IDENTIFICATION OF *THEILERIA PARVA* VACCINE CANDIDATE ANTIGENS RECOGNISED BY CYTOTOXIC T LYMPHOCYTES FROM ZEBU CATTLE

A THESIS IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN APPLIED VETERINARY PARASITOLOGY

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DECLARATION

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This thesis is dedicated to my beloved wife Fridah Asiko, and daughter Audrey for their distinguished encouragement and moral support which was the motivation that helped me to complete this work.

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ABSTRACT

East Coast fever (ECF), caused by *Theileria parva,* a tick-borne intracellular apicomplexan parasite, is a highly fatal lymphoproliferative disease of cattle. Immunity against *T. parva* has previously been shown to be mediated through lysis of schizont infected cells by Major histocompatibililty complex (MHC) class I restricted $CD8^+$ cytotoxic T lymphocytes (CTL). A strategy has recently been developed to identify CTL target schizont antigens and has provided a solid basis for the development of a subunit vaccine against ECF.

To date CTL target antigens have been identified using CTL derived from *Bos taurus* and Boran (*B. indicus)* cattle immunised with the Muguga stock of *T. parva.* It has been hypothesised that additional antigens are required to formulate a sub-unit vaccine that would protect the out bred cattle population at risk from a highly heterogeneous *T. parva* population. This study aimed to extend and expand the process of vaccine candidate antigen identification by employing CTL obtained from genetically diverse East African zebu cattle immunised with the cocktail of *T .parva* stocks that constitute the FAO1 live vaccine that protects cattle across the ECF endemic areas. *T. parva* specific $CD8^+$ polyclonal CTL lines were generated by repeated *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) from ITM immunised zebu cattle with *T. parva* infected lymphoblasts. CTL were confirmed to express a $CD3^+$ $CD8^+$ phenotype and specifically lyse autologous *T. parva* infected cells in ^{51}Chromium release cytotoxicity assays. CTL lines were tested for recognition of immortalised skin fibroblasts (iSF) infected with recombinant Modified Vaccinia Ankara strain (MVA) viruses expressing the previously

identified schizont antigens. Only CTL from one calf (BY120), showed specific recognition of these antigens; CTL responded specifically to iSF infected with MVA expressing antigen Tp2. Synthetic peptides were employed to identify a novel CTL epitope and analysis of the consensus sequences of positive peptides suggested that the minimal length antigenic peptide was the 10mer $Tp2_{138-147}$ (KTSIPNPCKW). CTL were next used to immunoscreen iSF transiently transfected with 96 cDNA encoding secreted/membrane bound proteins and 644 cDNA pools derived from a *T. parva* (Muguga) schizont cDNA expression library. CTL from calf BY126 specifically responded to two cDNA pools. All the other CTL failed to recognize transfected iSF.

This study has demonstrated that CTL isolated from Zebu cattle immunized with a cocktail of *T. parva* stocks recognize novel schizont antigens and continued immunoscreening is required to identify these antigens, which will constitute valuable additions to the vaccine candidates currently being evaluated.

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ABBREVIATIONS

BSA	Bovine Serum Albumin
BVD	Bovine Viral Diarrhea
CTL	Cytotoxic T Lymphocyte
cDNA	Complementary Deoxyribonucleic Acid
DMEM	Dulbecos Minimum Essential Medium
ECF	East Coast Fever
EDTA	Ethylene Diamine Tetra Acetic Acid
ELISpot	Enzyme Linked Immunospot
FACS	Fluorescent Activated Cell Sorter
FBS	Fetal Bovine Serum
FITC	Flouroisothiocyanate
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
IFN-γ	Gamma Interferon
iSF	Immortalized Skin Fibroblasts
IgG	Immunoglobulin G
IL	Interleukin
ILRI	International Livestock Research Institute
mAb	Monoclonal Antibody
MHC	Major Histocompatibililty Complex
Mg	Milligram
Min	Minute
ml	Milliliter

CHAPTER ONE

1.0 INTRODUCTION AND OBJECTIVES

1.1 Introduction

Tick-borne diseases are a major constraint to livestock improvement throughout the tropics. Worldwide, an estimated 600 million cattle are exposed to anaplasmosis and babesiosis and 200 million cattle are exposed to theileriosis. In sub-saharan Africa as many as 175 million cattle may be exposed to cowdriosis. In Eastern, Central and Southern Africa, theileriosis (East Coast Fever), caused by *Theileria parva*, is considered to be the most significant tick borne disease of cattle. The parasite infects cattle and buffalo (*Synerus caffer*) (Mukhebi *et al.,* 1992).

East Coast fever (ECF) is an acute and often fatal disease of cattle in Eastern, Central and Southern Africa that is caused by *T. parva*, an apicomplexan parasite transmitted by the three- host tick, *Rhipicephalus appendiculatus* (Norval *et al*, 1992). The principal losses from this disease are due to the death of susceptible cattle and substantial production losses reaching up to US$300 per annum table 1 (Mukhebi *et al.*, 1992).

Many of the farmers who suffer losses are smallholder dairy producers since improved high yielding exotic and crossbred dairy cattle are very susceptible to ECF (Dolan *et al.*, 1982; Ndung'u *et al.*, 2005).

The only effective control strategy at present involves an integrated approach combining, extensive use of acaricides, grazing management for tick control, selective breeding for ECF resistant stock and treatment using anti-theilerial drugs. These methods have a number of drawbacks including expense, development of resistance to acaricides and the negative impact on the environment caused by acaricides (Young *et al.*, 1998). One of the emerging methods of control is vaccination. The infection and treatment method (ITM) is a strategy based on the infection of susceptible cattle with sporozoite stabilates and simultaneous treatment with long acting tetracyclines (Radley *et al.*, 1975).

The ITM is very effective but faces challenges including dependence on an intact cold chain, variable control of the dose of inoculation, the high cost of tetracycline and concerns over the introduction of new vaccine strains to resident tick populations resulting into epidemics to previously naïve cattle (McKeever and Morrison, 1990). A cheap, efficacious and easy to deliver sub-unit vaccine would provide an attractive alternative means to contribute to the sustainable control of ECF (McKeever *et al.*, 1999).

Immunity induced by ITM is based on cell mediated mechanisms targeted at the schizont infected cell and is further associated with parasite-specific major histocompatibililty complex class I (MHC-I) restricted $CD8^+$ cytotoxic T lymphocytes (CTL) that kill schizont infected cells (McKeever and Morrison, 1990; Goddeeris *et al.*, 1990). Parasite

antigens recognized by MHC-I restricted $CD8^+$ CTLs are therefore logical candidates for a sub-unit vaccine against ECF.

A successful strategy has been developed to identify CTL target antigens from *T. parva*, which involves the *in vitro* screening of autologous bovine immortalised skin fibroblasts (iSF) transiently transfected with schizont complementary DNA (cDNA) using *T. parva* specific CTL (Graham *et al.*, 2006). To date, ten schizont antigens have been identified and are currently being evaluated for their immunogenicity and efficacy under laboratory conditions.

The CTL used to identify these antigens have been isolated from exotic breeds of cattle namely (Friesian and Jersey) and Boran or Boran/exotic crosses that had been immunized by ITM using the Muguga stock of *T. parva* (Graham *et al.*, 2006). There is need to identify additional antigens, restricted by multiple MHC-class I alleles, which are conserved between parasite stocks. Zebu animals have diverse MHC class I haplotypes (Ellis *et al.*, 1999).

This study therefore aimed to extend vaccine candidate antigen identification to CTLs generated from East African Zebu cattle (*B. indicus)* from three different geographical areas of Kenya. Cattle were vaccinated by ITM using the FAO1 cocktail (containing three stocks of *T. parva* Muguga, *T. parva* Kiambu 5, *T. parva* Serengeti transformed). Cattle were vaccinated with this cocktail to enhance the probability of isolating CTL that recognise antigens conserved between stocks. Incorporation of such antigens into a

subunit vaccine would help in protecting the region's diverse cattle population against the genetically diverse parasite population.

1.2 Objectives

1.2.1 General Objective

To identify *Theileria parva* vaccine candidate antigens for use in sub unit vaccine development by screening a schizont expression cDNA library using schizont specific MHC class 1 restricted cytotoxic T lymphocytes.

1.2.2 Specific Objectives

1. To generate and characterize the parasite stock specificity of MHC class I restricted $CD8^+$ CTL from Zebu cattle immunized by ITM using the FAO1 cocktail vaccine.
2. To use these CTL to screen vaccine candidate antigens, a random schizont cDNA library and selected genes to identify target antigens.
3. To confirm that antigens identified are targets of MHC class I restricted CTL and to map epitopes using synthetic peptides.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 East Coast fever

East Coast Fever (ECF), caused by the tick-borne protozoan parasite, *T. parva*, is a lymphoproliferative disease characterized by high fever, lymphadenopathy and high mortalities especially in exotic cattle (Dolan *et al.*, 1982; Ndung'u *et al.*, 2005). The clinical and pathological changes are associated with invasion of lymphoid and non-lymphoid tissues with parasitized lymphocytes (Irvin and Morrison, 1987). The disease is endemic in 11 countries of sub-Saharan Africa (Norval *et al.*, 1992) and is of major economic consequence; claiming over one million cattle each year in East, Central and Southern Africa (Mukhebi *et al.*, 1992). It has been calculated that the disease causes direct losses in beef, milk, traction and manure, in treatment, acaricides, research and extension costs in the 11 affected countries of US$300 million a year as shown in Table 1, in addition to an estimated mortality of 1.1 million cattle (Mukhebi *et al.*, 1992). The livelihood of smallholder farmers depends on one or two cattle and hence, the financial burden due to loss of income and livestock products due to this disease impacts on the quality of all aspects of family life.

Table 1.

Estimated losses in 1989 due to East Coast fever in 11 African countries affected by ECF.

Item	Loss in (000's) US$	% of Total loss
Beef loss, total	20,607	12
-mortality loss	17,232	-
-morbidity loss	3,375	-
Milk loss total	78,697	47
-mortality loss	7,495	-
-morbidity loss	71,202	-
Animal traction loss/ha	21,308	13
Manure loss	88	0
Treatment	8,114	5
Acaricide application	3,008	20
Research and extension	8,550	4
Total loss (000's) US$	168,402	100
ECF loss per cattle head	US$ 7.00	
ECF loss per hectare	US$1.10	

Source: Mukhebi *et al.*, (1992).

2.1.1 Life cycle of *T. parva*

The life cycle of *T. parva* occurs both in the tick and mammalian host (figure 1) (Norval *et al.*, 1992). *T. parva* sporozoite stages in the acinar cells of infected tick salivary glands are inoculated into the bovine host during tick feeding (Stagg *et al.*, 1981; Webster *et al.*, 1985). They move through the interstitial fluid and rapidly infect host lymphocytes, and develop into intracytoplasmic multinucleated macroschizonts. The presence of the parasite within the host lymphocyte induces the malignant transformation of the host cell (Mehlhorn and Schein, 1984). The host cell and the schizont divide synchronously resulting in the clonal expansion of the infected lymphocyte. Within the infected lymphocyte, schizonts are associated with microtubules involved in spindle formation during host cell division (Conrad *et al.*, 1986).

During host cell mitosis, the schizont uses the host cell mitotic spindle to become distributed over the two daughter cells. Infected animals develop a lymphoma-like disorder that can typically results in death within 2-3 weeks (Dobbelaere and Heussler, 1999). From day 14 after tick infection of cattle, individual schizonts undergo merogony to form merozoites (microschizonts) that rupture the host cell and are released into the blood stream where they invade erythrocytes and develop into piroplasms (Norval *et al*, 1992). Ticks ingest the piroplasms during a blood meal and following a sexual cycle in the gut, ookinetes migrate to the salivary glands (Schein *et al.*, 1977). Sporogony is initiated when the tick attaches to the host animal, resulting in the release of sporozoites into the saliva ready for transmission (Fawcett *et al.*, 1985).

