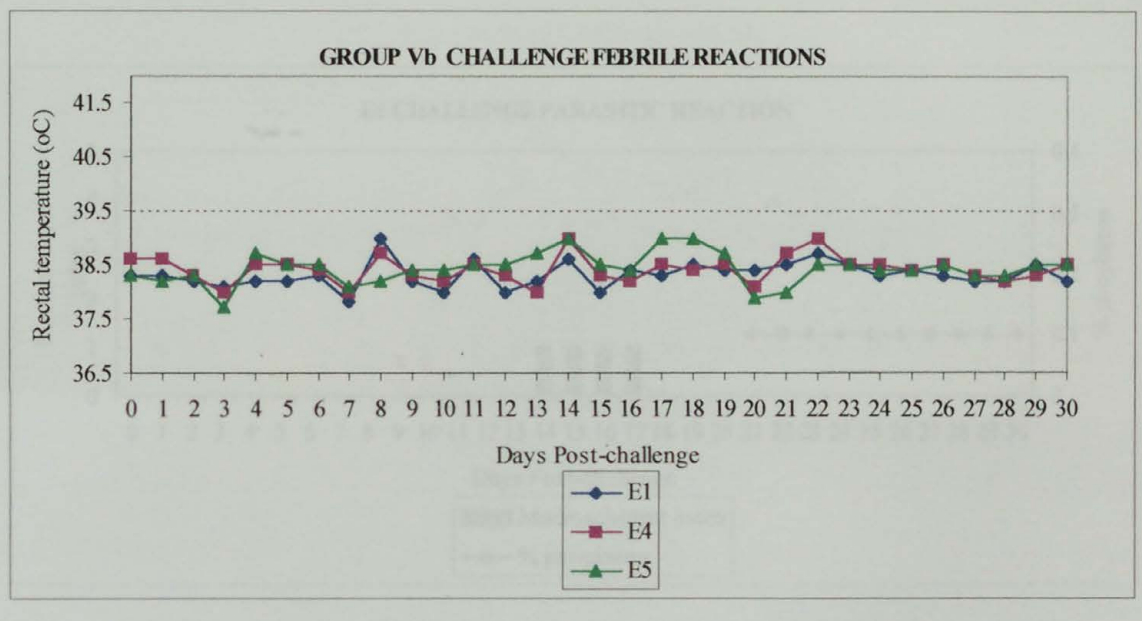


Figure 3.12(a): Febrile reaction to *T. Parva* Avery challenge



APPENDIX III

Figure 3.12(b): Parasitic reaction to *T. Parva* Avery challenge

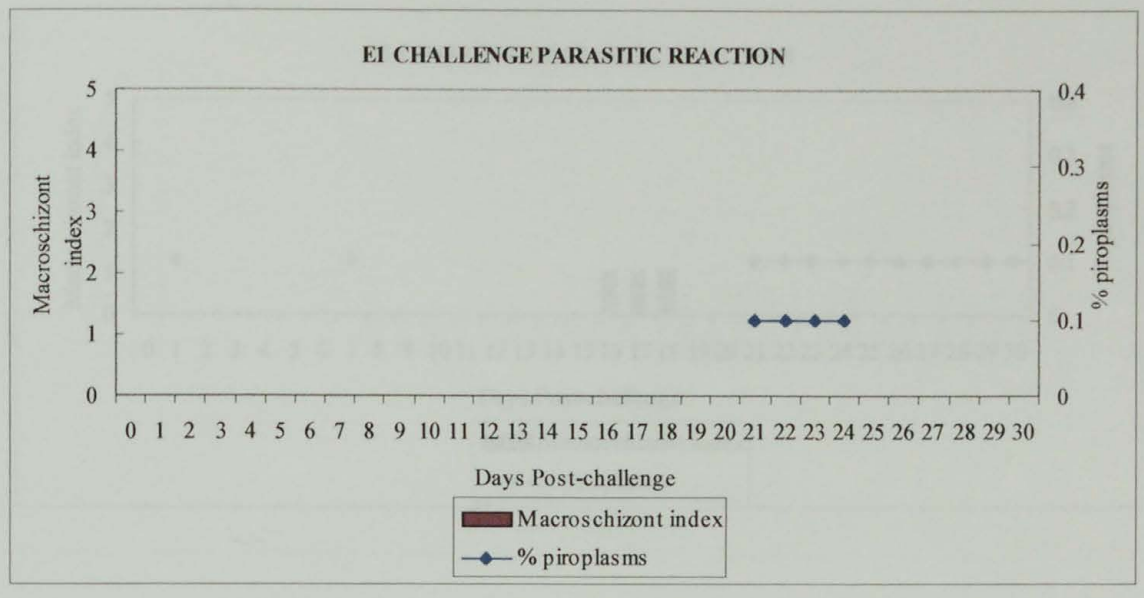
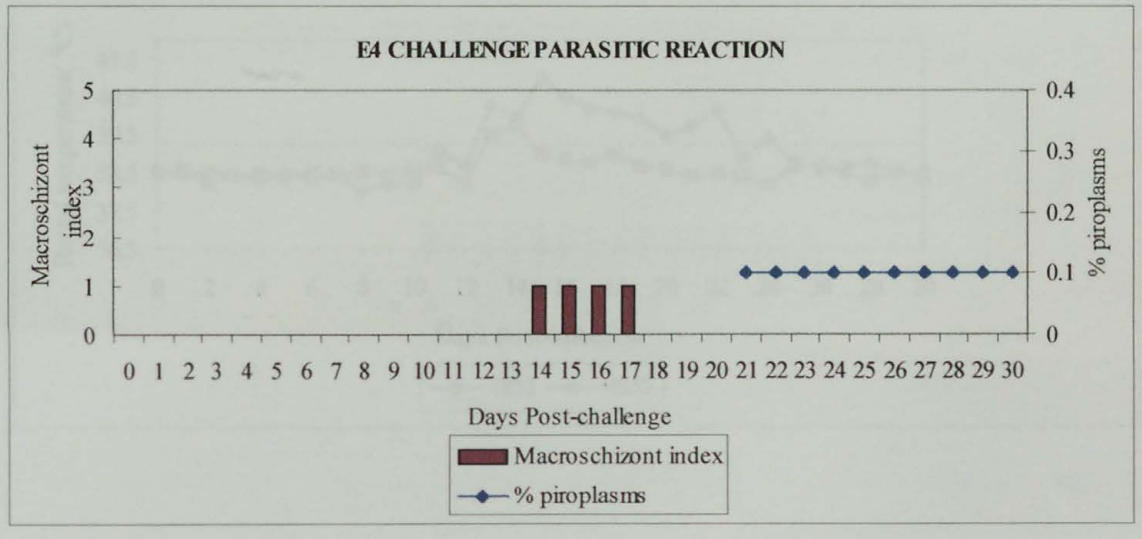


Figure 3.12(c): Parasitic reaction to *T. Parva* Avery challenge



APPENDIX III

Figure 3.12(d): Parasitic reaction to *T. Parva* Avery challenge

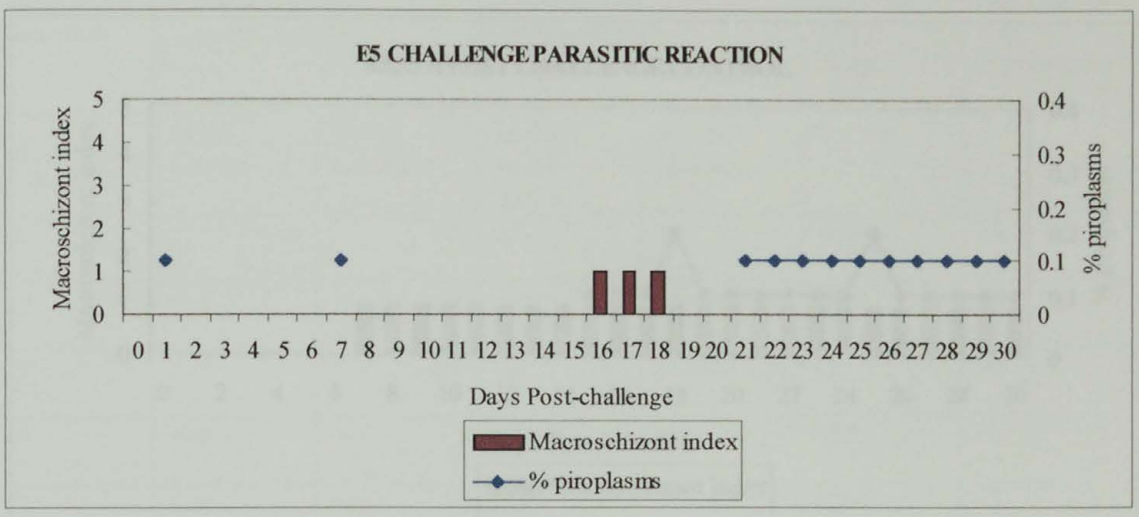
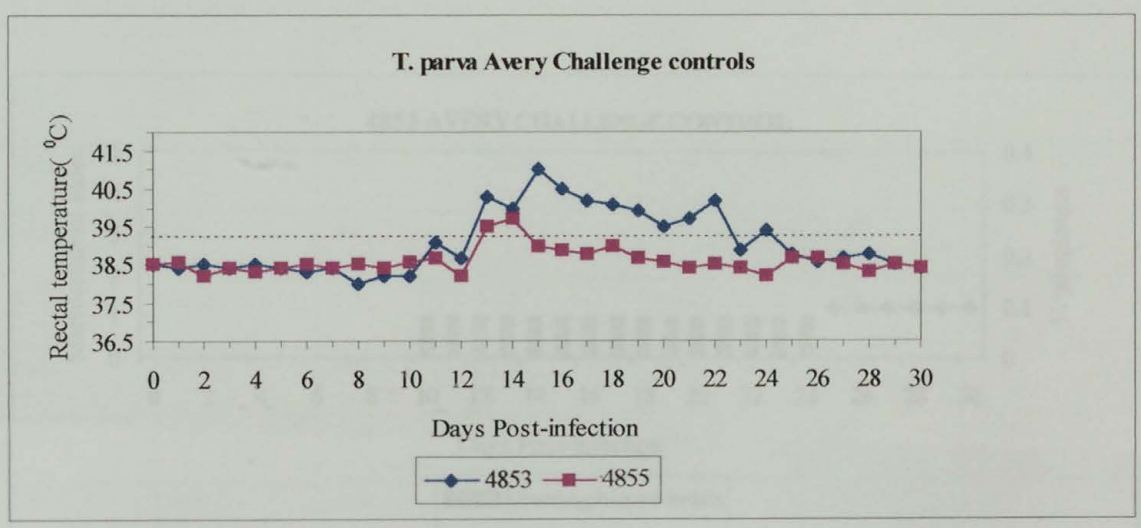