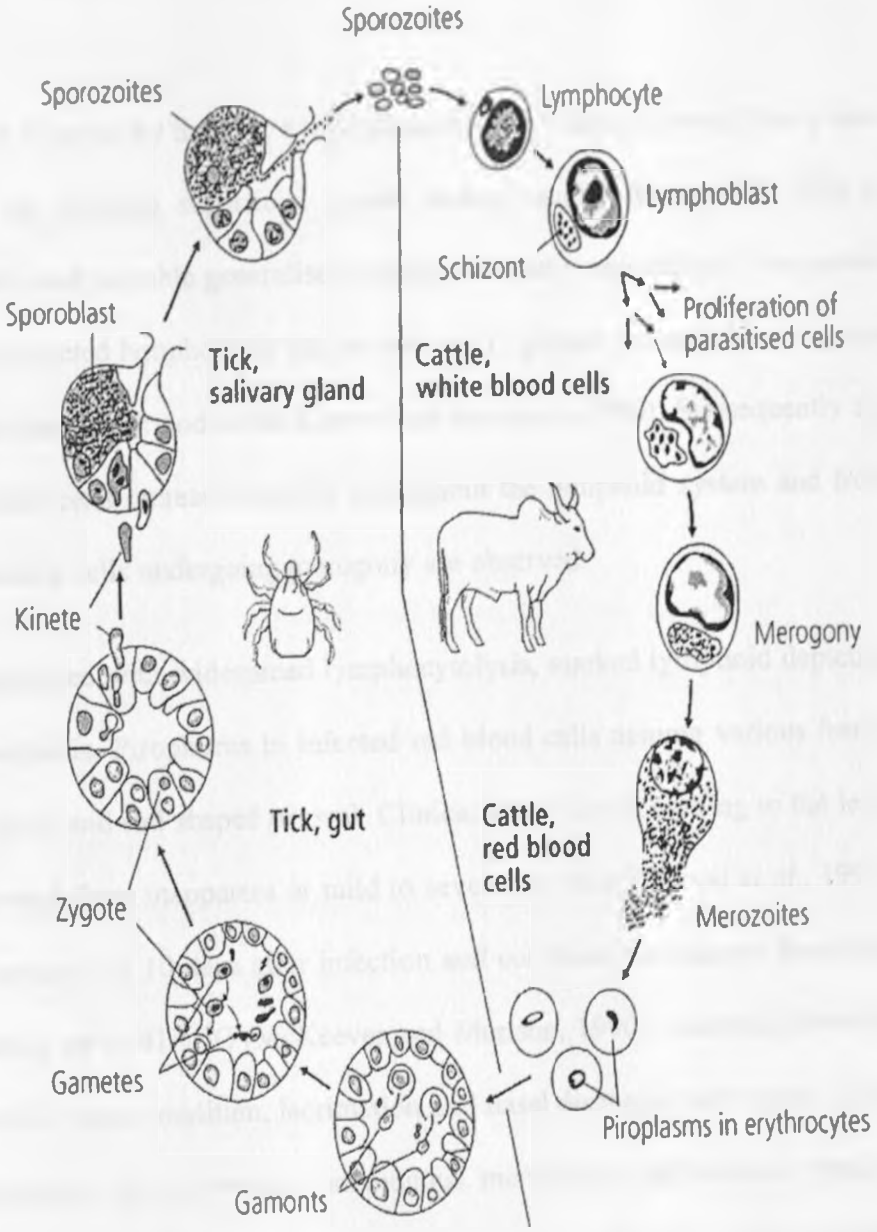


Figure 1. Life cycle of *T. parva* in the mammalian and arthropod hosts (Source: Norval *et al.*, 1992).

2.1.2 Pathogenesis and clinical signs of ECF

After inoculation of *T. parva* by the tick, a mild phase of 5 to 9 days follows. This is seen as a swelling of the draining superficial lymph nodes, usually the parotid. This is followed by a visible and palpable generalised lymphadenopathy especially of the parotid lymph node before infected lymphocytes can be detected in giemsa stained biopsy smears from the local draining lymph nodes (McKeever and Morrison, 1990). Subsequently the number of parasitised cells increases rapidly throughout the lymphoid system and from about 14 days onwards, cells undergoing merogony are observed.

The disease is associated with widespread lymphocytolysis, marked lymphoid depletion manifested as leucopenia. Piroplasms in infected red blood cells assume various forms, but are typically small and rod shaped or oval. Clinical signs vary according to the level of challenge and range from inapparent or mild to severe and fatal (Norval *et al.*, 1992). Typically, fever occurs 7 to 10 days after infection and continues throughout the course of infection, reaching up to 41.5 ^{o}C (McKeever and Morison, 1990). Anorexia develops and the animal rapidly loses condition, lacrimation and nasal discharge may occur. Other signs include petechiae and echymoses on mucous membranes and corneal opacity. Terminally, dyspnoea is common and just before death; there is a sharp fall in body temperature and bilateral nasal discharge and diarrhoea (Dolan *et al.*, 1982).

Post-mortem gross lesions include enlarged lymph nodes, genaralised haemorrhages, button ulcers in the abomasums, interlobular emphysema and severe pulmonary oedema, and infarcts in the kidneys. Histopathological findings include; evidence of proliferating

lymphoblastoid cells in various organs. Most animals that recover remain carriers of infection and are immune to subsequent challenge with the same strain but susceptible to some heterologous strains (Moll *et al.*, 1986).

2.1.3 Treatment and control of ECF

An integrated approach with preventive methods involving a combination of effective fencing, pasture management, acaricide application, selection of tick resistant cattle and immunization is the most rational way of controlling ECF (Young *et al.*, 1998). However once animals have been infected, anti-theilerial naphthaquinone derivatives can be administered. Parvaquone and buparvaqoune administered at dosages of 10 mg/kg body weight (bwt) and 2.5 mg/kg bwt respectively, both given intramuscularly as two doses 48 hours apart, and the lactate salt of the coccidiostat halofuginone (1.2 mg/kg bwt) can be used as chemotherapeutic agents. However, these drugs are expensive and early and accurate diagnosis is required for effective therapy (Norval *et al.*, 1992).

Rigid tick control programmes involving bi-weekly acaricide treatment are used by farmers to keep ECF-free exotic and improved herds. Similar efforts by poor smallholder farmers have often failed, because tick control is costly and improper application schedules have induced natural development of resistance by ticks. Besides environmental pollution, regular use of chemicals and drugs introduces residues in the food chain with food safety implications (Anon, 1968). Immunisation against ECF by the Infection and Treatment Method (ITM) is in high-demand and is now widely gaining

acceptance (Mutugi, *et al.*, 1989). The ITM is a strategy based on the infection of susceptible cattle with cryopreserved sporozoite stabilates derived from infected ticks and simultaneous treatment with long-acting tetracyclines (Radley *et al.*, 1975). ITM induces solid immunity to homologous and in certain instances heterologous stocks of the parasite. Whilst very efficacious several features of ITM including cost, cold chain requirements, induction of carrier state, and the risk of introducing new parasite strains are constraints to the widespread and sustainable adoption of ITM (Norval *et al.*, 1992; McKeever and Morrison, 1990). The development of a cheap, efficacious, easy to deliver sub-unit vaccine, would therefore have enormous positive benefits in combating the disease (McKeever et *al.*, 1999).

2.1.4 Immunity to ECF

2.1.4.1 Humoral immune responses against *T. parva*

Primary exposure of cattle to T. *parva* results in an antibody response, which although alone is not protective, contains antibodies that are capable of neutralizing sporozoites *in vitro* (Musoke *et al.*, 1992). The 67 kD major sporozoite surface coat protein, p67, has been shown to be the major neutralizing target of these antibodies (Nene *et al.*, 1995. Antibodies in sera from cattle hyperimmunised with lysates of *T. parva* sporozoites or cattle from endemic areas recognise p67, and characterization p67 with monoclonal antibodies shows that the B-cell epitopes on the antigen are conserved among different parasite stocks (Musoke *et al.*, 1984); Bishop *et al.*, 2003). p67 has been developed into

an experimental sub-unit vaccine against *T. parva* and has been evaluated under laboratory and field conditions. Fragments of the p67 protein have been generated in an attempt to overcome problems of protein stability and have been evaluated in field trials with a 50% reduction in field cases. Evaluation of the immunizing potential of a baculovirus expressed recombinant form of p67 in the laboratory has shown up to 85% protection under laboratory conditions (Musoke *et al.*, 2005) and this protein is now being further evaluated by a veterinary vaccine company for potential commercialisation.

2.1.4.2 Cellular immune responses against *T. parva*

Cattle that naturally recover from ECF or are immunized by ITM develop immunity to homologous and sometimes heterologous challenge. This immunity is mediated by cellular mechanisms that target schizont infected cells (McKeever and Morrison, 1990). Protection is not transferable through serum, immune animals invariably develop a schizont parasitosis following challenge, and protection can be induced by challenge with large numbers of autologous parasitised lymphoblasts propagated *in vitro*.

The T cell derived cytokines, IFN-γ and TNF-α, have been implicated in immunity after being shown *in vitro* to inhibit of development of *T. parva* infected cells, although neither has an effect on established cells. Dobbelaere and Heussler (1999) have demonstrated that gamma delta (γδ) T-cells respond to infected cells, their population expands during the exposure to infection and a proportion of these cells have the ability to lyse a number of parasite stocks in an MHC unrestricted manner. Killing of parasite infected cells that are not MHC restricted has also been attributed to NK cells (Goddeeris *et al.*, 1989).

Whilst these effector mechanism may all contribute to immunity, the major mechanism deployed by immune cattle against *T. parva* has been shown to be the killer function of parasite specific MHC class I restricted $CD8^+$ T cells. Lymphocytes taken directly from immune cattle after challenge and those re-stimulated *in vitro* by autologous *T. parva* infected cells are capable of recognizing and killing autologous but not allogeneic schizont infected cells (Eugui and Emery, 1981).

The effector cells responsible for this lytic activity reside in the T cell compartment of peripheral blood cells and express the cell surface marker CD8 (Eugui and Emery, 1981). Significantly, this response is detectable by direct ^{51}Chromium release assay of peripheral blood mononuclear cells for 2-3 days coinciding with remission of infection (Eugui and Emery, 1981). Direct evidence for $CD8^+$ T cell involvement in immunity to *T. parva* was demonstrated by an adoptive cell transfer experiment between pairs of monozygotic twin calves. Transfer of efferent lymph lymphocytes highly enriched for $CD8^+$ T cells from an immune calf resolving a challenge infection to its naïve twin that had been experimentally infected 1-2 days earlier resulted in attenuation of infection and recovery (McKeever *et al.*, 1994).

There is therefore a correlation between the appearance of $CD8^+$ CTL in the blood of immune cattle under challenge and the clearance of infection and evidence that these cells mediate protection (McKeever *et al.*, 1994). A strong $CD4^+$ T cell proliferative response to autologous *T. parva* infected cells has been detected in immune cattle and a proportion of responding cells have lytic activity (Baldwin *et al.*, 1992). *In vitro* studies have shown that the induction of *T. parva* specific $CD8^+$ CTL responses requires input from $CD4^+$ T cells. It has been proposed that $CD4^+$ T cells recognizing peptide fragments of the parasite in the context of MHC class II activate and licence antigen presenting cells

(APCs) e.g. dendritic cells to induce antigen-specific CTLs. Moreover $CD4^+$ T cell production of interleukin 2 (IL-2), supports the clonal expansion of $CD8^+$ T cells (Taracha *et al.*, 1997) while production of gamma interferon (IFN-γ) may activate macrophages for enhanced phagocytosis and intracellular killing of the parasites.

2.1.5 Parasite stock specificity of CTL responses

Immunity to one stock of *T. parva* does not necessarily confer protection against others. Studies carried out with the Muguga and the Marikebuni stocks of *T. parva* have shown that cattle immunized with *T. parva* Marikebuni are invariably protected against challenge by both stocks but only a proportion of cattle immunized with Muguga mount a protective response against the Marikebuni stock (Irvin *et al.*, 1983). Analyses of the stock-specificity of CTL responses in cattle immunized with these parasite stocks are consistent with this heterogeneity in protection being a reflection of CTL specificity (Taracha *et al.*, 1995). Hence only a proportion of cattle immunized with Muguga developed cross reactive CTL responses, and these animals were protected against Marikebuni challenge, whereas cattle that developed Muguga specific response were not. All Marikebuni immunised cattle developed cross-reactive CTL and were protected against a Muguga challenge.

Studies with cloned CTLs confirmed that cross reactivity arose from the presence of epitopes that are shared between the two stocks (Taracha *et al.*, 1995). The parasite strain specificity of CTL is determined by the phenotype of the restricting class I MHC

(Godderis, *et al.*, 1990). This was validated in an experiment where individual monozygotic twin calves were immunized with either the Muguga stock or a cloned Marikebuni stock. The Muguga immunized animal generated a strain specific response, whereas the Marikebuni immunized twin developed cross reactive CTLs, Both responses were restricted by the same MHC molecule (Taracha *et al.*, 1995). It has also been established that cattle with strain specific immunity can develop cross reactive CTL after heterologous challenge. These observations are consistent with the expression of a number of CTL antigens by *T. parva* parasite strains (several of which might be represented within a stock), which are variably immunodorminant in the context of different host MHC phenotypes and which vary in their degree of conservation between strains (McKeever and Morrison, 1998).

2.1.6 The Food and Agriculture Organization (FAO 1) cocktail ITM vaccine

In order to overcome the degree of parasite-stock heterogeneity of CTL mediated immunity three *T. parva* stocks have been combined to generate an ITM based vaccine that can provide excellent coverage against diverse parasite stocks (Anon, 1998). The three constituent parasite stocks, Muguga, Kiambu 5, and Serengeti transformed, have been prepared as one representative composite stabilate referred to as the Food and Agriculture Organisation (FAO1) cocktail. The stabilates were produced by infecting cattle and ticks with the appropriate seed stock. Infected ticks from each of the three

stocks were then processed in proportions ensuring an equal number of infected tick salivary gland acini from each of the component. Titration experiments were carried out to determine safety and efficacy for use in the field. The results of the experiments indicated that an immunizing dose of 1:80 was the safest (Anon, 1998). The FAO1 cocktail vaccine has therefore been tested extensively for ITM vaccination against ECF in cattle in eastern, central and southern Africa. Molecular characterization of the three stocks in the vaccine have been conducted using an indirect fluorescent antibody test with a panel of anti-schizont monoclonal antibodies (mAb), southern blotting with four *T. parva* repetitive DNA probes and polymerase chain reaction (PCR) based assays detecting polymorphism within four single copy loci encoding antigen genes. The data indicates that Serengeti transformed and Muguga are very closely related genetically, whereas the Kiambu 5 is quite distinct, (Bishop *et al.*, 2001).

2.1.7 Progress towards the development of a schizont based subunit vaccine against ECF

Research towards a sub-unit vaccine has used two complementary approaches to identify antigens that are targets of CTL from cattle immunized by ITM. Both approaches involve detecting CTL recognition of transiently transfected APCs. The first approach used *T. parva* schizont RNA to generate a unidirectional cDNA library, from which cDNA pools were screened. The second approach used genome data to select parasite genes encoding secreted or membrane bound proteins for screening. CTL recognition of cDNA pool or selected gene transfected APC has been assessed using an IFN- γ ELISpot assay.

Ten antigens, Tp1 to Tp10, have been identified using CTL generated from *Bos Taurus* (Friesian and Jersey) and Boran (*Bos indicus*) or Boran/Taurine crossed cattle that have been immunized by ITM using the Muguga stock of *T. parva.* In a preliminary vaccine trial utilising DNA attenuated viral vectors in prime boost vaccination regimes, these antigens induced $CD8^+$ T cell IFN-γ and CTL responses in 80% and 30% of cattle, respectively. Significantly, the CTL responses correlated with survival and reduced disease severity following a lethal challenge infection (Graham *et al.*, 2006). $CD8^+$ T cell responses in cattle vaccinated using Tp1 to Tp8 were also restricted to a small number of immunodominant epitopes and MHC class-1 alleles. Genetic polymorphism has also been observed for some of the antigens that affect CTL recognition (Pelle *et al.*, personal communication). There is therefore a need to identify additional antigens that are restricted by multiple MHC class-1 alleles and conserved amongst parasite stocks.

The East African zebu is an important source of protein for human food in East Africa. This is an indigenous genotype which is well adapted to environmental stress including endemic diseases of the tropics e.g. trypanosomosis and ECF (Rege and Tawa, 1999). Based on genetic distance estimates, and phylogenetic tree analysis, the indigenous zebu were found to be closely related, forming a relatively homogeneous and genetic group of unique populations, that were distinct from Boran (*Bos indicus*) and *Bos taurus* but more closely related to the former than the latter (Rege *et al.*, 2001). Due to the genetic uniqueness and distinction from Boran cattle, it is likely that the CTL from East African zebu will recognise additional schizont antigens that are likely to be restricted by novel

MHC class-1 alleles. The current study aims to extend vaccine candidate antigen identification to CTLs generated from zebu cattle from three geographical areas of Kenya and which were vaccinated by ITM using the FAO1 cocktail. Vaccination with this cocktail should enhance the chance of generating CTL that recognise antigens conserved between stocks. Such antigens would, if incorporated into a subunit vaccine, help in protecting the region's out bred cattle population against a genetically diverse parasite population.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Cattle Immunisation and Management

The cattle used in the study were Small East African Zebu *(Bos indicus)* male calves aged 18 to 24 months sourced from three regions of Kenya namely; Mariakani in Mombasa district (BY118, BY120), Kiboko in Makueni district (BY122, BY124) and Kakamega in Kakamega district (BY126, BY127). The animals were screened for Anaplasmosis, Babesiosis and Cowdroisis by examining Giemsa stained blood smears and also by serology. They were kept in-doors and fed with hay and concentrates. The calves were immunised by ITM with the FAO1 cocktail *T. parva* stocks (Muguga, Kiambu 5 and Serengeti transformed (1:80 dilution of FAO1 cocktail, ILRI, Nairobi, Kenya) by simultaneous inoculation of infective sporozoites and long-acting oxytetracycline at 20mg/kg body weight (Radley *et al.*, 1975). Calves were challenged three months post-immunisation with FAO1 cocktail (1:80 dilution of FAO1 cocktail, ILRI, Nairobi, Kenya) and boosted after a further 2 months with the Muguga stock of *T. parva* (1:20 dilution of stabilate no. 4133, ILRI, Nairobi, Kenya). After immunisation and challenge calves were monitored for three weeks for parasitosis by examining Giemsa stained lymphoid aspirates and blood smears taken from the ear vein and pyrexia by taking rectal temperatures.

3.2 Generation, characterization and maintenance of cytotoxic T lymphocytes

3.2.1 Establishment of *T. parva* infected cell lines

Prior to immunisation, venous blood was collected from the six calves, peripheral blood mononuclear cells (PBMCs) purified and infected *in vitro* with *T. parva* sporozoites as described by Godeeris & Morrison (1986). PBMC were infected with Muguga (TpM) and Kiambu 5 (TpK5) stocks of *T. parva* and with a mixture of the 3 stocks that constitute the FAO1 cocktail (TpFAO1) Infected cell lines were maintained in RPMI-1640 supplemented with 10% Fetal Bovine Serum (FBS) (HyClone, UK; tested for BVDV & *Mycoplasma spp.*), 100 iU/ml penicillin, 100µg/ml streptomycin, 50µg/ml gentamycin, $5x10^{-5}$M 2-mercaptoethanol and 2mM L-glutamine) and passaged 1/5 three times a week.

3.2.2 Generation of *T. parva* specific bulk T-cell cultures

Venous blood was collected from calves 2 weeks post-immunisation. PBMC were prepared as described by Goddeeris & Morrison (1989). PBMC were adjusted to $4x10^{6}$/ml in RPMI-1640 without HEPES supplemented with 10% FBS (HyClone; tested for BVDV & mycoplasma spp.), L-glutamine, 2-mercaptoethanol and penicillin and

streptomycin as described above (CTL medium) and 1ml/well added to 24 well plates (Costar, Corning, NY, USA). PBMCs were co-cultured with irradiated (50Gy cesium) autologous *T. parva* infected cells (TpM, TpFAO1 or TpK5) at 2 x 10^5 /well and incubated for 7 days at 37°C in a 5% CO_2 humidified atmosphere. Cells were harvested by aspiration and dead cells removed by centrifugation over Ficoll-Paque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden). After washing in CTL medium, cells were added to 24 well plates (3x10^6/well) and co-cultured with irradiated autologous PBMC (filler cells) at 1x10^6/well and either irradiated TpM, TpFAO1 or TpK5 at 2 x10^5/well for 7 days as before. Viable cells were harvested as described above, the cells were counted and adjusted to 2 x10^6/well and stimulated with 4 x10^5/well of either irradiated TpM, TpFAO1 or TpK5, and 2 x10^6/well filler cells.

3.2.3 Generation and maintenance of *T. parva* specific $CD8^+$ polyclonal CTL lines

Viable cells from TpM, TpFAO1, TpK5 stimulated cultures were harvested day 7 post-stimulation and $CD8^+$ T cells were purified by magnetic activated cell sorting (MACS). In this technique the $CD8^+$ T cells were labeled with ILA 105 monoclonal antibody specific for CD8 antigens (mAb; IL-A105; ILRI, Nairobi, Kenya Refs) and anti mouse IgG 2a magnetic micro beads (Miltenyi Biotec) were added. The suspension was loaded into a column placed in the magnetic field of a MACS separator. The magnetically labeled $CD8^+$ cells were retained in the column while the unlabeled cells ran through and this cell fraction was depleted of $CD8^+$ T cells. After removal from the magnetic field,

the labeled $CD8^+$ T cells retained in the column were eluted as the positively selected fraction. (Miltenyi Biotec Inc, Gergisch Gladbach, Germany). Sorted $CD8^+$ T cells were re-suspended in CTL medium at a $5x10^4$ and 100µl of cell suspension distributed into 96-well, round bottom culture plates. 100µl of either autologous irradiated TpM, TpFAO1 or TpK5 at $5 x10^4$ in medium containing 10U/ml recombinant human interleukin 2 (HuIL-2) were added to each well.. Cells were maintained by re-stimulation every 14 days as described above

3.3 Testing and characterizing cytotoxic activity

Autologous and allogeneic TpM, TpFAO1, TpK5 in log phase of growth were re-suspended at $2x10^7$/ml in RPMI-1640 medium supplemented with 5% FBS (cytotoxicity medium). 100µl of the target cells were mixed with 100µl (100µCi) of ^{51}Cr-sodium chromate and incubated for 1 hour at 37°C. Cells were washed 3 times in 7ml of cytotoxicity medium by centrifugation at 1500 rpm for 7 min at room temperature and re-suspended at $1 x 10^6$/ml. Viable effector $CD8^+$ T cells were harvested from *Theileria parva* stimulated bulk T cell cultures 7 days post-stimulation (effector cells) and re-suspended in cytotoxicity medium at $2x10^7$/ml.

Two-fold doubling dilutions of effector cells were distributed in duplicate (100µl/well) to 96-well half area (A/2) flat-bottom culture plates (Costar) resulting in a range of effector cell concentrations of $4x10^6$ to $2.5x10^5$/well. Target cells were added to each well containing effector cells (50µl/well) resulting in target cell ratios ranging from 80:1 to 5:1. In separate triplicate wells; target cells were added to 100µl cytotoxicity medium or

1% Tween20 (Sigma, Germany) to measure spontaneous and maximum release of the label, respectively. Plates were incubated for 4 hours at 37°C in a 5% CO_2 humidified atmosphere. Cells were resuspended in wells by repeated pipetting and pelleted by centrifugation at 180 x *g* at room temperature. 75µl of supernatant was transferred from each well into sample vials (Milian, Geneva, Switzerland) and gamma emissions counted in a gamma counter (Micromedic MEplus, TiterTek, Huntsville, AL, USA). Results were calculated and expressed as % lysis (= 100 x (test release – spontaneous release) / (maximum release – spontaneous release).

3.4 Phenotypic analysis of T cells

T cells were resuspended in RPMI-1640 supplemented with 2% horse serum and 0.02% sodium azide (FACS medium) and the cell concentration was adjusted to 1-2x10^7/ml. 50µl of the cell suspension was dispensed into wells of a 96-well round bottom plate (Costar). 50µl of medium or test mAbs (Table 2) were added to wells containing cells. and incubated for 30 minutes at 4°C on ice. The cells were pelleted by centrifugation at 180x*g* for 3 minutes at 4°C, the medium was flicked off and the cell pellet broken on a mixer. The cells were re-suspended in 200µl of FACS medium and washed twice as before. Cells were re-suspended in 50µl of FITC-conjugated goat anti mouse Ig (Sigma) diluted at 1:200 in FACS media and incubated for 30 minutes on ice at 4°C. The plates were then washed twice as described above. Cells were re-suspended in 100µl of FACS medium and analysed with the FACScan (Becton Dickinson). Cells which could not be analysed on the same day were washed twice with PBS containing 2% sodium azide and fixed in 2% formalin in PBS and stored at 4^0C in the dark.

Table 2. Monoclonal Antibodies for Fluorescent Activated Cell Sorting (FACS)

Monoclonal Antibody	Working Dilution	Isotype	Specificity	Origin
ILA-12	1/500	IgG2a	bovine CD4	ILRI (Baldwin *et al.*, 1986)
ILA-105	1/500	IgG2a	bovine CD8	ILRI (MacHugh et al., 1993)
GB-21A	1/200	IgG2b	bovine γδ TCR	VMRD* (Davis *et al.*, 1993, MacHugh *et al.*, 1993)
MM1A	1/100	IgG1	bovine CD3	VMRD (Davis *et al.*, 1993)

*VMRD Inc., Pullman, WA, USA

3.5 Generation and maintenance of bovine skin fibroblasts

Autologous bovine skin fibroblasts (SF) had been isolated aseptically from ear skin biopsies and established as previously described (Graham *et al.*, 2006). Cultures were examined microscopically after every 4 days. To sub-culture, cultures were rinsed with 0.02% EDTA in $Ca^{2+}Mg^{2+}$-free PBS and detached by 5 min incubation at 37°C with Trypsin-EDTA solution containing 2.5 mg/ml Trypsin and 0.2 mg/ml EDTA in HBSS (Sigma, Poole, UK). SF were aspirated and washed in complete DMEM containing 10% FBS by centrifugation at 1500 rpm for 5 min. SF were resuspended in complete DMEM and 1/3 of the cells returned to the flask for continued culture. SF had been immortalized by transfection of pSV3-neo, (ATCC 37150, ATCC, Rockville MD, USA) using Fugene 6 transfection reagent as described by the manufacturer (Roche Diagnostics GmbH, Mannheim, Germany) (Graham *et al.*, 2006). SF were maintained under selection media containing 0.5μg/ml of geneticin G418 (Sigma) and expression of SV40 large T-antigen assessed using immunoperoxidase staining (SV40 T Ag Ab2, Oncogene Research Products, San Diego, CA, USA).

3.6 Transfection of immortalised skin fibroblasts with schizont cDNA library and selected genes

Immortalised SF (iSF) were maintained in 75cm^3 flasks with complete DMEM as described above. Twenty four hours prior to transfection, iSF were harvested and adjusted to 2x10^5/ml, and dispensed, 100µl/well, into 96 well flat-bottom plates and incubated overnight at 37°C in a 5% CO_2 humidified atmosphere. Plasmid miniprep DNA was prepared from 644 cDNA pools (each containing 50 colonies) from a *T. parva* (Muguga) schizont cDNA library and from 96 genes predicted to encode secreted/membrane-bound proteins (Promega, Madison, WI, USA) (Graham *et al.*, 2006). iSF were transiently transfected in 96-well plates with selected genes or pools of schizont cDNA (100ng/well) using Fugene-6 (Roche).The efficiency of transfection was estimated by transfecting a reporter gene, JSP-1, into iSF and determining the surface expression by FACS staining with a specific mAb (IL-A10, ILRI).