Figure 3.13(a): Control febrile reaction to *T. Parva* infection



APPENDIX III

Figure 3.13(b): Control parasitic reaction to *T. Parva* infection

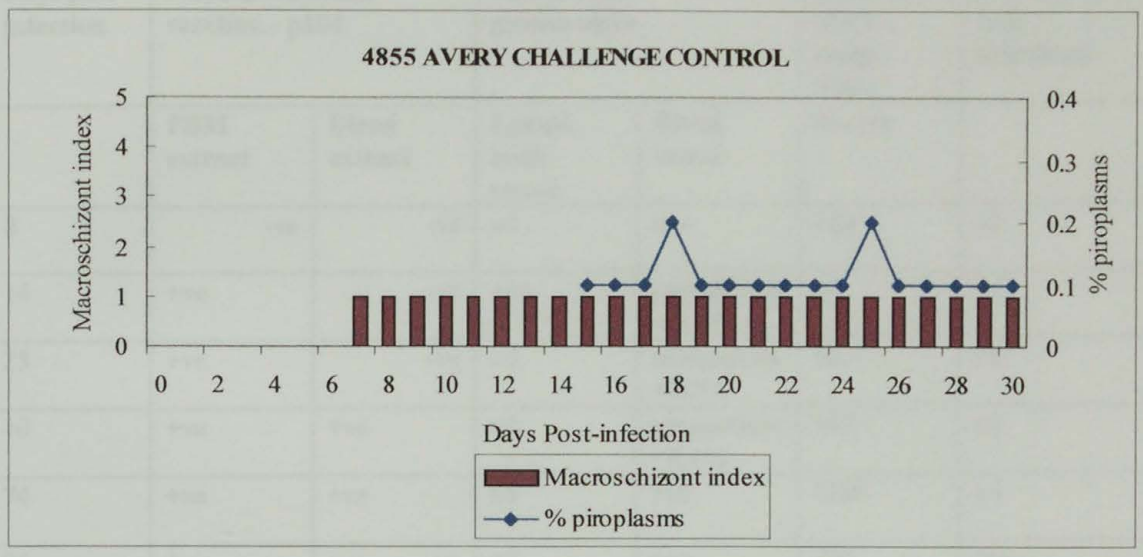
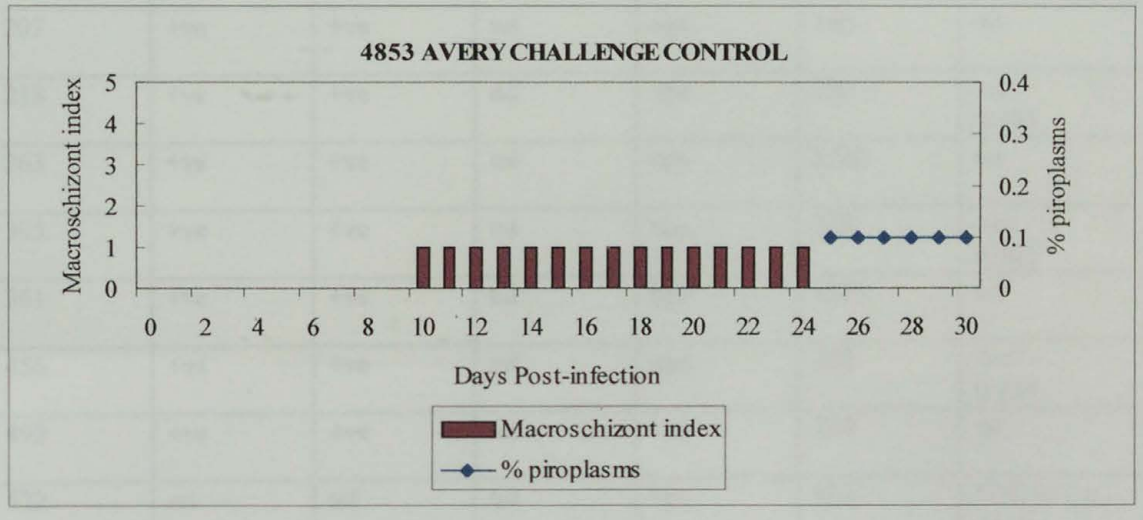


Figure 3.13(c): Control parasitic reaction to *T. Parva* infection



APPENDIX IV

Table 1 (group I) Summary of results obtained with different methods used to detect carrier status.
Calf number: W70 *T. parva* Boleni infected Day 0:31/03/94

Days post infection	**Polymerase chain reaction - p104**		**Microscopy giemsa stain**		***T. parva* IFAT-. recip. Titre**	**Prevalence of tick-infections**
	PBM extract	**Blood extract**	**Lymph node smear**	**Blood smear**	**Serum**	
0	-ve	-ve	nd	nps	160	nd
14	**+ve**	-ve	**+ve**	**piroplasms <0.1%**	nd	nd
25	**+ve**	-ve	nd	**piroplasms <0.1%**	nd	nd
40	**+ve**	**+ve**	nd	**piroplasms <0.1%**	640	nd
74	**+ve**	**+ve**	nd	nps	1280	nd
95	**+ve**	-ve	nd	nps	640	**+ve 5.96%**
109	**+ve**	**+ve**	nd	nps	320	nd
137	**+ve**	**+ve**	nd	**piroplasms <0.1%**	640	nd
179	**+ve**	**+ve**	nd	nps	320	nd
207	**+ve**	**+ve**	nd	nps	160	nd
218	**+ve**	**+ve**	nd	nps	320	-ve 0/193
263	**+ve**	**+ve**	nd	nps	5120	nd
305	**+ve**	**+ve**	nd	nps	320	-ve 0/200
361	**+ve**	**+ve**	nd	nps	1280	nd
456	**+ve**	**+ve**	nd	nps	320	-ve 0/134
493	**+ve**	**+ve**	nd	nps	320	nd
522	nd	nd	nd	nps	320	*NR to T.p. Avery challenge
Prop. +ve	15/15	12/15		4 / 16	6 / 14	1 / 4

+ve -positive -ve -negative
IFAT -cut off is 640 nd -not done
nps -no parasites seen NR -no reaction
T. p. -*T. parva* recip. -reciprocal
Prop. +ve -proportion of positive results
PBM -peripheral blood mononuclear cells

APPENDIX IV

Table 2: (group I) Summary of results obtained with different methods used to detect carrier status.
Calf number: W73 *T. parva* Boleni infected Day 0:31/03/94

Day post infection	**Polymerase chain reaction - p104**		**Microscopy giemsa stain**		***T. parva* IFAT-recip. Titre**	**Prevalence of tick-infections**
	PBM extract	**Blood extract**	**Lymph node smear**	**Blood smear**	**Serum**	
0	-ve	-ve	nd	nps	80	nd
14	**+ve**	**+ve**	**+ve**	**piroplasms <0.1%**	nd	nd
25	-ve	-ve	nd	**piroplasms <0.1%**	1280	nd
40	**+ve**	**+ve**	nd	**piroplasms <0.1%**	1280	nd
74	**+ve**	-ve	nd	nps	320	nd
95	**+ve**	-ve	nd	nps	5120	**+ve 0.4%**
109	**+ve**	**+ve**	nd	nps	160	nd
137	-ve	-ve	nd	nps	640	nd
179	-ve	**+ve**	nd	nps	320	nd
207	-ve	**+ve**	nd	nps	320	nd
218	-ve	**+ve**	nd	nps	5120	**+ve 3.29%**
263	-ve	**+ve**	nd	nps	2560	nd
305	-ve	**+ve**	nd	**piroplasms <0.1%**	320	-ve 0/144
361	-ve	**+ve**	nd	nps	320	nd
456	-ve	**+ve**	nd	nps	640	-ve 0/124
493	**+ve**	**+ve**	nd	nps	640	nd
522	nd	nd	nd	nd	1280	NR to T.p.Avery challenge
Prop. +ve	6/15	11/15		4 / 15	9 / 15	2 / 4

+ve -positive
-ve -negative
nps -no parasites seen
T. p. -*T. parva*
recip. -reciprocal
NR -no reaction
IFAT -cut off is 640
nd -not done
PBM -peripheral blood mononuclear cells
Prop. +ve -proportion of positive results

APPENDIX IV

Table 3:(group I) Summary of results obtained with different methods used to detect carrier status.
Calf number: W74 *T. parva* Boleni infected Day 0:31/03/94

Day post-infection	**Polymerase chain reaction - p104**		**Microscopy giemsa stain**		***T. parva* IFAT-recip. Titre**	**Prevalence of tick-infections**
	PBM extract	**Blood extract**	**Lymph node smear**	**Blood smear**	**Serum**	
0	-ve	-ve	nd	nps	80	nd
14	**+ve**	-ve	mac **+ve**	**piroplasms <0.1%**	nd	nd
25	-ve	**+ve**	nd	**piroplasms <0.1%**	nd	nd
40	**+ve**	**+ve**	nd	nps	2560	nd
74	**+ve**	**+ve**	nd	nps	2560	nd
95	**+ve**	nd	nd	nps	640	-ve 0/218
109	**+ve**	**+ve**	nd	nps	320	nd
137	**+ve**	**+ve**	nd	nps	640	nd
179	-ve	**+ve**	nd	nps	320	nd
218	-ve	**+ve**	nd	**piroplasms <0.1%**	320	**+ve 2.22%**
221	-ve	**+ve**	nd	nps	80	nd
263	-ve	**+ve**	nd	nps	5120	nd
305	-ve	-ve	nd	nps	320	-ve 0/145
Died before challenge	nd	nd	nd	nd	nd	nd
Prop. +ve	6 / 12	9 / 11		3 / 12	5 / 10	1/ 3