3.7 Detection of CTL recognition of transfected iSF by IFN-γ ELISpot assay

Twenty-four hours post-transfection, transfected iSF were washed with PBS (200µl/well), detached with 100µl/well 0.25% Trypsin-EDTA and transferred to 96 well round-bottom plates (Costar) containing 100µl/well cold RPMI supplemented with 10% FCS. Cells were centrifuged at 1200 rpm for 3 min, supernatant removed and cells re-suspended in 100µl of RPMI-1640 supplemented as described above. Schizont-specific

CTL, generated and maintained as described above, were harvested 7-14 days post-stimulation, and re-suspended at $2x10^5$/ml in RPMI-1640 medium supplemented with 10% FBS and 5U/ml recombinant human IL-2 (Sigma). ELISpot plates (Millipore Corporation, Bedford, MA, USA) were coated with 50µl/well of 1µg/ml of murine anti-bovine IFN-γ mAb (CC302; Serotec, Oxford, UK) and incubated overnight at 4°C. Wells were washed twice with un-supplemented RPMI-1640 and blocked using 200µl/well with RPMI-1640 supplemented with 10% FBS by incubating at 37°C for 2 hours.

The blocking medium was removed, replaced with 50µl/well CTL and 100µl/well transfected cells. As a positive control, irradiated TpM, TpK5 or TpFAO1 were serially diluted in iSF with each at a density of $4x10^5$/ml to give populations containing 20000, 10000, 5000, 2500, 1250, 625, 312 infected cells when added 50µl/well to wells containing CTL. Plates were incubated in a humidified incubator at 37°C for 20 hours. After incubation, the contents of the wells were removed and wells washed four times with distilled water containing 0.05% Tween 20 with the plate shaken for 30 seconds between washes.

The process was repeated an additional four times, using PBS containing 0.05% Tween 20. Rabbit anti-bovine IFN-γ antisera diluted 1/1500 in PBS-T supplemented with 0.2% BSA (PBS/BSA) was added 50µl/well and the plate incubated for 1 hour at RT. Plates were washed 4 times with PBS-T before being incubated for 1 hour at RT with 100µl/well murine monoclonal anti-rabbit IgG conjugated to alkaline phosphatase (Sigma) diluted 1/2000 in PBS-T/BSA and 0.2µm filtered Sigma Fast BCIP/NBT

buffered substrate (Sigma) added. Sigma Fast BCIP/NBT buffered substrate was prepared by dissolving 1 tablet/10ml distilled water and passing it through a 0.2μm filter. Plates were washed for 10 min at RT with PBS-T, 100μl/well BCIP/NBT substrate added and plates incubated for 10minutes at room temperature in the dark. The substrate was removed, wells washed with copious amounts of water and plates air-dried at room temperature in the dark. Plates were read on an automated ELISpot reader (AID Diagnostica, Strasberg, Germany).

3.8 Synthetic peptide libraries and recombinant MVA viruses

Two sets of overlapping synthetic peptides covering the full length of antigen Tp2 were used; 12mer peptides offset by 2 residues (Mimetopes, Clayton, Australia) and 16mer peptides offset by 4 residues (Pepscan Systems B.V., Lelystad, The Netherlands). Peptides were dissolved in 50% (v/v) DNA synthesis grade acetonitrile/water (Applied Biosystems, Warrington, UK) aliquoted and stored at -20°C. Autologous iSF were pulsed with titrations of peptides or infected, at a multiplicity of infection of 10, with recombinant MVA viruses expressing the complete open reading frames of *T. parva* antigens Tp1, Tp2, Tp3, Tp4, Tp5, Tp6, Tp7 and Tp8 (Graham *et al.*, 2006), co-cultured with CTL and recognition assessed by IFN-γ ELISpot as described above.

CHAPTER FOUR

4.0 RESULTS

4.1 Generation and Characterization of CTL

Six zebu animals were vaccinated by ITM with the FAO1 cocktail containing Muguga, Kiambu 5 and Serengeti transformed *T. parva* stocks to induce $CD8^+$ CTL responses against all the three stocks. *T. parva* specific bulk T cell cultures were generated from cattle four months after vaccination by repeated *in vitro* stimulation with autologous *T. parva* infected cell lines. *T. parva* specific CTL activity was detected in 8/9 cultures and therefore animals were challenged with the FAO1 cocktail in order to boost immune responses and increase the chances of isolating *T. parva* specific CTL populations. Following boosting (after three months and also after two months), the same 8/9 *T. parva* stimulated bulk T cell cultures showed CTL activity, Moreover, only 5 out of the 8/9 cultures showed lysis of cells infected with the Muguga stock. Since the material to be screened for antigens was derived from the Muguga stock (Table 3), all cattle were challenged with *T. parva* Muguga to enhance the chances of isolating Muguga-specific or cross-reactive CTL. The Muguga challenge had no observable effect on the CTL activity of bulk cultures. Cultures which exhibited CTL activity were enriched for $CD8^+$ T cells by MACS sorting and phenotype confirmed by FACS analysis (Fig 2 and 3). All cell lines were >90% $CD3^+$ $CD4^+$ T cells with small contaminating populations of $CD4^+$ T cells and γδ TCR^+ T cells.

Table 3. Cytotoxic T Lymphocytes (CTL) activity detected after ITM Immunization and challenge

Animal Identity	*T. parva* stock for *in vitro* stimulation	CTL activity*		
		Post-ITM immunisation with FAO 1 cocktail	Post-challenge with FAO1 cocktail	Post-challenge with Muguga stock
BY118	Muguga	Low	Low	Low
BY120	Muguga	High	High	High
BY122	Muguga	Low	Low	Low
	FAO 1	Medium	Medium	Medium
BY124	Muguga	High	High	High
	Kiambu 5	Low	Low	Low
BY126	Muguga	Low	Low	Low
	FAO 1	Negative	Negative	High
BY127	Muguga	Medium	Medium	Medium

*CTL activity measured against the *T. parva* infected cell line used for *in vitro* stimulation was graded as high (>60% lysis), medium (41-60% lysis), low (10-40% lysis) or negative (<10% lysis)

Cytolytic activity of bulk T cell and polyclonal $CD8^+$ T cell lines was measured after stimulation with autologous cells infected with Muguga, Kiambu 5 or the FAO1 cocktail. Parasite-specific, MHC class I restricted CTL activity was assessed against autologous and allogeneic *T. parva* infected lymphoblasts and autologous uninfected lymphoblasts. Figures 4 to 9 shows representative CTL data from the T cell lines generated from the six

animals. *T. parva* specific CTL from BY118 consistently exhibited weak lytic activity against autologous schizont infected cells and no lytic activity against allogeneic targets (Fig. 4). BY118 was later discovered to have sub-clinical trypanosomosis which causes immunosuppression and may have resulted in the failure to expand CTL *in vitro*. CTL obtained from BY120 and stimulated by the Muguga stock exhibited strong lytic activity against autologous schizont infected cells and no lytic activity against allogeneic targets and uninfected targets (Fig. 5).

CTL obtained from BY122 following stimulation with the Muguga stock exhibited weak lytic activity against cells infected with the Muguga stock and the FAO1 cocktail, and no lytic activity against allogeneic targets and uninfected blasts. FAO-1 stimulated T cell lines from BY122 exhibited strong lytic activity against FAO1 infected cells but minimal lytic activity against Muguga infected cells and no lytic activity against allogeneic targets and blasts (Fig. 6). Muguga stimulated cultures from BY124 exhibited good lytic activity against Muguga infected targets that was greater than that against Kiambu 5 infected cells. In contrast, Kiambu 5 stimulated CTL showed equivalent activity against both Kiambu 5 and Muguga infected cells (Fig. 7). T cell lines obtained from BY126 that were stimulated by FAO1 or Muguga both exhibited weak lytic activity against the two infected cell lines (Fig. 8). Muguga stimulated T cells from BY127 displayed strong lytic activity against autologous Muguga infected cells and no lytic activity against allogeneic targets and blasts (Fig 9).

Figure 2. Expression of major surface markers; $CD3^+$ $CD8^+$ phenotype on *T parva* specific polyclonal $CD8^+$ T cell lines (CTL) from cattle numbers BY118, BY120, BY122 and BY124. PBMCs were stimulated by either *T. parva* Muguga or *T. parva* FAO 1, fractionated and enriched for $CD8^+$ T cells then stained with monoclonal antibodies specific for CD3, CD4, CD8 and γδ cells. Expression was determined by flow cytometry

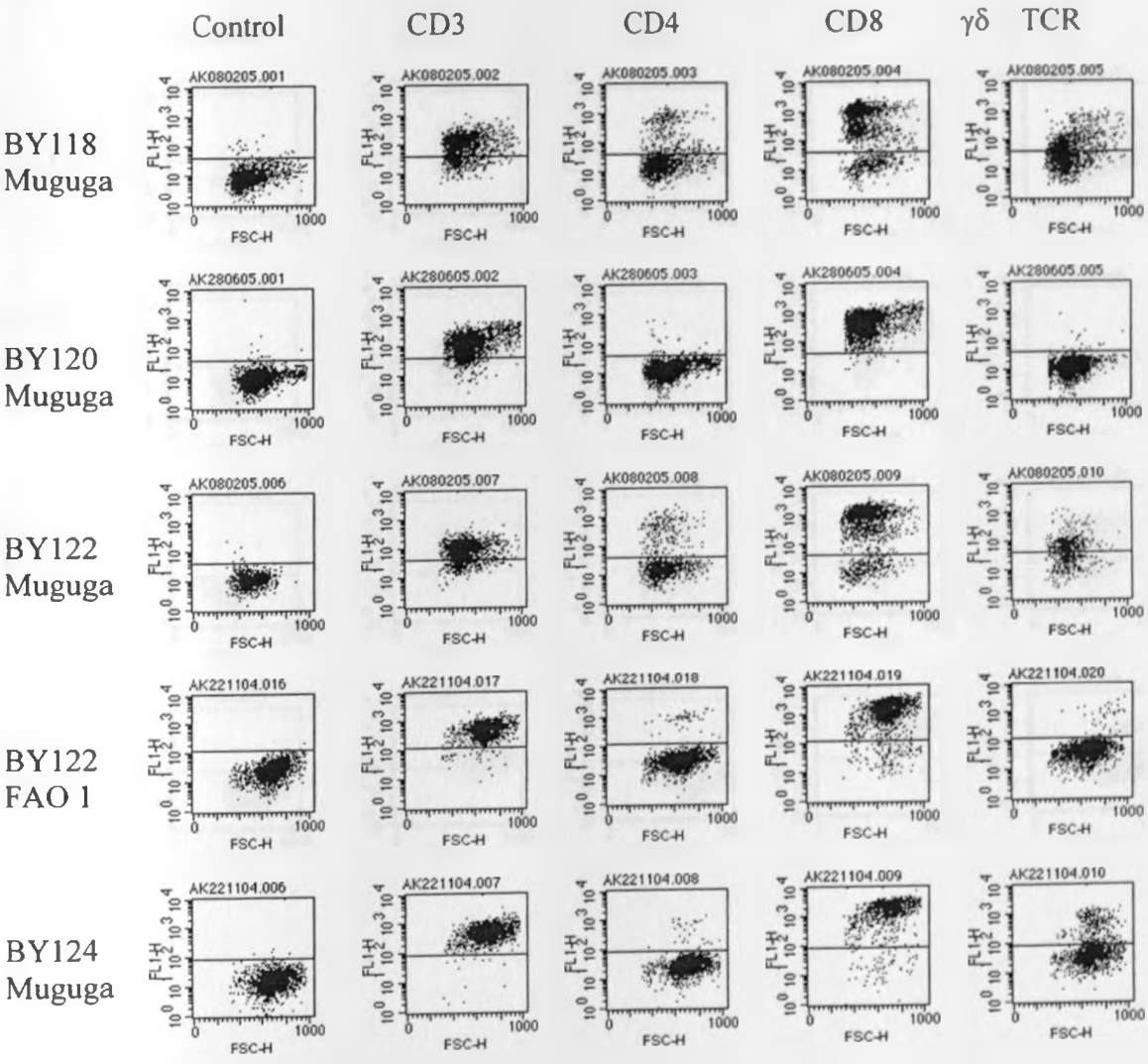


Figure 3. Expression of major surface markers; $CD8^+CD3^+$ phenotype on *T.parva* specific polyclonal $CD8^+$ T cell lines (CTL) from animals number BY124, BY126 and BY127. PBMCs were stimulated by either *T. parva* (Muguga) or *T. parva* (FAO 1) and fractionated and enriched for $CD8^+$ T cells, they were stained with monoclonal antibodies specific for CD3, CD4,CD8 and γδ cells and expression determined by flow cytometry.

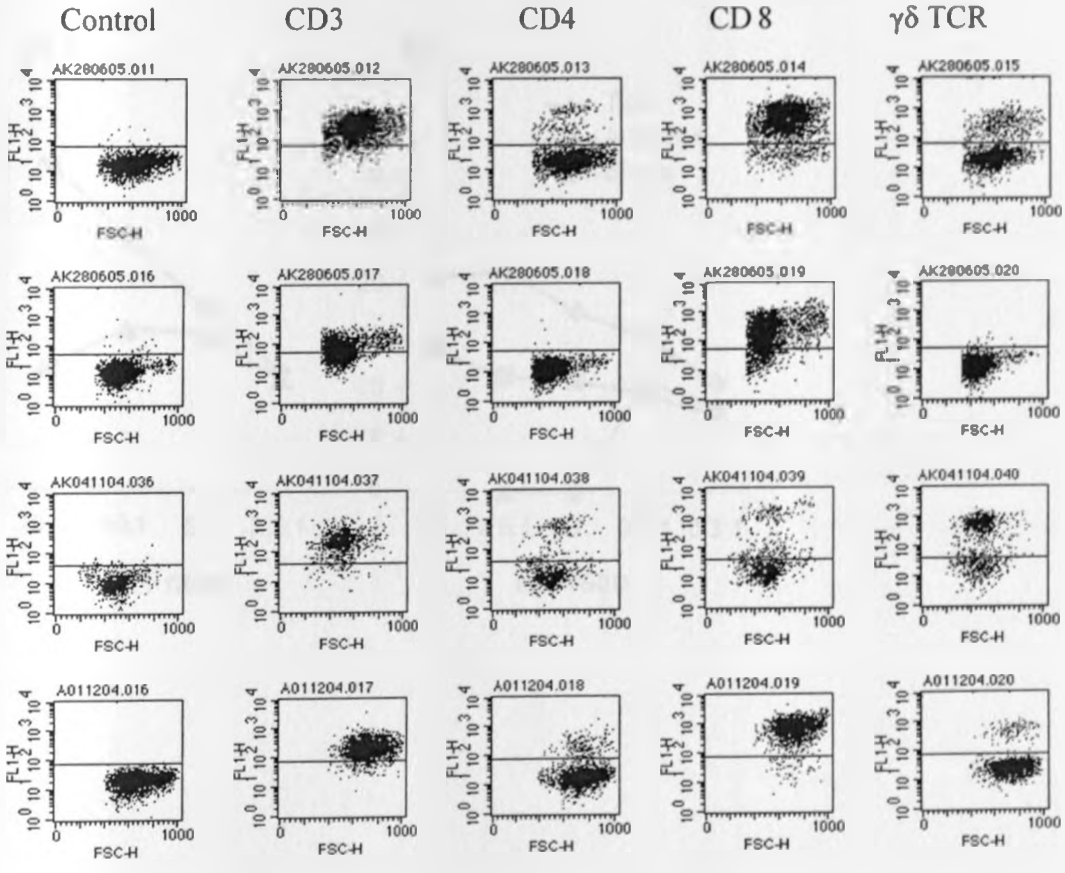


Figure 4. Cytotoxic activity of *T. parva* -specific T cells from BY118. Lytic responses of a bulk T cell line (A) and polyclonal $CD8^+$ T cell line (B) was determined against autologous *T. parva* (Muguga) infected lymphoblasts (TpM), allogeneic *T. parva* (Muguga) infected lymphoblasts (AlloTpM)-from animal F100 and autologous uninfected lymphoblasts (Blasts). E: T – Effector target ratio.

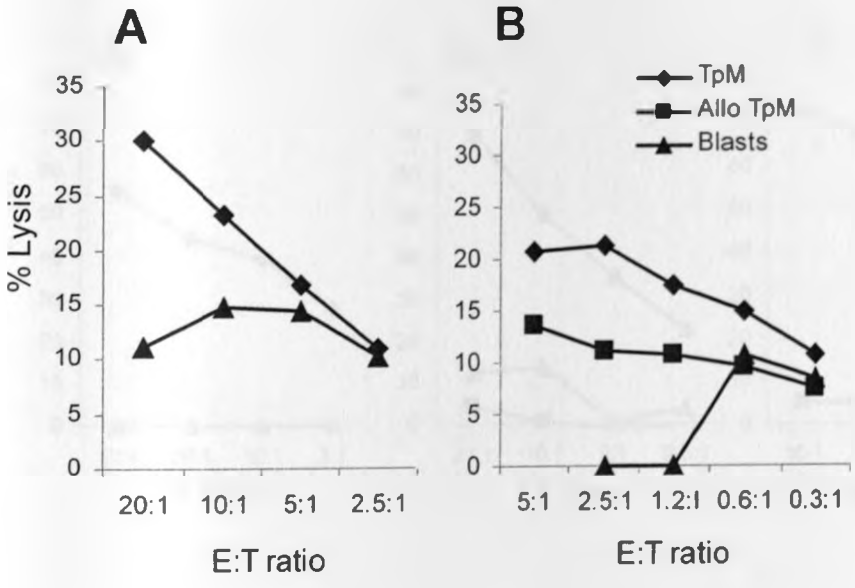


Figure 5. Cytotoxic activity of *T parva* specific T cells from BY120. Lytic responses of a bulk T cell line after one (**A**) and two (**B**) *in-vitro* stimulations, and a polyclonal $CD8^+$ T cell line (**C**) was determined against autologous *T. parva* (Muguga) infected lymphoblasts (TpM), allogeneic *T. parva* (Muguga) infected lymphoblasts from F100 (AlloTpM) and autologous uninfected lymphoblasts (Blasts).

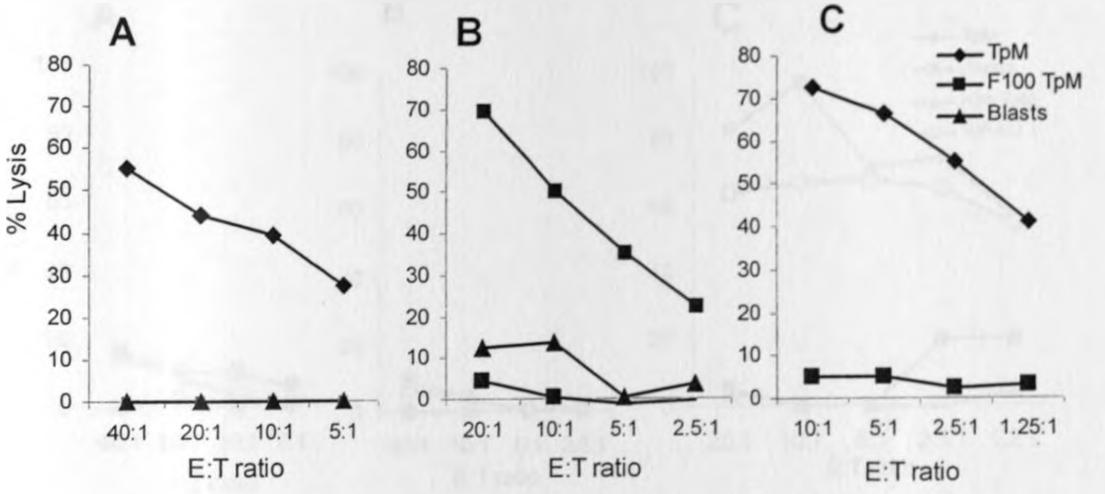


Figure 6. Cytotoxic activity of *T. parva* specific T cells from BY122. Lytic responses of bulk cultures stimulated with *T. parva* (Muguga) (**A-B**), *T. parva* (FAO1) (**C-E**) following one (**A, C**) and two (**B, D**) *in-vitro* stimulations and the FAO1 stimulated a polyclonal $CD8^+$ T cell line (**E**). CTL activity was determined against autologous *T. parva* (Muguga) infected lymphoblasts (TpM), autologous *T. parva* (FAO1) infected lymphoblasts (TpFAO1), allogeneic *T. parva* (Muguga) infected lymphoblasts (AlloTpM) and autologous uninfected lymphoblasts (Blasts).

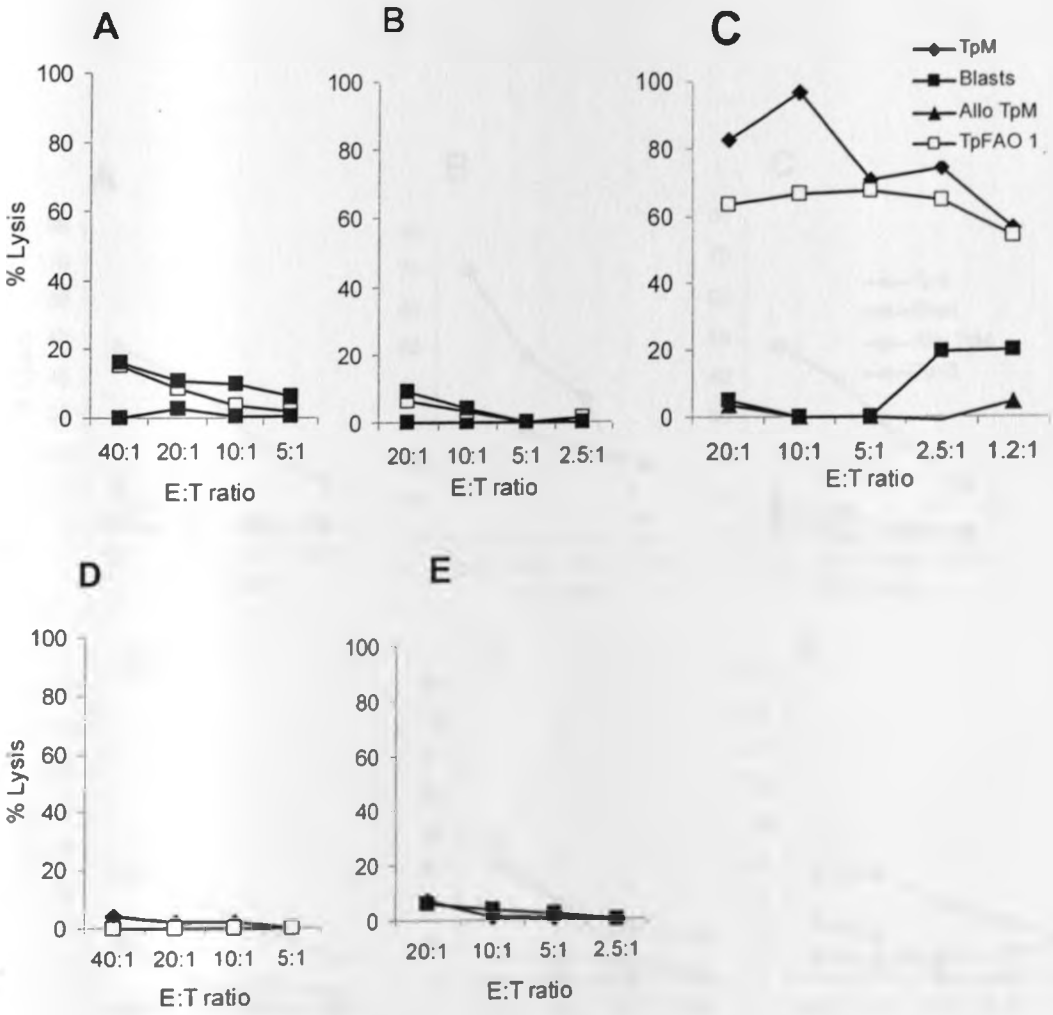


Figure 7. Cytotoxic activity of *T. parva* specific T cells from BY124. Lytic responses of bulk cultures stimulated with *T. parva* (Muguga) (**A-C**) and *T. parva* Kiambu 5 (**D-F**) following one (**A, D**) and two (**B, E**) *in-vitro* stimulations and polyclonal $CD8^+$ T cell lines (**C and F**). CTL activity was determined against autologous *T. parva* (Muguga) infected lymphoblasts (TpM), autologous *T. parva* Kiambu 5) infected lymphoblasts (TpK5, allogeneic *T. parva* (Muguga) infected lymphoblasts (AlloTpM) and autologous uninfected lymphoblasts (Blasts).