+ve -positive
-ve -negative
nps -no parasites seen
PBM -peripheral blood mononuclear cells
recip. -reciprocal
IFAT -cut off is 640
Prop. +ve -proportion of positive results

APPENDIX IV

Table 4: (group I)Summary of results obtained with different methods used to detect carrier status.
Calf number: W75 *T. parva* Boleni infected Day 0:31/03/94

Day post - infection.	**Polymerase chain reaction - p104 primers**		**Microscopy Giemsa stain**		***T. parva* IFAT-recip. titre**	**Prevalence of tick-infections**
	PBM extract	**Blood extract**	**Lymph node smear**	**Blood smear**	**Serum**	
0	-ve	-ve	nd	nps	160	nd
14	**+ve**	**+ve**	**mac +ve**	**piroplasms <0.1%**	320	nd
25	-ve	-ve	nd	**piroplasms <0.1%**	nd	nd
40	-ve	-ve	nd	nps	1280	nd
74	**+ve**	**+ve**	nd	**piroplasms <0.1%**	640	nd
95	**+ve**	**+ve**	nd	nps	320	**+ve 7.14%**
109	**+ve**	**+ve**	nd	**piroplasms <0.1%**	320	nd
137	**+ve**	-ve	nd	nps	640	nd
179	-ve	-ve	nd	nps	160	nd
207	-ve	**+ve**	nd	nps	160	nd
218	-ve	**+ve**	nd	nps	320	**+ve 1.05%**
253	-ve	**+ve**	nd	nps	2560	nd
305	-ve	**+ve**	nd	nps	640	-ve 0/190
361	-ve	**+ve**	nd	nps	640	nd
446	nd	-ve	nd	nd	nd	nd
493	nd	-ve	nd	nd	nd	nd
Died before challenge	nd	nd	nd	nd	nd	nd
Prop. +ve	5 /13	9 / 13		4 / 13	6 / 12	2 / 3

+ve -positive
-ve -negative
recip. -reciprocal
PBM -peripheral blood mononuclear cells
Prop. +ve -proportion of positive results
nd -not done
nps -no parasites seen
IFAT -cut off is 1:640

APPENDIX IV

Table 5: (group II) Summary of results obtained with different methods used to detect carrier status.

Calf number: 1442 *T. parva* Avery Stabilate Day 0:31/03/94

Day post infection	**Polymerase chain reaction - p104**		**Microscopy Giemsa stain**		***T. parva* IFAT-recip. Titre**	**Prevalence of tick-infections**
	PBM extract	**Blood extract**	**Lymph node smear**	**Blood smear**	**Serum**	
0	-ve	-ve	nd	nps	160	nd
14	+ve	+ve	nps	nps	nd	nd
25	+ve	-ve	nd	nps	nd	nd
40	-ve	-ve	nd	nps	160	nd
74	+ve	-ve	nd	nps	320	nd
95	+ve	+ve	nd	nps	160	-ve 0/131
109	+ve	-ve	nd	nps	160	nd
137	+ve	-ve	nd	nps	320	nd
179	+ve	-ve	nd	nps	160	nd
207	-ve	-ve	nd	nps	160	nd
218	-ve	-ve	nd	nps	320	-ve 0/186
263	-ve	-ve	nd	nps	640	nd
305	-ve	+ve	nd	nps	320	-ve 0/137
361	-ve	+ve	nd	nps	320	nd
456	-ve	-ve	nd	nps	160	-ve 0/23
493	-ve	+ve	nd	nps	nd	nd
522	nd	nd	nd	nps	320	NR to T. p. Avery challenge
Prop. +ve	7 / 15	5 /15		0 / 16	1 / 13	0 / 4

+ve	-positive	recip.	-reciprocal
-ve	-negative	nps	-no parasites seen
nd	-not done	IFAT	-cut off is 1:640
PBM	-peripheral blood mononuclear cells	T. p.	-*T. parva*
Prop. +ve	-proportion of positive results	NR	-no reaction

APPENDIX IV

Table 6 (group II) Summary of results obtained with different methods used to detect carrier status.
Calf number 1454 *T. parva* Avery Stabilate Day 0:31/03/94

Day post infection	**Polymerase chain reaction - p104 primers**		**Microscopy Giemsa stain**		***T. parva* IFAT-recip. Titre**	**Prevalence of tick-infections**
	PBM extract	**Blood extract**	**Lymph node smear**	**Blood smear**	**Serum**	
0	-ve	-ve	nd	nps	160	nd
14	**+ve**	**+ve**	**macs+ve**	nps	nd	nd
15 *	**+ve**	**+ve**	**macs+ve**	nps	nd	nd
25	**+ve**	**+ve**	nd	nps	nd	nd
40	**+ve**	-ve	nd	nps	10240	nd
74	**+ve**	**+ve**	nd	nps	1280	nd
95	-ve	nd	nd	nps	640	-ve 0/152
109	**+ve**	**+ve**	nd	nps	640	nd
137	**+ve**	**+ve**	nd	nps	1280	nd
179	-ve	**+ve**	nd	nps	320	nd
207	-ve	-ve	nd	nps	320	nd
218	-ve	**+ve**	nd	nps	160	-ve 0/244
263	-ve	-ve	nd	nps	2560	nd
305	-ve	**+ve**	nd	nps	1280	-ve 0/137
361	**+ve**	**+ve**	nd	nps	1280	nd
456	-ve	-ve	nd	nps	640	-ve 0/147
493	nd	**+ve**	nd	nps	640	nd
522	nd	nd	nd	nps	640	NR to T.p. Avery challenge
Prop. +ve	8 / 17	11 / 15		0 / 17	11 / 14	0 / 4

+ve	-positive	-ve	-negative
nd	-not done	*	**-Butalex® treatment**
T.p.	-*T. parva*	IFAT	-cut off is 640
NR	-no reaction	Prop. +ve	-proportion of positive results
recip.	-reciprocal	nps	-no parasites seen
PBM	-peripheral blood mononuclear cells		

APPENDIX IV

Table 7: (group III) Summary of results obtained with different methods used to detect carrier status.

Calf number : 1447 *T. parva* Bally Vaughan stabilate 17 Day 0:31/03/94

Day post infection	**Polymerase chain reaction - p104**		**Microscopy Giemsa stain**		***T. parva* IFAT-recip. Titre**	**Prevalence of tick-infections**
	PBM extract	**Blood extract**	**Lymph node smear**	**Blood smear**	**Serum**	
0	-ve	-ve	nd	nps	80	nd
14	**+ve**	**+ve**	**mac +ve**	nps	160	nd
25	-ve	-ve	nd	**piroplasms <0.1%**	160	nd
40	-ve	-ve	nd	**piroplasms <0.1%**	2560	nd
74	**+ve**	-ve	nd	nps	640	nd
95	-ve	-ve	nd	nps	160	-ve 0/143
109	**+ve**	**+ve**	nd	nps	320	nd
137	**+ve**	-ve	nd	nps	1280	nd
179	-ve	**+ve**	nd	nps	320	nd
207	-ve	**+ve**	nd	nps	320	nd
218	-ve	**+ve**	nd	nps	160	-ve 0/204
263	-ve	-ve	nd	nps	2560	nd
305	-ve	**+ve**	nd	nps	1280	**-ve**
361	-ve	-ve	nd	nps	2560	nd
456	-ve	-ve	nd	nps	2560	-ve 0/228
493	-ve	**+ve**	nd	nps	640	nd
522	nd	nd	nd	nd	1280	NR to T.p. Avery challenge
Prop. +ve	4 /15	7 /15		2 / 15	9 /16	0 / 4

+ve	-positive	nd	-not done
-ve	-negative	nps	-no parasites seen
recip.	-reciprocal	IFAT	-cut off is 640
PBM	-peripheral blood lymphocytes	NR	-no reaction
T. p.	-*T. parva*		
Prop. +ve	-proportion of positive results		

APPENDIX IV

Table 8: (group III) Summary of results obtained with different methods used to detect carrier status.

Calf number: 1459 *T. parva* Bally Vaughan stabilate 17 Day 0:31/03/94

Day post infection	**Polymerase chain reaction - p104**		**Microscopy Giemsa stain**		***T. parva* IFAT-recip. Titre**	**Prevalence of tick-infections**
	PBM extract	**Blood extract**	**Lymph Node**	**Blood smear**	**Serum**	
0	-ve	-ve	nd	nps	160	nd
14	**+ve**	**+ve**	nps	nps	nd	nd
25	**+ve**	-ve	nd	nps	nd	nd
40	-ve	-ve	nd	nps	2560	nd
74	nd	**+ve**	nd	nps	640	nd
95	**+ve**	-ve	nd	nps	640	-ve 0/148
109	nd	**+ve**	nd	nps	160	nd
137	-ve	-ve	nd	nps	1280	nd
179	-ve	-ve	nd	nps	320	nd
204	ve-	**+ve**	nd	nps	160	nd
218	-ve	**+ve**	nd	nps	320	-ve 0/24
263	-ve	**+ve**	nd	nps	2560	nd
305	-ve	**+ve**	nd	nps	1280	-ve 0/76
361	-ve	**+ve**	nd	nps	640	nd
456	-ve	**+ve**	nd	nps	640d	-ve 0/231
493	-ve	nd	nd	nps	320	nd
522	nd	nd	nd	nd	640	NR to T.p. Avery challenge
Prop. +ve	3 / 13	9 /14		0 / 15	9 / 15	0 / 4

+ve	-positive	recip.	-reciprocal
-ve	-negative	nps	-no parasites seen
IFAT	-cut off is 640	T. p.	-*T. parva*
nd	-not done	NR	-no reaction
PBM	-peripheral blood mononuclear cells		
Prop. +ve	-proportion of positive results		

APPENDIX IV

Table 9: (group Iva) Summary of results obtained with different methods used to detect carrier status.