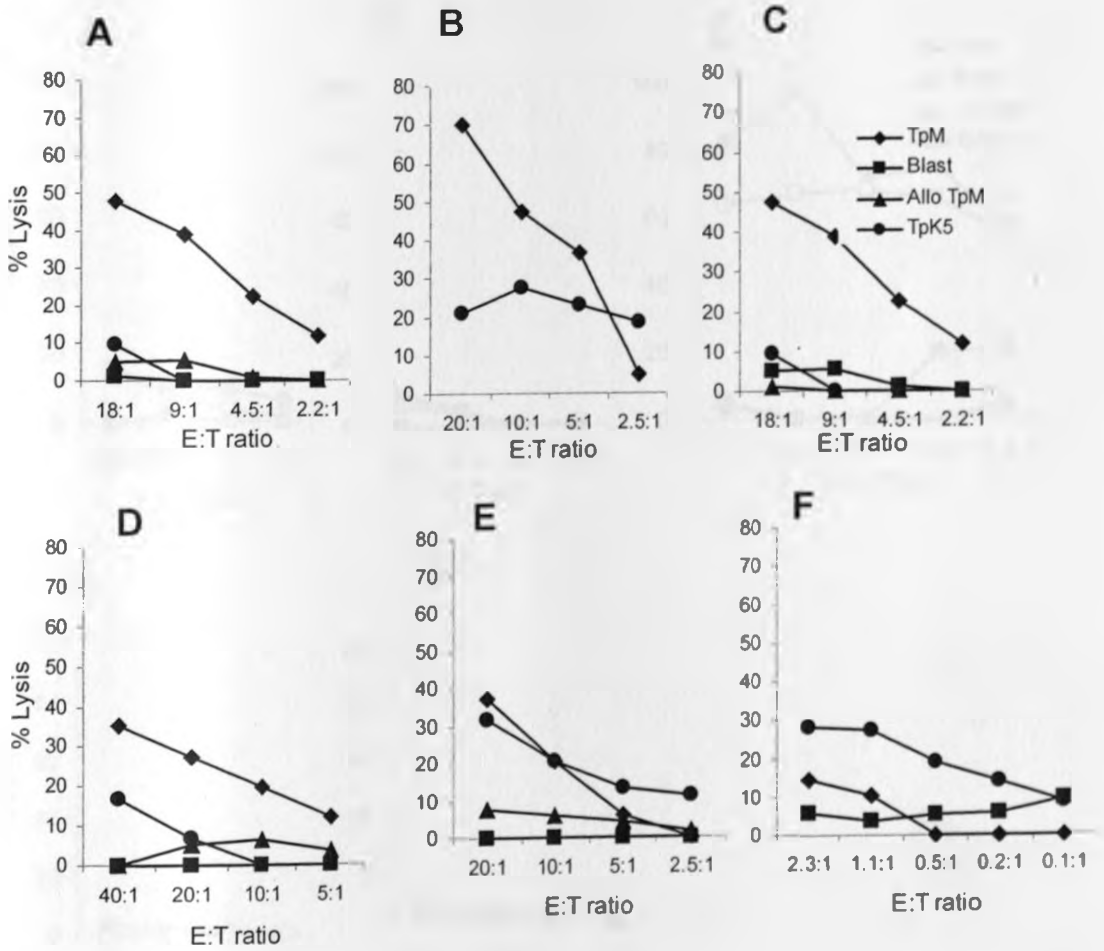


Figure 8. Cytotoxic activity of *T. parva* specific T cells from BY126. Lytic responses of bulk cultures stimulated with *T. parva* (Muguga) (**A-C**) and *T. parva* (FAO1) (**D-E**) following one (**A, D**) and two (**B, E**) *in-vitro* stimulations and the polyclonal $CD8^+$ T cell line (**C**). CTL activity was determined against autologous *T. parva* (Muguga) infected lymphoblasts (TpM), autologous *T. parva* (FAO1) infected lymphoblasts (TpFAO1), allogeneic *T. parva* (Muguga) infected lymphoblasts (AlloTpM) and autologous uninfected lymphoblasts (Blasts).

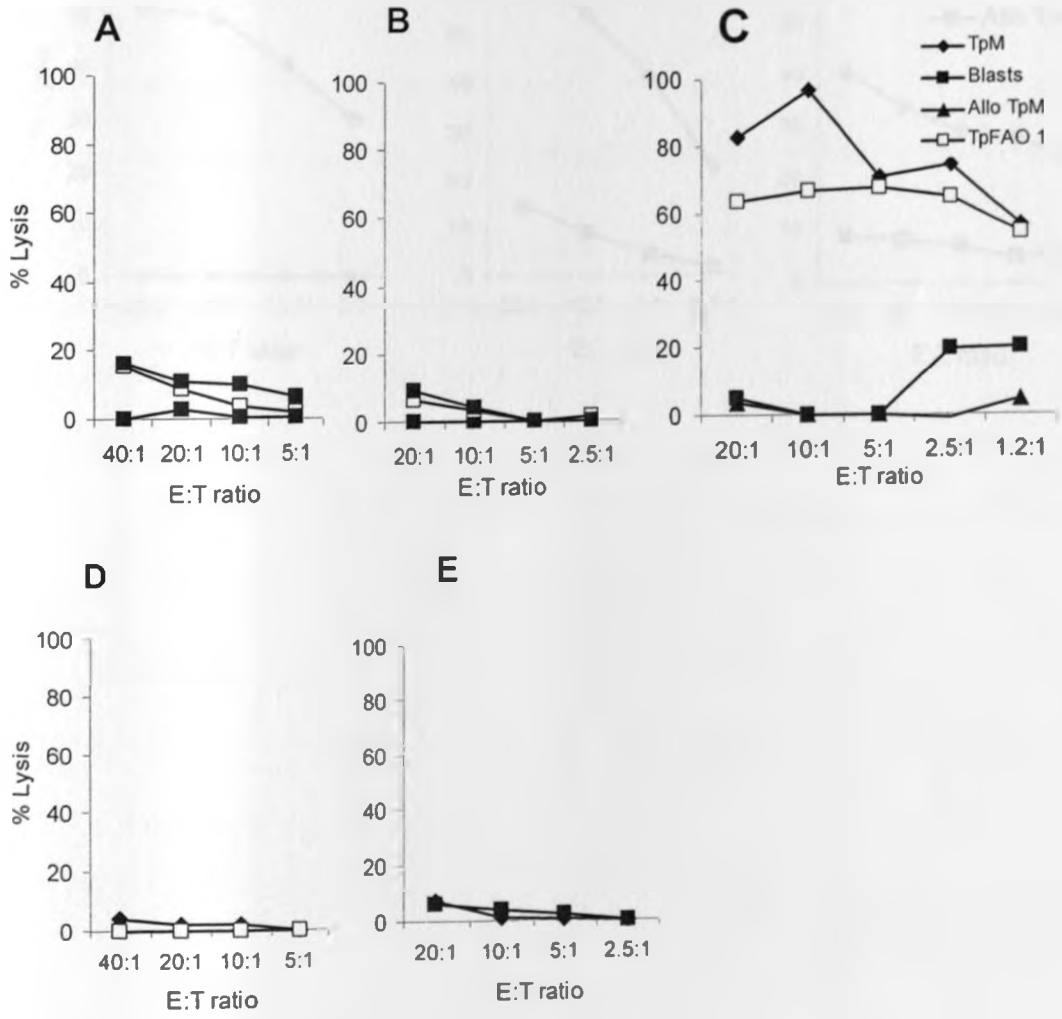
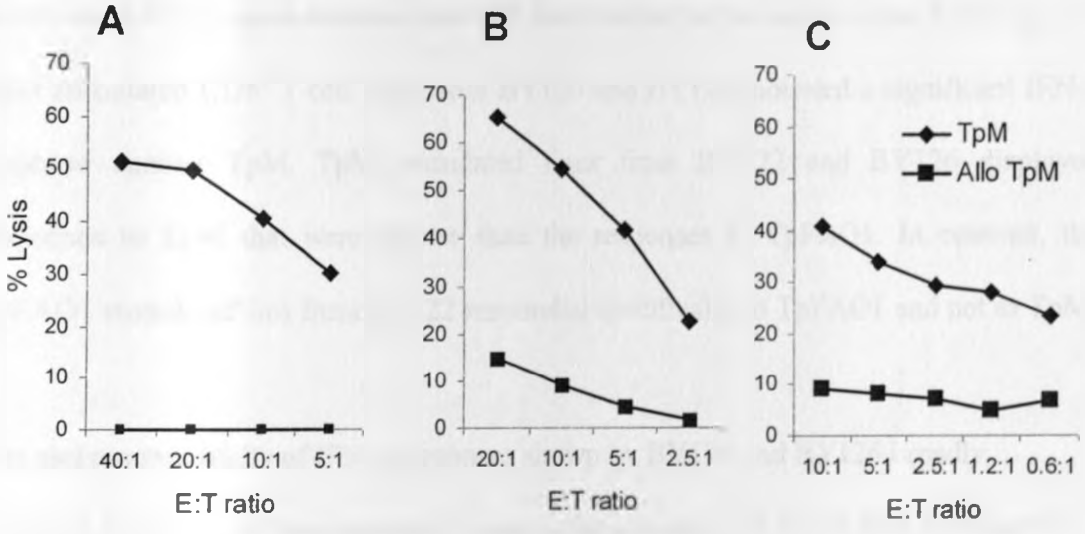


Figure 9. Cytotoxic activity of *T. parva* specific T cells from BY127. Lytic responses of a bulk T cell line after one (**A**) and two (**B**) *in-vitro* stimulations, and a polyclonal $CD8^+$ T cell line (**C**) was determined against autologous *T. parva* (Muguga) infected lymphoblasts (TpM) and allogeneic *T. parva* (Muguga) infected lymphoblasts (AlloTpM).

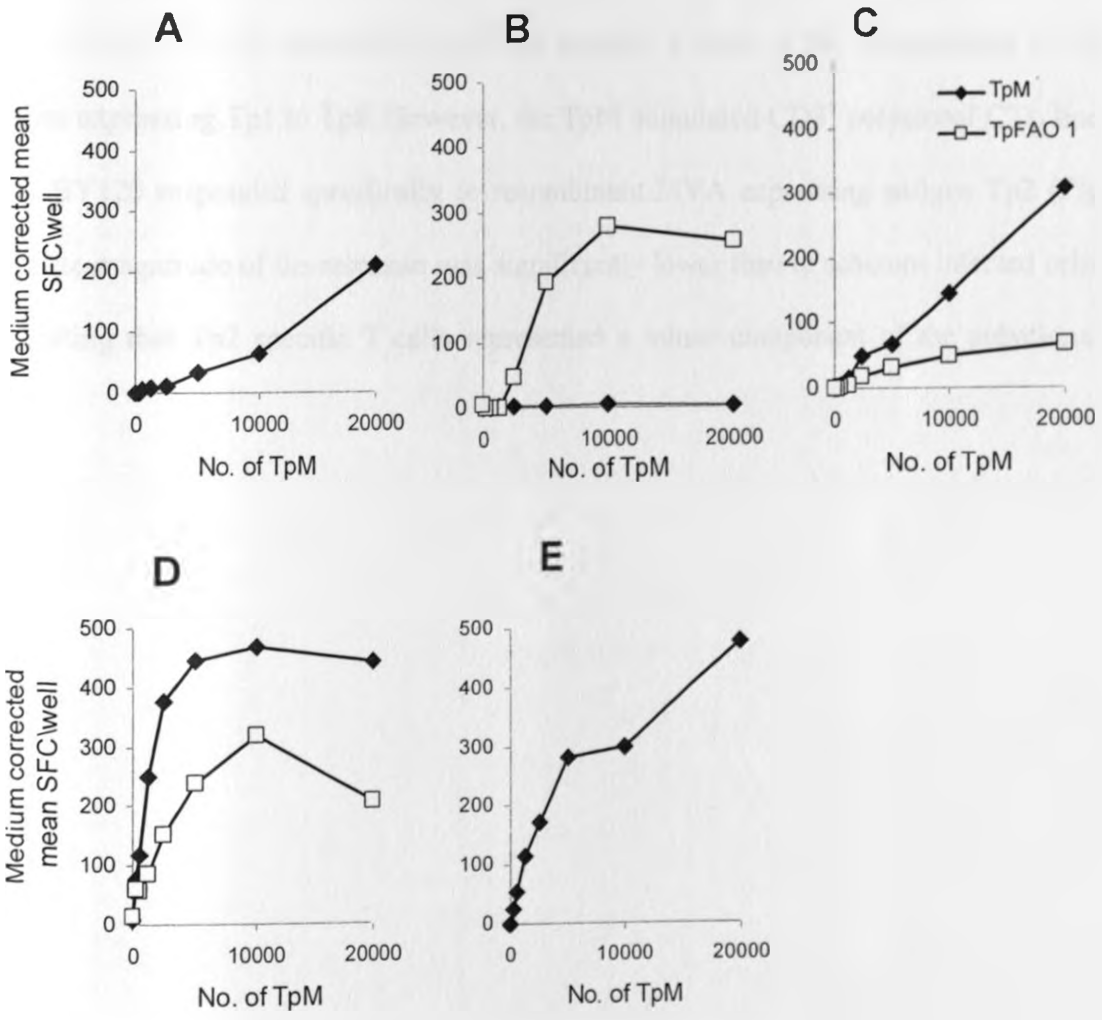


4.2 Recognition of *T. parva* infected lymphoblasts with *T. parva* Specific $CD8^+$ polyclonal CTL lines using an IFN-γ ELISpot assay

Prior to commencement of antigen screening, the ability to detect responses of *T. parva* specific $CD8^+$ polyclonal CTL lines to *T. parva* infected cells were evaluated in an IFN- γ ELISpot assay. Fourteen days post stimulation, CTL were added to pre-coated ELISpot wells containing doubling dilutions of autologous schizont infected cells and the secretion of IFN-γ spots assessed after 20 hour incubation as can be seen from Fig. 10. TpM stimulated $CD8^+$ T cell lines from BY120 and BY127 mounted a significant IFN-γ response against TpM. TpM stimulated lines from BY122 and BY126 displayed responses to TpM that were greater than the responses to TpFAO1. In contrast, the TpFAO1 stimulated line from BY122 responded specifically to TpFAO1 and not to TpM.

The antigen specificity of IFN-γ responses shown by BY120 and BY126 broadly reflected the results of the cytotoxicity assays. Significantly, all T cell lines responded to <150 TpM which represented <1% of the starting TpM population. Given that the expected transfection efficiencies of iSF in the immunoscreen should exceed 1% it was concluded that the polyclonal CTL lines were potent enough to be used for immunoscreening. Polyclonal T cell lines from BY124 non specifically secreted IFN-γ when cultured in the presence of iSF and were not used in any further immunoscreens (data not shown). All the other polyclonal lines had low background IFN-γ release when co-cultured with autologous iSFs.