Calf number 1456 Ayrshire Ticks. (150 adult *R. append.* ticks).Day 0:16.02.94

Day post tick application .	**Polymerase chain reaction - p104**		**Microscopy Giemsa stain**		***T. parva* IFAT-recip. titre**	**Prevalence of tick-infections**
	PBM extract	**Blood extract**	**Lymph node smear**	**Blood smear**	**Serum**	
0	-ve	-ve	nd	nps	160	nd
14	nd	nd	mac +ve	nps	nd	nd
25	nd	nd	nps	**piroplasms <0.1%**	nd	nd
82	-ve	-ve	nd	**piroplasms <0.1%**	640	nd
117	-ve	-ve weak +/-	nd	**piroplasms <0.1%**	2560	nd
138	**+ve**	-ve	nd	**piroplasms <0.1%**	160	**+ve 11.6%**
152	-ve	**+ve**	nd	**piroplasms <0.1%**	80	nd
180	**+ve**	**+ve**	nd	**piroplasms <0.1%**	320	nd
222	-ve	-ve	nd	**piroplasms <0.1%**	160	nd
250	-ve	**+ve**	nd	**piroplasms <0.1%**	160	nd
264	-ve	**+ve**	nd	**piroplasms <0.1%**	160	**+ve 10.24%**
306	-ve	-ve	nd	**piroplasms <0.1%**	640	nd
321	-ve	-ve	nd	**piroplasms <0.1%**	320	nd
402	-ve	-ve	nd	nps	320	nd
488	-ve	**+ve**	nd	nps	320	**+ve 5.41%**
534	-ve	**+ve**	nd	nps	160	nd
566	nd	nd	nd	nd	nd	NR to T.p. Avery challenge
Prop. +ve	2 / 13	6 / 13		11 / 15	3 / 13	3 / 3

+ve	-positive	NR	-no reaction
-ve	-negative	T. p.	-*T. parva*
nd	-not done	nps	-no parasites seen
PBM	-peripheral blood mononuclear cells		
recip.	-reciprocal IFAT (-cut off is 640)		
Prop. +ve	-proportion of positive results		

APPENDIX IV

Table 10: (group IVb) Summary of results obtained with different methods used to detect carrier status.

Calf number: 1437 Chikeya Tick application (150 adult *R. app.* ticks) Day 0:16.02.96

Day post tick application	**Polymerase chain reaction - p104**		**Microscopy giemsa stain**		***T. parva* IFAT-recip. titre**	**Prevalence of tick-infections**
	PBM extract	**Blood extract**	**Lymph Node**	**Blood smear**	**Serum**	
0	-ve	nd	nd	nps	80	nd
14	nd	nd	**mac +ve**	nps	nd	nd
25	nd	-ve	nd	**piroplasms <0.1%**	nd	nd
82	**+ve**	-ve	nd	nps	1280	nd
117	**+ve**	-ve	nd	nps	5120	nd
138	**+ve**	-ve	nd	nps	640	**+ve 0.38%**
152	**+ve**	**+ve**	nd	nps	320	nd
180	**+ve**	-ve	nd	nps	320	
222	-ve	**+ve**	nd	**piroplasms <0.1%**	320	nd
250	-ve	-ve	nd	nps	320	nd
264	-ve	**+ve**	nd	nps	80	**+ve 0.5%**
Died before challenge	nd	nd	nd	nd	nd	nd
Prop. +ve	5 / 8	3 / 9		2 / 10	3 / 8	2 / 2

+ve positive
-ve negative
nd -not done
PBM -peripheral blood mononuclear cells
recip. -reciprocal
nps -no parasites seen
IFAT -cut off is 640
Prop. +ve -proportion of positive results

APPENDIX IV

Table 11: (group V) Summary of results obtained with different methods used to detect carrier status.
Calf number: 1372 Hunyani Estates Exposure DAY 0 18/02/94

Day post exposure	POLYMERASE CHAIN REACTION - P104		MICROSCOPY GIEMSA STAIN		*T. parva* IFAT-recip. Titre	Prevalence of tick-infections
	PBM extract	Blood extract	Lymph node smear	Blood smear	Serum	
0	-ve	-ve	nd	nps	80	nd
11	-ve	nd	**macs -ve**	nps	nd	nd
19	-ve	nd	**macs +ve**	**piro<0.1%**	nd	nd
26	-ve	-ve	nps	nps	nd	nd
37	-ve	nd	nd	nps	nd	nd
41	-ve	nd	nd	nps	nd	nd
66	-ve	**+ve**	nd	nps	nd	nd
80	-ve	**+ve**	nd	**piro<0.1%**	1280	nd
115	-ve	-ve	nd	**piro<0.1%**	640	nd
132	-ve	-ve	nd	**piro<0.1%**	2560	**+ve 68.03%**
150	-ve	**+ve**	nd	**piro<0.1%**	320	nd
178	-ve	**+ve**	nd	**piro<0.1%**	640	nd
220	-ve	-ve	nd	**piro<0.1%**	640	**+ve 60.6%**
248	-ve	**+ve**	nd	**piro<0.1%**	640	nd
262	-ve	**+ve**	nd	**piro<0.1%**	640	nd
304	-ve	**+ve**	nd	**piro<0.1%**	10240	nd
319	-ve	**+ve**	nd	**piro<0.1%**	2560	**+ve 27.2%**
488	-ve	nd	nd	nd	640	nd
501	-ve	**+ve**	nd	nd	640	**+ve 37.82%**
535	-ve	**+ve**	nd	nd	640	nd
563	nd	nd	nd	nd	1280	NR to T.p. Avery challenge
Prop. +ve	0 / 19	10 /14		11 / 16	13 / 14	4 / 4

+ve -positive ifat -cut off is 640
-ve -negative piro -piroplasms seen
PBM -peripheral blood mononuclear cells NR -no reaction
nps -no parasites seen T. p -*T. parva*
Prop. +ve -proportion of positive results

APPENDIX IV

Table 12a: (group Vb) Summary of results obtained with different methods used to detect carrier status. Calf numberE1 exposed at Hunyani Estates

Day post exposure	POLYMERASE CHAIN REACTION - P104		MICROSCOPY GIEMSA STAIN		*T. parva* IFAT-recip. titre	Prevalence of tick-infections
	PBM extract	Blood extract	Lymph node smear	Blood smear	Serum	
0	-ve	-ve	nd	nps	80	nd
42	+ve	+ve	nd	piro<0.1%	1280	nd
128	+ve	+ve	nd	piro<0.1%	1280	nd
137				piro<0.1%	1280	**18.88%**
175	nd	-ve	nd	piro<0.1%	1280	nd
182				piro<0.1%	640	NR to T.p. Avery challenge

Table 12b: (group Vb) Summary of results obtained with different methods used to detect carrier status. Calf number E4 Exposed at Hunyani Estates

DAY post exposure	POLYMERASE CHAIN REACTION - P104		MICROSCOPY GIEMSA STAIN		*T. parva* IFAT-recip. titre	Prevalence of tick-infections
	PBM extract	Blood extract	Lymph Node	Blood smear		
0	-ve	-ve	nd	nps	80	nd
42	-ve	+ve		piro<0.1%	1280	nd
128	+ve	+ve		piro<0.1%	640	nd
137				piro<0.1%	320	**12.39%**
175	-ve	+ve	nd	piro<0.1%	320	nd
176	+ve (weak)	nd		piro<0.1%	320	
182				piro<0.1%	320	NR to T.p. Avery challenge

Table 12c: (group Vb) Summary of results obtained with different methods used to detect carrier status. Calf number E5 Exposed at Hunyani Estates

DAY post exposure	POLYMERASE CHAIN REACTION - P104		MICROSCOPY GIEMSA STAIN		*T. parva* IFAT-recip. titre	Prevalence of tick-infections
	PBM extract	Blood extract	Lymph Node smear	Blood smear s		
0	-ve	-ve	nd	nps	80	nd
42	-ve	-ve	nd	piro<0.1%	640	nd
128	+ve	+ve	nd	piro<0.1%	640	nd
137	nd	nd		piro<0.1%	320	**90.44%**
175	+ve	-ve	nps	piro<0.1%	640	nd
182				piro<0.1%	320	NR to T.p. Avery challenge

LN -Lymph node
PBM -peripheral blood mononuclear cells
nps -no parasites seen
NR -no reaction
piro -piroplasms seen
+ve -positive
-ve -negative
IFAT -cut off is 640
T. p. -*T. parva*

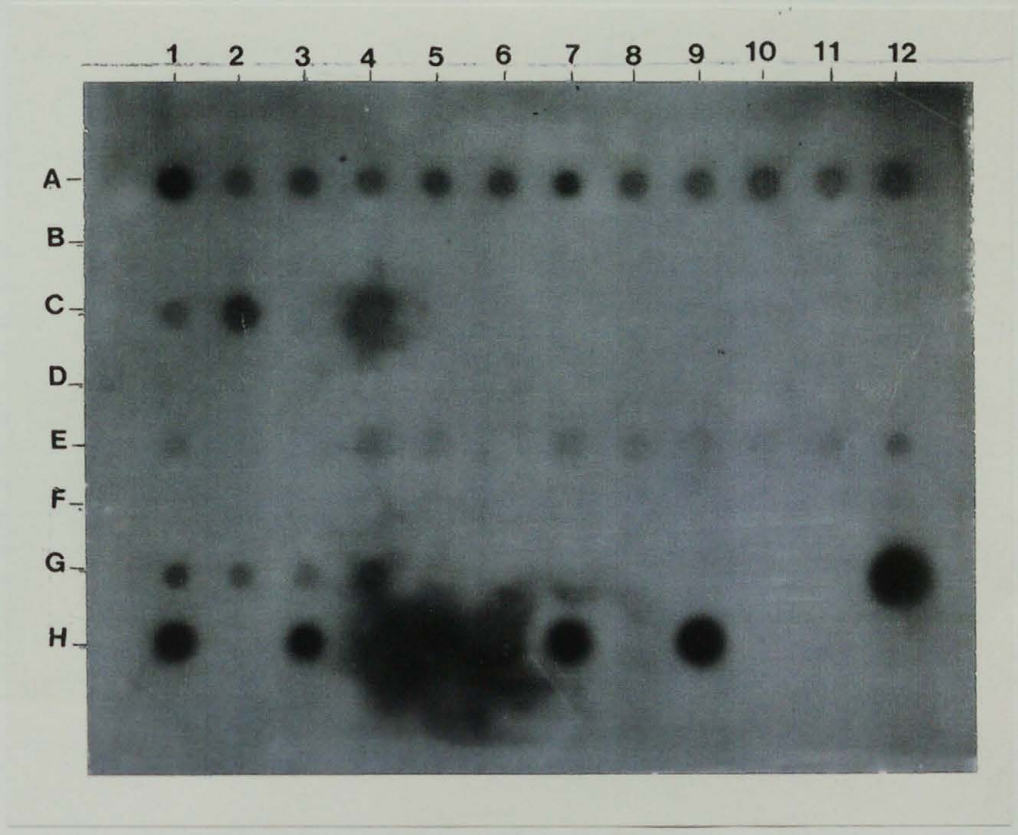
APPENDIX V

Figure 1

The results of dot blot hybridisation to confirm PCR products from samples from

Animal No: W70

PBM phenol chloroform extraction		**Result**		
A1	Positive control 1.0ng amplified			
T. parva Muguga piros DNA		+ve	H9	positive control
A2	Day 14 p.i.	+ve	H10	blank H11
A3	Day 25 p.i	+ve	H12	blank
A4	Day 40 p.i.	+ve	H8	blank
A5	Day 74 p.i.	+ve	pta	post tick application
A6	Day 95 p.i.	+ve	+ve	positive
A7	Day 109 p.i.	+ve	-ve	negative
A8	Day 137 p.i.	+ve	P/K	proteinase K digestion
A9	Day 179 p.i.	+ve	p.i.	post infection
A10	Day 204 p.i.	+ve		
A11	Day 218 p.i.	+ve		
A12	Day 263 p.i.	+ve		
C1	Day 278 p.i.	+ve		
C2	Day 361 p.i.	+ve		
C3	Day 446 p.i.	weak +ve		
C4	Day 493 p.i.	+ve		
C5	Day 0	-ve		
EDTA saponin lysed-/ PK digested extracts				
E1	Day 14 p.i.	+ve		
E2	Day 14 p.i.	-ve		
E3	Day 25 p.i.	-ve		
E4	Day 40 p.i.	+ve		
E5	Day 74 p.i.	+ve		
E6	Day 95 p.i.	-ve		
E7	Day 109 p.i.	weak +ve		
E8	Day 137 p.i.	weak +ve		
E9	Day 179 p.i.	weak +ve		
E10	Day 204 p.i.	weak +ve		
E11	Day 218 p.i.	weak positive		
E12	Day 263 p.i.	weak positive		
G1	Day 278 p.i.	+ve		
G2	Day 361 p.i.	weak +ve		
G3	Day 446 p.i.	weak +ve		
G4	Day 493 p.i.	+ve		
G6	Day 0	-ve		
G12	Positive control	+ve		
H1	Positive control 0.1 ng Piroplasm			
DNA amplified		+ve		
H2	BLANK	-ve		
H3	positive control	+ve		
H4	positive control	+ve		
H5	positive control	+ve		
H6	blank			
H7	positive control	+ve		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APPENDIX V

Figure 2

The results of dot blot hybridisation to confirm PCR products from samples from Animal No: **W73 and W74** using p104 primers.

W73

PBMs Phenol chloroform extracts.

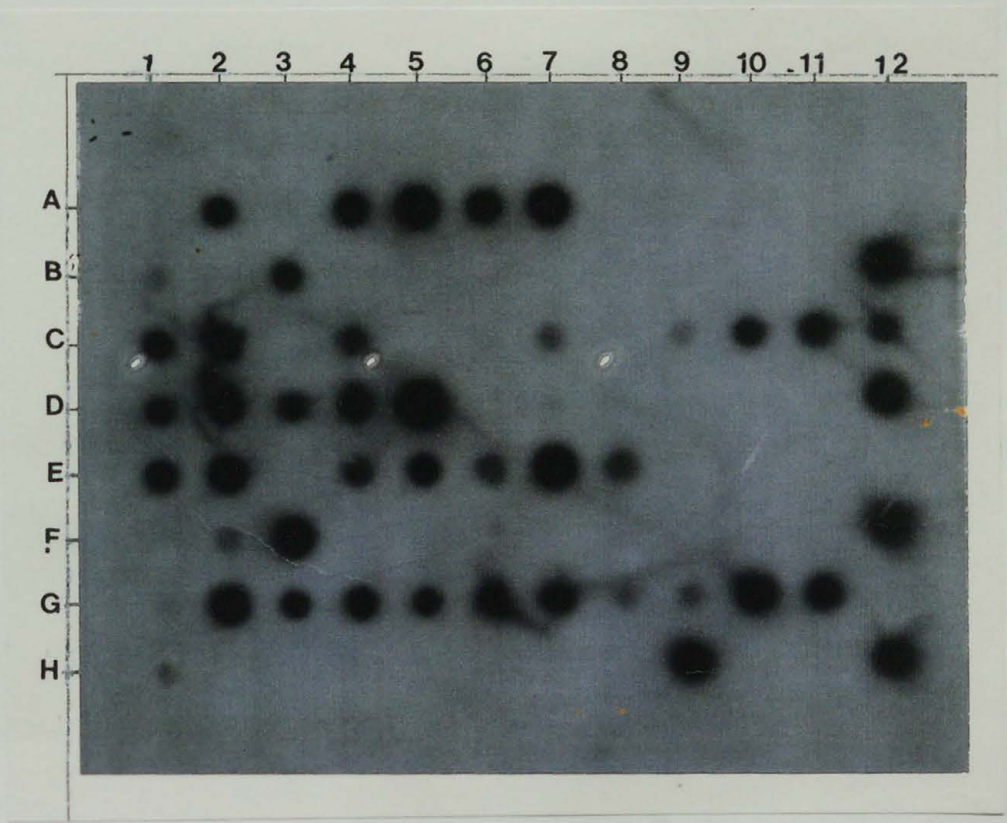
		Result
A1	Day 0	-ve
A2	Day 14 p.i	+ve
A3	Day 25 p.i	-ve
A4	Day 40 p.i	+ve
A5	Day 74 p.i	+ve
A6	Day 95 p.i	+ve
A7	Day 109 p.i	+ve
A8	Day 137 p.i	-ve
A9	Day 179 p.i	-ve
A10	Day 204 p.i	-ve
A11	Day 218 p.i	-ve
A12	Day 263 p.i	-ve
B1	Day 305 p.i	(weak +ve)
B2	Day 361 p.i	-ve
B3	Day 493 p.i	+ve
B12	+ve control	
C1	+ve control	+ve
W73	**EDTA saponin lysed extracts**	
C2	Day 14 p.i	+ve
C3	Day 25	-ve
C4	Day40 p.i	+ve
C5	Day 74 p.i	-ve
C6	Day 95 p.i	-ve
C7	Day 109 p.i	+ve
C8	Day 137 p.i	-ve
C9	Day 179 p.i	+ve
C10	Day 204 p.i	+ve
C11	Day 218 p.i	+ve
C12	Day 263 p.i	+ve
D1	Day 305 p.i	+ve
D2	Day 361 p.i	+ve
D3	Day 456 p.i	+ve
D4	Day 493 p.i	+ve
D5	Day 15 RPG +ve	
D12	+ve control	

W74

PBMs Phenol chloroform extracts

		Result
E1	positive control	+ve
E2	Day 14 p.i.	+ve
E3	Day 25 p.i.	-ve
E4	Day 40 p.i.	+ve
E5	Day 74 p.i.	+ve
E6	Day 95 p.i.	+ve
E7	Day 109 p.i.	+ve
E8	Day 137 p.i.	+ve
E9	Day 179 p.i.	-ve
E10	Day 203 p.i.	-ve
E11	Day 218 p.i.	-ve
E12	Day 263 p.i.	-ve
F1	Day 305 p.i.	-ve
F3	Day 14 RPG(phenol)	+ve
F4	Day 0	-ve
Edta saponin lysed P/K digested		
G1	Day 0	-ve
G2	Day 14 p.i.	+ve
G3	Day 25 p.i.	+ve
G4	day 40 p.i.	+ve
G5	Day 74 p.i.	+ve
G6	Day 95 p.i.	+ve
G7	Day 109 p.i.	+ve
G8	Day 137 p.i.	+ve
G9	Day 179 p.i.	+ve
G10	Day 213 p.i.	+ve
G11	Day 221 p.i.	+ve
G12	Day 263 p.i.	-ve
H9	Boleni +ve control	
H3	Bovine DNA	-ve control
H12	Muguga piros	+vecontrol

pta	post tick application	P/K	proteinase K digestion
p.i.	post infection	-ve	negative
nd	not done	+ve	positive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APPENDIX V

Figure 3 The results of dot blot hybridisation to confirm PCR products from samples from Animal No: **W75 and 1372** using p104 primers.

W75 PBMs Phenol chloroform extracts

		Result
A1	Day 0	-ve
A2	Day 14	+ve
A3	Day 25	-ve
A4	Day 40	-ve
A5	Day 74	+ve
A6	Day 95	nd
A7	Day 109	+ve
A8	Day 137	+ve
A9	Day 179	-ve
A10	Day 204	-ve
A11	Day 218	-ve
A12	Day 263	-ve
B1	Day 305	-ve
B2	Day 361	-ve
B3	Day 13 RPG	+ve
B4	Day 14 RPG	+ve
B5	Day 15 RPG	+ve
B12	+ve control	