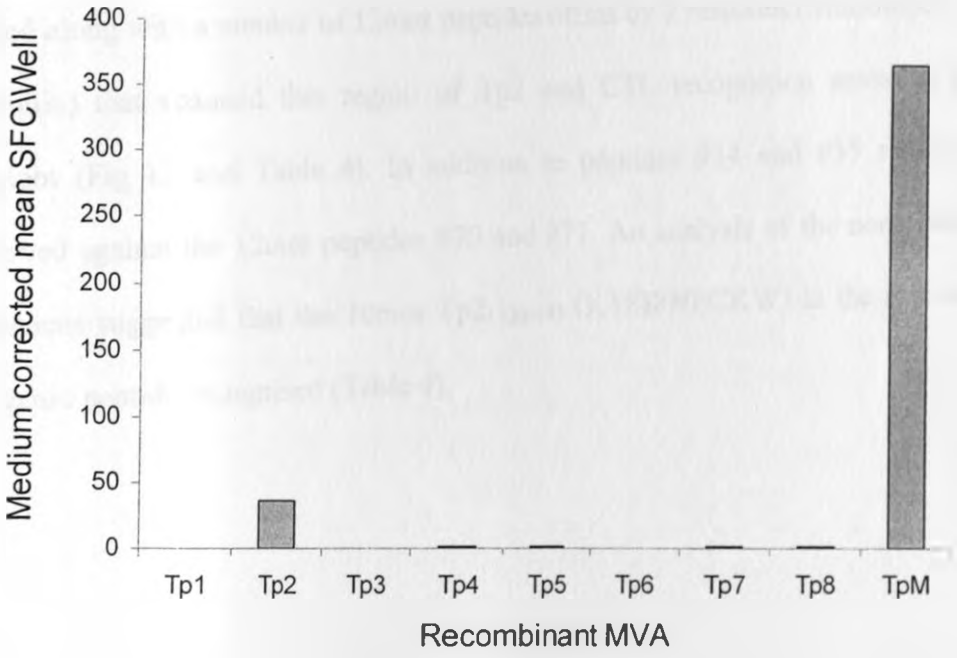
Figure 10. Assessment of CTL recognition of autologous schizont infected by IFN-γ ELISpot. *T. parva* infected cells (TpM and TpFAO1) were co-cultured with TpM stimulated $CD8^+$ polyclonal T cell lines from BY120 (A), BY122 (C), BY126 (D) and BY127 (E) and a TpFAO1 stimulated $CD8^+$ polyclonal T cell line from BY122 (B) and recognition assessed by IFN-γ ELISpot. Cultures containing only TpM controls did not show any responses. Responses are presented as medium corrected mean number of spot forming cells (SFC)/well.



4.3 Detection of Recognition of vaccine candidate *T. parva* schizont antigens by Zebu CTL

Prior to immunoscreening for novel vaccine candidate antigens, CTL were used to screen the existing eight CTL target antigens using IFN-γ ELISpot assays. Autologous iSF infected with eight recombinant modified vaccinia virus Ankara strain (MVA) viruses expressing antigens Tp1 – Tp8 were co-cultured with polyclonal CTL raised from BY120, BY122, BY1267 and BY127 and recognition assessed by IFN-γ ELISpot. CTL from BY122, BY126, and BY127 did not respond to any of the recombinant MVA viruses expressing Tp1 to Tp8. However, the TpM stimulated $CD8^+$ polyclonal CTL line from BY120 responded specifically to recombinant MVA expressing antigen Tp2 (Fig 11). The magnitude of the response was significantly lower than to schizont infected cells suggesting that Tp2 specific T cells represented a minor component of the polyclonal line.

Figure 11. Recognition of *T. parva* vaccine candidate antigens by a TpM stimulated polyclonal $CD8^+$ T cell line from BY120. T-cell recognition of immortalized skin fibroblasts infected with recombinant MVA viruses expressing Tp1 to Tp8 was assessed by IFN-γ ELISpot assay. TpM were included as a positive control. Responses are presented as medium corrected mean number of spot forming cells (SFC)/well.



4.4 Mapping of the $CD8^+$ T cell epitope on Tp2 recognised by BY120

Autologous iSF were pulsed with 41 overlapping synthetic peptides (16 residues overlapping by 12 residues) representative of the full length of the Tp2 protein (Pepscan, Lelystad, Netherlands) (Mimotopes, Clayton, Australia) and recognition by BY120 CD8+ polyclonal CTL line was assessed by IFN-γ ELISpot (Fig. 12). Weak responses were observed against two overlapping peptides (# 34 and # 35) suggesting a single epitope. In order to help define the minimal length epitope peptide #34 and #35 were titrated along with a number of 12mer peptides offset by 2 residues (Mimotopes, Clayton, Australia) that spanned this region of Tp2 and CTL recognition assessed by IFN-γ ELISpot (Fig 12 and Table 4). In addition to peptides #34 and #35 responses were observed against the 12mer peptides #70 and #71. An analysis of the consensus peptide sequences suggested that the 10mer Tp2 $_{138\text{-}147}$ (KTSIPNPCKW) is the minimal length antigenic peptide recognised (Table 4).

Figure 12. Mapping of the $CD8^+$ T cell epitope on Tp2 recognized by TpM stimulated BY120 polyclonal $CD8^+$ T cell line. Forty one overlapping 16-mer synthetic peptides covering the full length of Tp2 were co-cultured with BY120 (TpM) $CD8^+$ polyclonal line and autologous immortalized skin fibroblasts and recognition assessed by IFN-γ ELISpot assay. Responses are presented as mean number of spot forming cells (SFC)/well with medium controls subtracted.

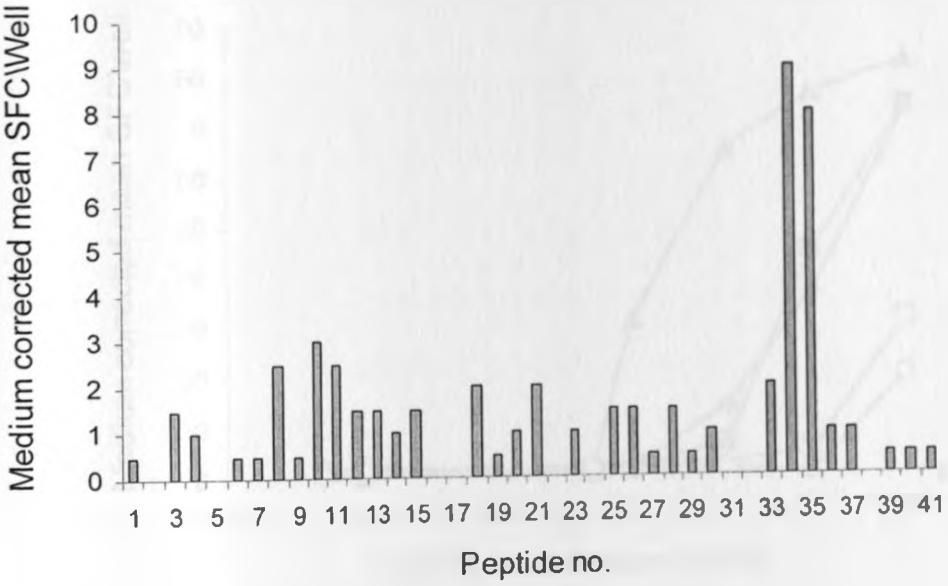


Figure 13. Fine mapping of the $CD8^+$ T cell epitope on Tp2 recognized by TpM stimulated BY120 polyclonal $CD8^+$ T cell line. Titrations of overlapping 16mer (33, 34, 35) and 12mer (69, 70, 71, 72) Tp2 peptides co-cultured with BY120 (TpM) $CD8^+$ polyclonal line and autologous immortalized skin fibroblasts as assessed using IFN-γ ELISpot assay. Responses are presented as medium corrected mean number of spot forming cells (SFC)/well.

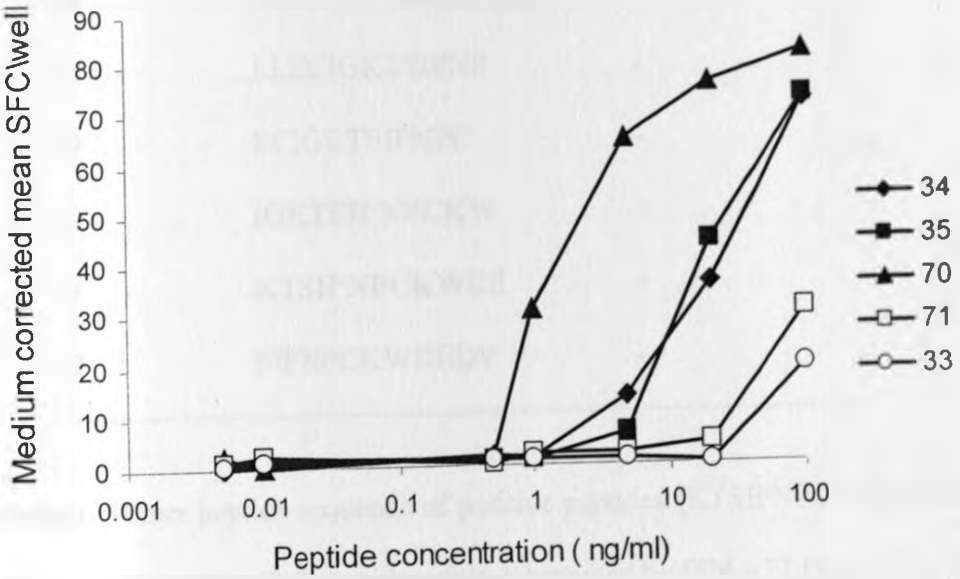


Table 4. Deduction of the minimal length Tp2 peptide recognized by TpM stimulated $CD8^+$ polyclonal T cell line from BY120

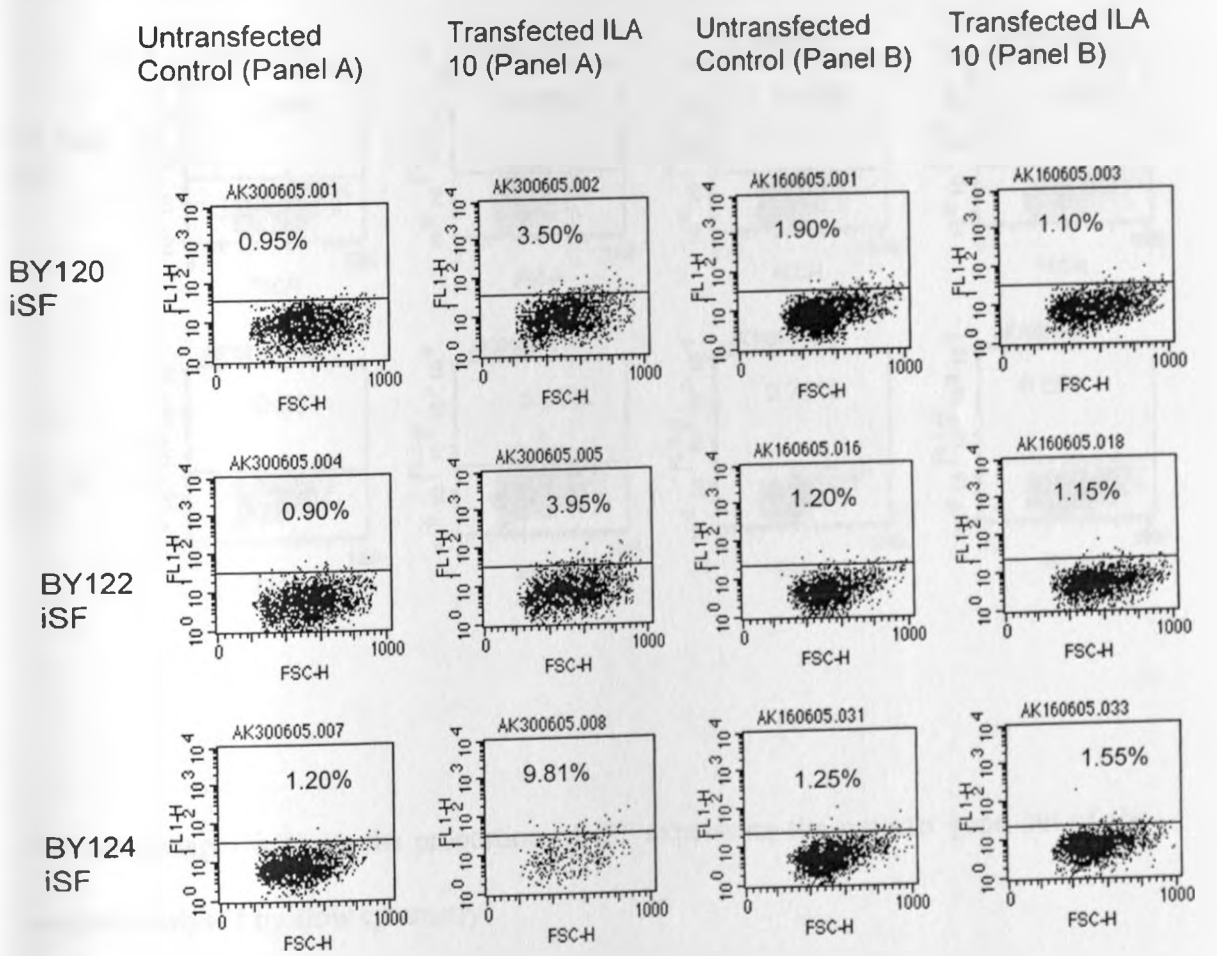
Peptide No.	Peptide Sequence~	T cell response*
33	KSALLECIGKTSIPNP	-
34	LECIG**KTSIPNPCKW**K	+
35	G**KTSIPNPCKW**KEDYL	+
36	IPNPCKWKEDYLKYKF	-
68	LLECIGKTSIPNP	-
69	ECIGKTSIPNPC	-
70	IG**KTSIPNPCKW**	+
71	**KTSIPNPCKW**KE	+
72	SIPNPCKWKEDY	-

~ The consensus 10mer peptide sequence of positive peptides (KTSIPNPCKW) is shown in **bold.** *T cell responses to Tp2 peptides were determined by IFN-γ ELISpot (Fig. 11).