1372 PBMs Phenol chloroform extracts

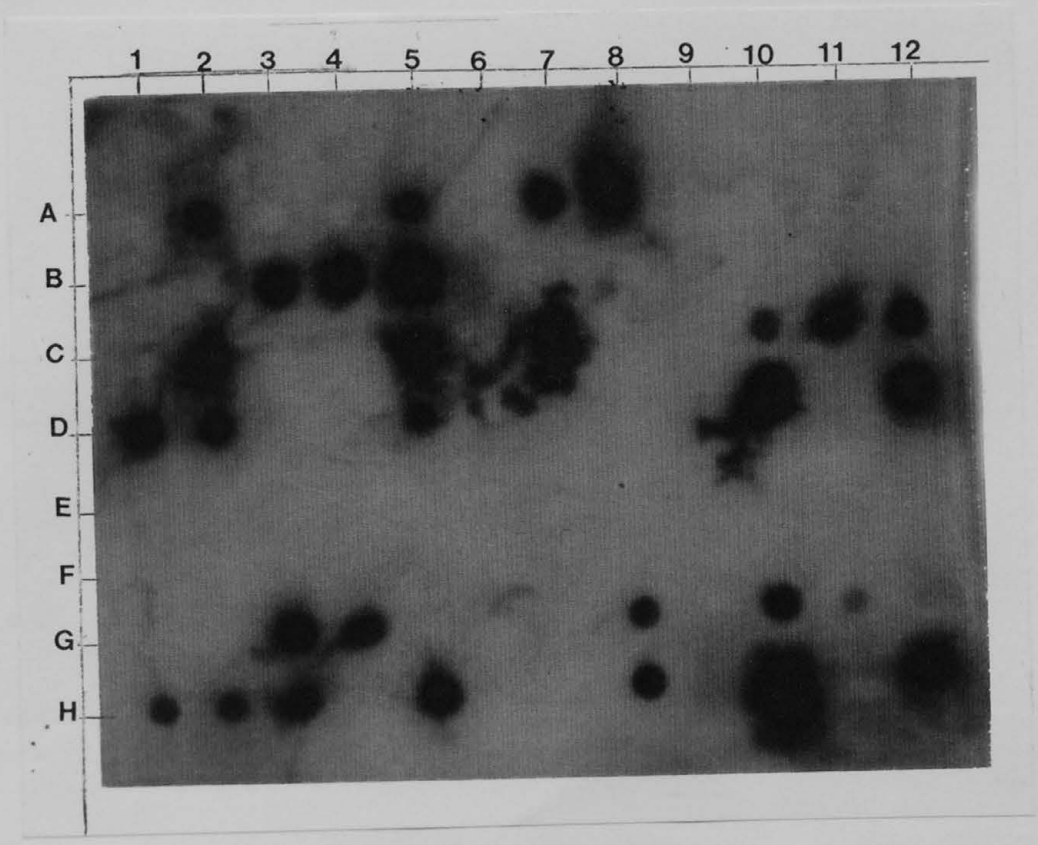
		Result
E1	Day 0	-ve
E2	Day 11 p.e.	-ve
E3	Day 19 p.e.	-ve
E4	Day 26 p.e.	-ve
E5	Day 37 p.e.	-ve
E6	Day 41 p.e.	-ve
E7	Day 66 p.e.	-ve
E8	Day 80 p.e.	-ve
E9	Day 115 p.e.	-ve
E10	Day 132 p.e.	-ve
E11	Day 150 p.e.	-ve
E12	Day 178 p.e.	-ve
F1	Day 220 p.e.	-ve
F2	Day 248 p.e.	-ve
F3	Day 262 p.e.	-ve
F4	Day 304 p.e.	-ve
F5	Day 319 p.e.	-ve
F6	Day 320 p.e.	-ve
F7	Day 402 p.e.	-ve
F8	Day 535 p.e.	-ve

W75	**EDTA saponin lysed digest**	
C1	Day 0	-ve
C2	Day 14	+ve
C3	Day 25	-ve
C4	Day 40	-ve
C5	Day 74	+ve
C6	Day 95	nd
C7	Day 109	+ve
C8	Day 137	-ve
C9	Day 179	-ve
C10	Day 204	+ve
C11	Day 218	+ve
C12	Day 263	+ve
D1	Day 305	+ve
D2	Day 361	+ve
D3	Day 456	-ve
D4	Day 493	-ve
D5	Boleni +ve control	
D10	+ve control	
D12	+ve control	
H8	Muguga piros DNA	

1372 EDTA saponon lysed P/K digest

G1	Day 0	-ve
G2	Day 26 p.e.	-ve
G3	Day 66 p.e.	+ve
G4	Day 80 p.e.	+ve
G5	Day 115 p.e.	-ve
G6	Day 132 p.e.	-ve
G7	Day 150 p.e.	-ve
G8	Day 248 p.e.	+ve
G9	Day 220 p.e.	-ve
G10	Day 262 p.e.	+ve
G11	Day 304 p.e.	+ve
G12		
H1	Day 319 p.e.	+ve
H2	Day 402 p.e.	+ve
H3	Day 535 p.e.	+ve
H4		
H5	Boleni positive control	
H6	Blank	
H7	Bovine DNA	

-ve	negative	+ve	positive
P/K	proteinase K digestion	p.i.	post infection
H10	Muguga piros DNA H12	Muguga piros DNA H9	
pta	post tick application		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APPENDIX V

Figure 4

The results of dot blot hybridisation to confirm PCR products from samples from **Animal No:1437 and 1447 using p104 primers.**

1437

Pbms Phenol chloroform extracts.

		Result
A1	Day 0	-ve
A2	Day 14	nd
A3	Day 25	nd
A4	Day 82	+ve
A5	Day 117	+ve
A6	Day 138	+ve
A7	Day 152	+ve
A8	Day 180	+ve
A9	Day 215	-ve
A10	Day 243	-ve
A11	Day 257	-ve
A12	Blank	
B1	Blank	
B2	Blank	
B3	Blank	
B4 -B12	Blank	

EDTA saponin lysed P/K digested extracts

		Result
C1	Day 0	nd
C2	Day 14	nd
C3	Day 14 pta	-ve
C4	Day 25 pta	-ve
C4	Day 82 pta	-ve
C5	Day 117 pta	-ve
C6	Day 138	-ve
C7	Day 152	+ve
C8	Day 180	-ve
C9	Day 215	+ve
C10	Day 243	-ve
C11	Day 257	+ve
C12	blank	
D1 - D12	Blank	

1447

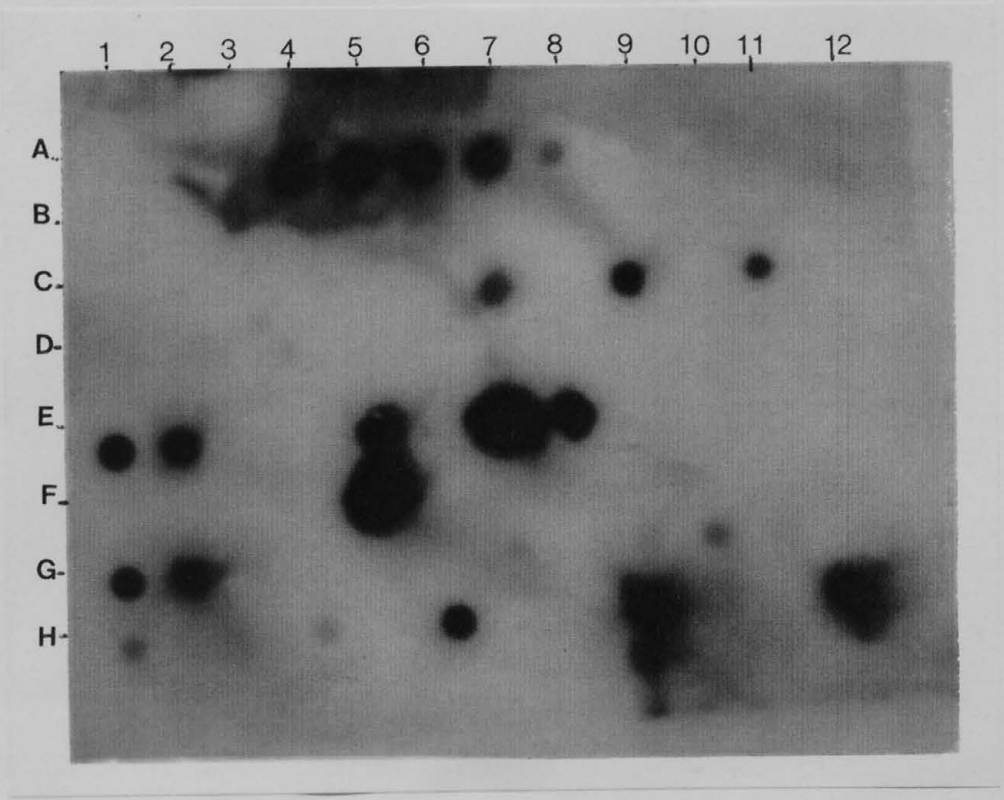
Pbms Phenol chloroform extracts

		Result
E1	Day 14 p.i. RPG	+ve
E2	Day 14 p.i.	+ve
E3	Day 25 p.i.	-ve
E4	Day 40 p.i.	-ve
E5	Day 74 p.i.	+ve
E6	Day 95 p.i.	-ve
E7	Day 109 p.i.	+ve
E8	Day 137 p.i.	+ve
E9	Day 179 p.i.	-ve
E10	Day 203 p.i.	-ve
E11	Day 218 p.i.	-ve
E12	Day 263 p.i.	-ve
F1	Day 305 p.i.	-ve
F2	Day 361 p.i.	-ve
F3	Day 456 p.i.	-ve
F4	Day 493p.i.	-ve
F5	Day 14 RPG	+ve

EDTA saponin lysed P/K digested extracts

		Result
G1	Day 14 p.i.	+ve
G2	Day 15 p.i.	+ve
G3	Day 25 p.i.	-ve
G4	Day 40 p.i.	-ve
G5	Day 74 p.i.	-ve
G6	Day 95 p.i.	-ve
G7	Day 109 p.i.	weak +ve
G8	Day 137 p.i.	-ve
G9	Day 179 p.i.	weak +ve
G10	Day 203 p.i.	weak +ve
G11	Day 218 p.i.	weak +ve
G12	Day 263 p.i.	-ve
H1	Day 305 p.i.	+ve
H2	Day 361 p.i.	-ve
H3	Day 456 p.i.	-ve
H4	Day 493 p.i.	+ve
H6	Boleni +ve control	
H9	Muguga +ve control	
H12	Muguga +ve control	

pta	post tick application	P/K	proteinase K digestion
-ve	negative	+ve	positive
p.i.	post infection	nd	not done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APPENDIX V

Figure 5

The results of dot blot hybridisation to confirm PCR products from samples from **Animal No: 1454 and 1456 using p104 primers.**

1454

Pbms Phenol chloroform extracts.

		Result
A1	D 14 RPG p.i.	+ve
A2	Day 14 p.i.	+ve
A3	Day 25 p.i.	+ve
A4	Day40 p.i.	+ve
A5	Day 74 p.i.	+ve
A6	Day 95 p.i.	-ve
A7	Day 109 p.i.	+ve
A8	Day 137 p.i.	+ve
A9	Day 179 p.i.	-ve
A10	Day 207 p.i.	-ve
A11	Day 218 p.i.	-ve
A12	Day 263 p.i.	-ve
B1	Day 305 p.i.	-ve
B2	Day 361 p.i.	+ve
B3	Day 456 p.i.	-ve
B4	Day 493 p.i.	nd

Edta saponin lysed P/K digested extracts

		Result
C1	Day 14 p.i.	+ve
C2	Day 15 p.i.	+ve
C3	Day 25 p.i.	+ve
C4	Day 40 p.i.	-ve
C5	Day 74 p.i.	+ve
C6	Day 95 p.i.	nd
C7	Day 109 p.i.	+ve
C8	Day 137 p.i.	+ve
C9	Day 179 p.i.	+ve
C10	Day 207 p.i.	-ve
C11	Day 218 p.i.	+ve
C12	Day 263 p.i.	-ve
D1	Day 305 p.i.	+ve
D2	Day 361 p.i.	+ve
D3	Day 456 p.i.	-ve
D4	Day 493 p.i.	+ve

1456

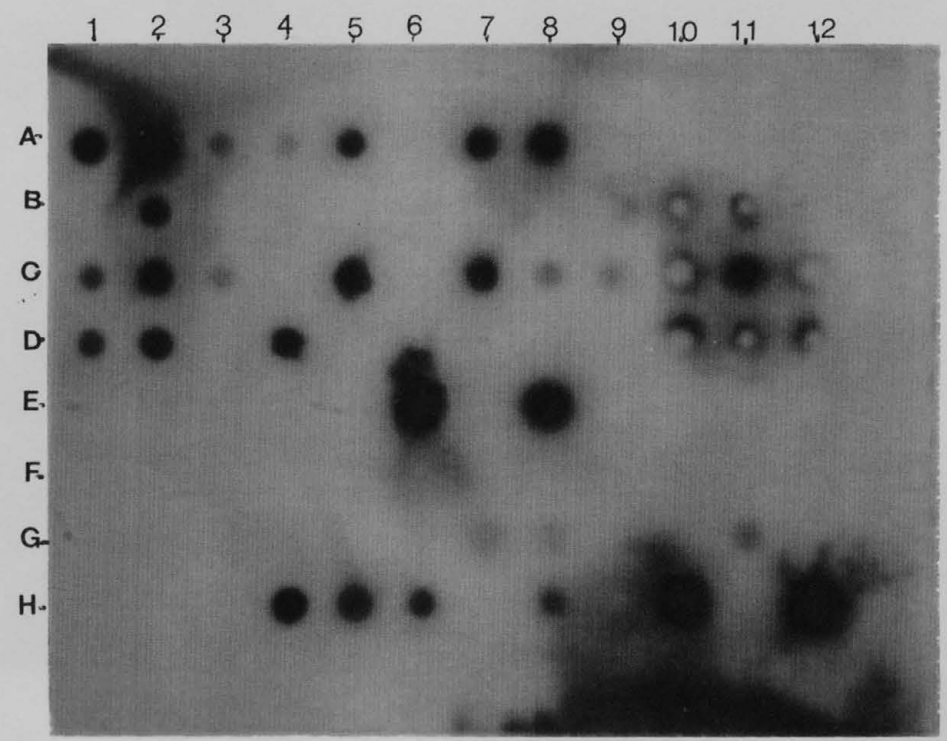
Pbms Phenol chloroform extracts

		Result
E1.	Day 0	-ve
E2.	Day 14 pta	nd
E3	Day 25 pta.	-ve
E4	Day 82 pta	-ve
E5	Day 117 pta	-ve
E6	Day 138 pta	+ve
E7	Day 152 pta	-ve
E8	Day 180 pta.	+ve
E9	Day 222 pta	-ve
E10	Day 250 pta	-ve
E11	Day 264 pta	-ve
E12	Day 306 pta	-ve
F1	Day 321 pta	-ve
F2	Day 402 pta	-ve
F3	Day 488 pta	-ve
F4	Day 534 pta	-ve

Edta saponin lysed P/K digested extracts

		Result
G1	Day 0	-ve
G3	Day 14 pta	nd
G4	Day 25 pta	nd
G5	Day 82 pta	-ve
G6	Day 117 pta	-ve
G7	Day 138 pta	weak +ve
G8	Day 152 pta	weak+ve
G9	Day 222 pta	-ve
G10	Day 250 pta	+ve
G11	Day 264 pta	weak+ve
G12	Day 306 pta	-ve
H1	Bov DNA -ve control	
H2	Day 321 pta	-ve
H3	Day 402 pta	-ve
H4	Day 488 pta	+ve
H5	Day 534 pta	+ve
H6 & H8	Boleni +ve control	
H10	Boleni +ve control	
H12	Muguga +ve control	

pta	post tick application
p.i.	post infection
nd	not done
+ve	positive
-ve	negative
P/K	proteinase K digestion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APPENDIX V

Figure 6

The results of dot blot hybridisation to confirm PCR products from samples from **Animal No: 1459 and 1442 using p104 primers.**

1459

Pbms Phenol chloroform extracts.

		Result
A1	Day 0 p.i.	-ve
A2	Day 14 p.i.	+ve
A3	Day 25 p.i.	+ve
A5	Day 40 p.i.	-ve
A6	Day 95 p.i.	+ve
A7	Day 74 p.i.	nd
A8	Day 109 p.i.	nd
A9	Day 137 p.i.	-ve
A10	Day 204 p.i.	-ve
A11	Day 213 p.i.	-ve
A12	Day 263 p.i.	-ve
B1	Day 305 p.i.	-ve
B2	Day 361 p.i.	-ve
B3	Day 456 p.i.	-ve
B4	Day 493 p.i.	nd
B5	Boleni positive control	
B11	Bov DNA -ve control	
B12	Bov DNA -ve control	

Edta saponin lysed P/K digested extracts

		Result
C1	Day 0 p.i.	-ve
C2	Day 14.p.i.	+ve
C3	Day 25 p.i.	-ve
C4	Day 40 p.i.	-ve
C5	Day 74 p.i.	+ve
C6	Day 95 p.i.	-ve
C7	Day 109 p.i.	+ve
C8	Day 137 p.i.	-ve
C9	Day 179 p.i.	-ve
C10	Day 204 p.i.	+ve
C11	Day 218 p.i.	+ve
C12	Day 263 p.i.	+ve
D1	Day 305 p.i.	+ve
D2	Day 361 p.i.	+ve
D3	Day 456 p.i.	+ve
D4	Day 493 p.i.	nd

1442

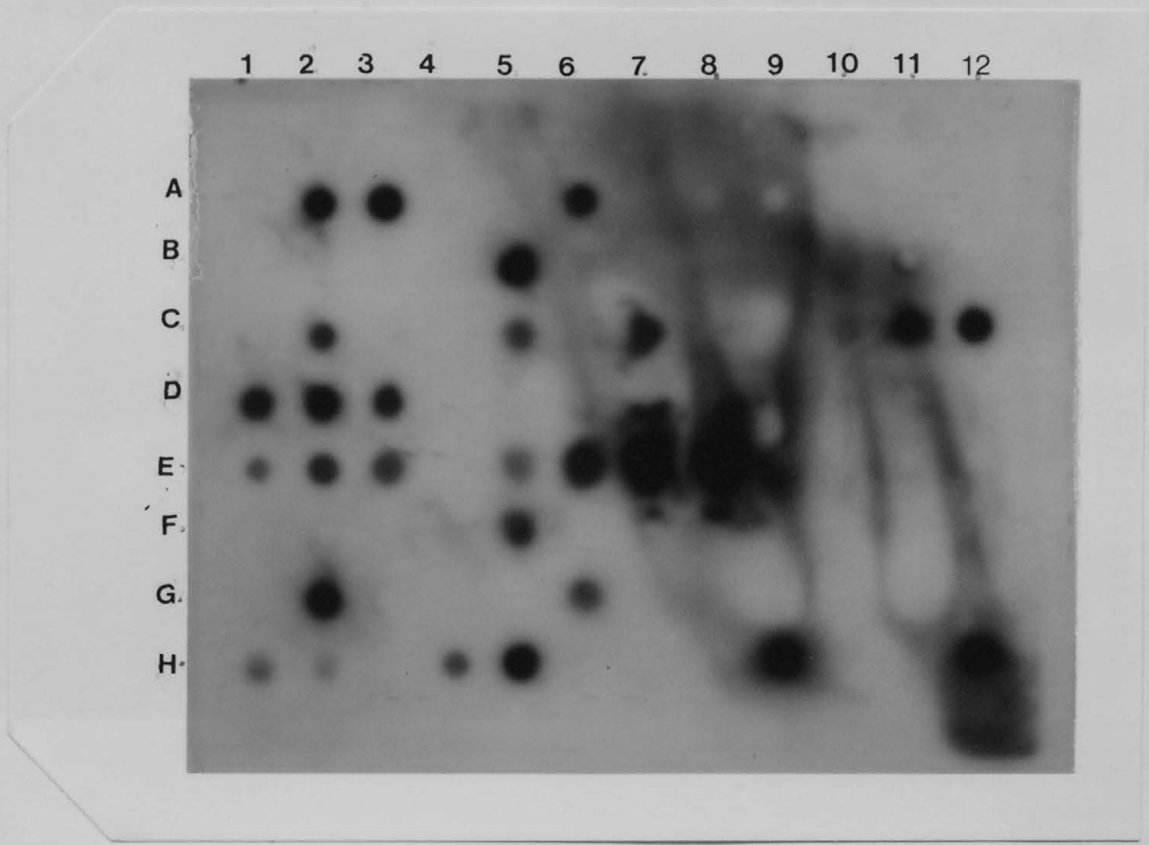
Pbms Phenol chloroform extracts

		Result
E1.	Day 14 p.i.	+ve
E2.	Day 14 p.i.	+ve
E3	Day 25. p.i.	+ve
E4	Day 40 p.i.	-ve
E5	Day 74 p.i.	+ve
E6	Day 95 p.i.	+ve
E7	Day 109 p.i..	+ve
E8	Day 137 p.i.	+ve
E9	Day 179 p.i.	+ve
E10	Day 207 p.i.	-ve
E11	Day 213 p.i.	-ve
E12	Day 263 p.i.	-ve
F1	Day 305 p.i.	-ve
F2	Day 361 p.i.	-ve
F3	Day 456 p.i.	-ve
F4	Day 493 p.i.	-ve
F5	Boleni +ve control	

Edta saponin lysed P/K digested extracts

		Result
G1	Day 0	-ve
G2	Day 14 p.i.	+ve
G3	Day 25 p.i.	-ve
G4	Day 40 p.i.	-ve
G5	Day 74 p.i.	-ve
G6	Day 95 p.i.	weak +ve
G7	Day 109 p.i.	-ve
G8	Day 137 p.i.	-ve
G9	Day 179 p.i.	-ve
G10	Day 207 p.i.	-ve
G11	Day 213 p.i.	-ve
G12	Day 263 p.i.	-ve
H1	Day 305 p.i.	weak +ve
H2	Day 361 p.i.	weak +ve
H3	Day 456 p.i.	-ve
H4	Day 493 p.i.	+ve
H5	Boleni +ve control	
H7	Bovine DNA -ve control	
H9	Boleni +ve control	
H12	Muguga +ve control	

pta	post tick application	-ve	negative	+ve	positive
P/K	proteinase K digestion	nd	not done	p.i.	post infection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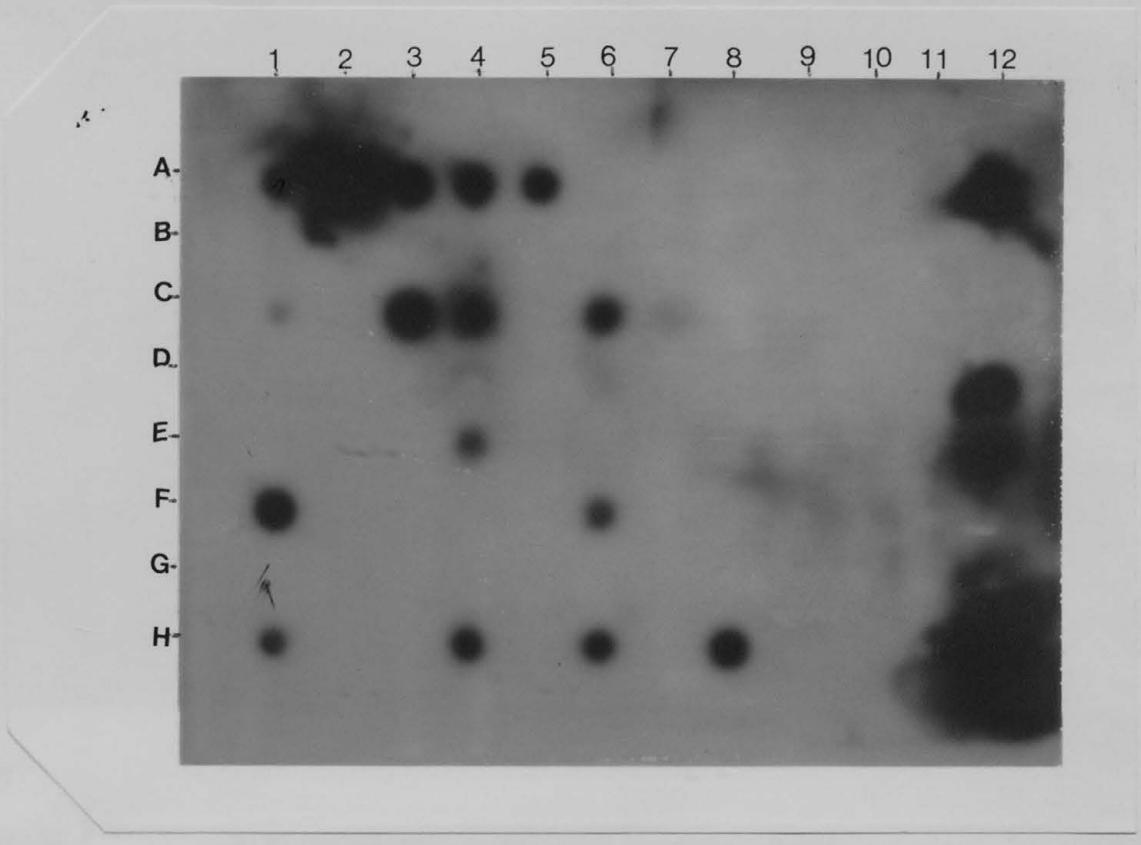
APPENDIX V

Figure 7 PCR results for second Hunyani Estates exposure

		Result
Calf E1		
A1	Day 42 post exposure PBM	+ve
A2	Day 42 post exposure EDTA blood	+ve
A3	Day 128 post exposurePBM	+ve
A4	Day 128 post exposure EDTA	+ve
A5	Day 175 post exposure EDTA	-ve
Calf E4		
C1	Day 42 post exposure PBM	+ve
C2	Day 42 post exposure EDTA blood	-ve
C3	Day 128 post exposurePBM	+ve
C4	Day 128 post exposure EDTA	+ve
C5	Day 175 post exposure EDTA	-ve
C6	Day 175 post exposure EDTA	+ve
C7	Day 176 post exposure PBM	weak +ve
C10	Day 0 EDTA	-ve
C11	Day 0 PBM	-ve
Calf E5		
E1	Day 42 post exposure PBM	+ve
E2	Day 42 post exposure EDTA blood	-ve
E3	Day 42 post exposurePBM	-ve
E4	Day 128 post exposure EDTA	weak +ve
E5	Day 175 post exposure EDTA	-ve
F1	Day 42 post exposure PBM	-ve
G6	day 175 post exposure PBM	+ve
H6 :, H7	*T. parva* Boleni macroschizont DNA	+ve
H8	*T. parva* Muguga piroplasm DNA	+ve
G12	*T. parva* Muguga piroplasm DNA	+ve
A12	*T. parva* Muguga piroplasm DNA	+ve
C12	*T. parva* Muguga piroplasm DNA	+ve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APPENDIX VI ; IFAT RESULTS
INDIRECT FLUORESCENT ANTIBODY TEST
RECIPROCAL SERUM DILUTIONS
Group I

Day p.i.	W70	W73	W74	W75
0	160	80	80	160
17	640	1280	640	320
40	640	1280	2560	1280
46	5120	2560	5120	2560
53	5120	2560	5120	5120
60	1280	2560	5120	1280
67	640	1280	2560	2560
74	1280	320	2560	320
81	640	640	640	640
88	5120	2560	2560	5120
95	640	5120	640	320
109	320	160	320	320
123	640	640	640	640
137	640	640	640	640
151	320	640	320	320
165	320	1280	320	320
179	320	320	320	160
193	160	640	320	640
218	320	320	320	320
221	320	320	80	320
235	320	2560	1280	2560
249	1280	5120	640	5120
263	5120	2560	5120	2560
278	320	320	320	640
291	320	640	320	640
305	320	320	320	640
319	640	640		640
333	640	640		640
347	640	320		640
361	320	320		640
375	320	320		160
389	320	160		
403	160	320		
427	320	640		
447	320	640		
456	160	320		
522	320	640		
550	640	1280		
562	640	1280		
572		1280		

INDIRECT FLUORESCENT ANTIBODY TEST
RECIPROCAL SERUM DILUTIONS

	Group II		**Group III**	
Day p.i.	Tag 1454	Tag 1442	Tag 1447	Tag 1459
0	160	160	160	160
17	320	320	160	160
40	5120	320	2560	1280
46	5120	320	2560	2560
53	2560	320	2560	1280
60	2560	160	2560	2560
67	5120	160	2560	640
74	1280	320	640	640
81	1280	160	1280	1280
88	5120	10240	1280	2560
95	640	160	160	640
109	640	160	320	160
123	1280	320	1280	1280
137	1280	320	1280	1280
151	1280	320	640	640
165	640	160	320	320
179	320	160	320	320
193	640	160	640	640
207	160	320	160	320
218	320	320	320	320
235	2560	640	1280	1280
249	10240	640	5120	2560
263	2560	640	2560	2560
278	1280	320	1280	1280
291	2560	640	1280	1280
305	1280	320	1280	1280
319	1280	320	2560	1280
333	1280	640	1280	640
347	1280	640	640	640
361	1280	320	2560	640
375	640	160	1280	160
389	640		640	160
403	640	160	640	320
427	640	160	640	320
447	640	160	320	320
456	1280	640	640	640
522	640	640	640	640
550	640	1280	320	320
562	1280	1280	1280	640
572	1280	1280	1280	640

INDIRECT FLUORESCENT ANTIBODY TEST
RECIPROCAL SERUM DILUTIONS

GroupIV			Group Va	
Day post tick application			Day post exposure.	
Day pta	**Tag1456**	**Tag 1437**	**Days post i**	**Tag 1372**
0	160	80	0	160
40	640	1280	40	1280
81	320	320	83	640
87	640	1280	89	1280
95	640	1280	97	1280
101	640	1280	103	1280
108	320	640	110	1280
115	2560	5120	117	640
122	1280	640	124	640
129	1280	10240	131	2560
138	160	640	140	640
150	80	80	152	320
164	160	160	166	640
178	320	320	180	320
192	320	320	194	640
206	160	160	208	640
220	160	320	222	320
234	320	320	236	1280
248	160	320	250	640
264	160	80	266	640
276	320		278	2560
290	1280		292	5120
304	640		306	10240
319	320		321	2560
332	640		334	1280
348	320		350	1280
360	320		362	5120
374	640		376	1280
388	320		390	640
402	320		404	640
416	160		418	320
430	160		432	640
444	160		446	320
468	320		470	640
488	320		501	1280
518	320		520	640
534	640		566	1280
591	1280		593	640
603	1280		605	640
613	1280		61[illegible]	640

INDIRECT FLUORESCENT ANTIBODY TEST
RECIPROCAL SERUM DILUTION

Group Vb

Days post exposure at Hunyani estates.

	E1	**E2**	**E3**	**E4**	**E5**
0	80	160	80	160	80
28	640	640	1280	1280	320
40	1280			1280	640
56	640			320	320
70	1280			320	640
128	640			640	640
137	1280			320	320
158	**640**			**320**	**640**
182	**640**			**320**	**640**
203	**640**			**320**	**640**
231	320			640	320
243	640			1280	2560