4.5 Testing transfection efficiencies of immortalized skin fibroblasts from Zebu cattle

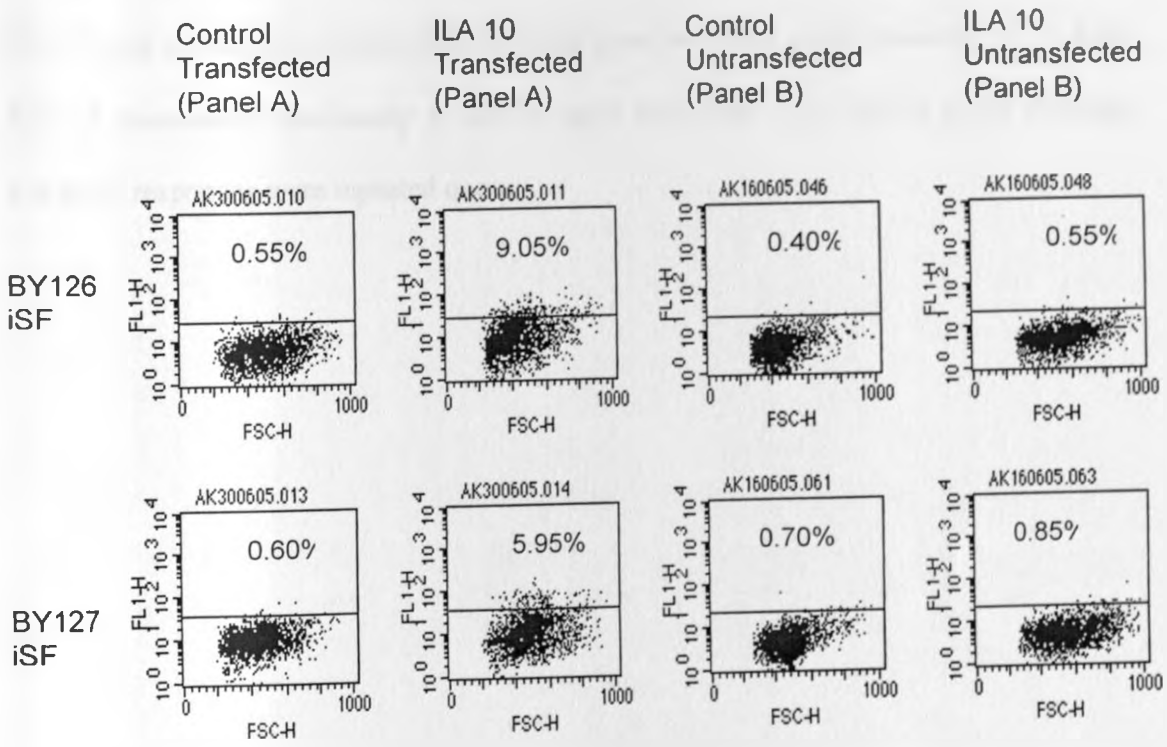
In advance of the initiation of screening for CTL target antigens by transient transfection of iSF, the transfection efficiencies of iSF from BY120, BY122, BY124, BY126 and BY127 were assessed using the bovine MHC class I gene JSP-1 as a reporter gene. There was considerable variation in transfection efficiencies between the iSF lines and experiments; a representative result is shown in Fig. 14 and 15. Transfection efficiencies as assessed by surface expression of JSP-1 ranged between 3% and 9% and this was assessed to be good enough to be used in the immunoscreens (Figures 14 & 15).

Figure 14. Transient transfection efficiencies of immortalized skin fibroblasts from zebu cattle numbers BY120, BY122, BY124. iSF were transfected with a eukaryotic expression vector containing the bovine MHC class I gene JSP-1. Surface expression of JSP-1 was determined by flow cytometry 24 hours post-transfection by staining with a specific monoclonal antibody (IL-A10). As a negative control, untransfected iSF were also stained with IL-A10. Two panels of iSF were transfected A, Stained with mAB IL-A10, B- Unstained) the percentages indicate the proportion of iSF expressing the reporter gene out of the number analysed by flow cytometry.



The percentages indicate the proportion of iSF expressing the reporter gene out of the number analysed by flow cytometry.

Figure 15. Transient transfection efficiencies of immortalized skin fibroblasts from zebu cattle numbers BY126, BY127. iSF were transfected with a eukaryotic expression vector containing the bovine MHC class I gene JSP-1. Surface expression of JSP-1 was determined by flow cytometry 24 hours post-transfection by staining with a specific monoclonal antibody (IL-A10). As a negative control, untransfected iSF were also stained with IL-A10.

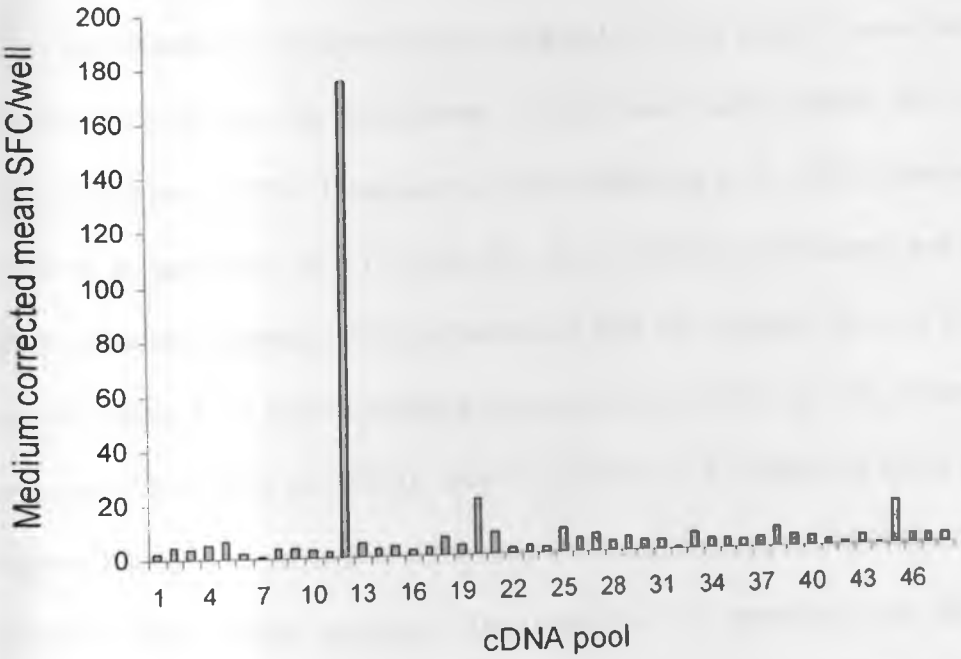


The percentages indicate the proportion of iSF expressing the reporter gene out of the number analysed by flow cytometry.

4.6 Screening the cDNA library and selected genes with CTL from Zebu cattle

Autologous iSF were transiently transfected with 96 selected genes and 480 cDNA pools and recognition by *T. parva* Muguga stimulated $CD8^+$ polyclonal CTL lines from BY120, BY122, BY126, and BY127 assessed by IFN-γ ELISpot. CTL from BY120, BY122, and BY127 did not respond to any of the selected genes or cDNA pools. However, CTL from BY120 responded specifically to cDNA pool B12 (Fig. 16). cDNA pools showing marginal responses were repeated once.

Figure 16. Screening of schizont cDNA pools with TpM stimulated $CD8^+$ polyclonal T cell lines from BY126. Forty-eight pools (B1-B48), each containing 50 cDNA clones, were transfected into autologous iSF. CTL recognition of transfected cells was assessed using IFN-γ ELISpot assay. Recognition of B12 is shown as the peak. Results are presented as medium corrected mean spot forming cells (SFC)/well.



CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 DISCUSSION

The identification of schizont antigens targeted by CTLs from *T. parva* immune cattle should pave the way for development of a sub unit vaccine against East Coast Fever (McKeever *et al.*, 1994, Taracha *et al.,* 1995, McKeever *et al.*, 1999). Previous work has resulted in generation of CTL from *Bos taurus* (Friesian and Jersey) and *Bos indicus* (Boran) cattle following ITM immunisation with the Muguga stock of *T. parva* and applied them in a high-throughput immunoscreen to identify ten vaccine candidate antigens (Graham *et al.,* 2006). Initial evaluation of a number of these antigens has shown that 30% of vaccinated cattle mounted antigen-specific CTL responses and were protected from lethal challenge. The specificity of responses was limited to the previously identified immunodominant epitopes. Research is now focussing on inducing these protective responses in a greater proportion of vaccinated cattle and in cattle of diverse haplotypes. Some of the efforts directed at achieving this include optimising the vaccine formulation/delivery system and continuing the search for additional vaccine candidate antigens. This study aimed to address this by extended antigen identification to CTL generated from genetically diverse zebu cattle immunised with a cocktail of *T. parva* stocks that can protect cattle throughout the East and Central African region.

The Kenyan zebu is found throughout the country and their wide distribution over diverse ecological zones implies that over the years, they have adapted selective characteristics because of natural selection under different environmental conditions and the various breeds are named after the tribes that breed them. The Kenyan zebu appear to be a homogeneous group of African cattle genetically distinct from pure *Bos indicus* and *Bos taurus* but more closely related to the former than the latter. This would enhance the chances of gencrating CTL that would identify antigens different from the ones earlier identified which would protect more animals in the out bred population.

The isolations imposed by tribal boundaries, whether physical and/or cultural, and those due to ecological restrictions are partially responsible for the genetic differentiation leading to the existence of different breeds and strains (Rege and Tawa, 1999). The Kenyan zebu breeds can be classified into three genetically distinct groups, thus 'Boran group' 'Coastal group' and 'Inland group'. In this work the cattle used were the coastal and inland groups. There has been little or no systematic selective breeding in the Kenyan zebu populations. Interbreeding and cross breeding in these populations is mainly as a result of geographical proximity. This is why the cattle used in this work were sourced from areas that were geographically distant to increase the chances of genetic distinctness between the experimental animals therefore increasing the chances of obtaining CTL that would recognise different antigens.

Attempts to generate CTL lines utilised an established protocol that employs *T. parva* infected cell lines as antigen presenting cells (Goddeeris and Morrison, 1989). The T cell lines generated displayed varying degrees of CTL activity with evidence that these were

parasite specific and MHC class I restricted as has been previously described (Morrison *et al.*, 1987). The varying degree of lysis observed between polyclonal lines may reflect different frequencies of cytotoxic populations within the lines or differences in antigen specificities between the lines as has been described (Graham *et al.*, 2006). Previous studies have shown that cattle immunised with one stock of *T. parva* show variations in the degree of protection against heterologous challenge and can also affect the parasite stock specificity of CTL responses. Moreover, a close association has been established between stock specificity of CTL responses and cross-protection profiles (Taracha *et al.*, 1995). In this study, zebu cattle were vaccinated using a cocktail of *T. parva* stocks in an attempt to generate CTL that would recognise antigens that are not only conserved but also non-conserved between stocks. In order to generate CTL with broad stock specificity, peripheral blood mononuclear cells (PBMC) were stimulated with autologous cells infected with different stocks of *T. parva* including cells infected with the cocktail of stocks (TpFAO1).

The CTL activity was assessed against different parasite stocks. A limitation of this analysis was caused by the incomplete set of infected cell lines available for each animal (Muguga, Kiambu 5, Serengeti transformed and FAO1 cocktail) which was due to technical difficulties in establishing the lines. The generation of infected cell lines with a number of *T. parva* stock stabilates proved to be technically challenging, particularly with the Serengeti transformed stock. The Muguga stimulated T cell lines from BY122 and BY126 showed equivalent lysis of TpM and TpFAO1 whereas stimulation with TpFAO1 resulted in a CTL population that lysed TpFAO1 but not TpM suggesting that despite possessing epitopes conserved in the Muguga stock, the TpFAO1 line was

preferentially stimulating CTL specific for an unconserved epitope. Interestingly, stimulation of PBMC of BY126 with TpFAO1 failed to induce a detectable CTL response. The stock-specificity of CTL from BY124 stimulated with Muguga and Kiambu 5 infected cells appeared to fluctuate but the resultant $CD8^+$ polyclonal CTL lines appeared to be stock specific. This result confirms molecular analyses that have shown that Kiambu 5 and Muguga stocks are markedly distinct (Bishop *et al.*, 2001). The stock specificities shown in ^{51}Cr-release assays were confirmed by IFN-γ ELISpot assays. Since the parasite material available for screening was derived from the Muguga stock, the CTL lines that did not recognise TpM could not be used.

Prior to immunoscreening the cDNA library enriched for schizont sequences and genes predicted to encode secreted or membrane bound proteins, the CTL were used to screen the previously identified CTL target antigens in order to determine whether CTL target antigens obtained from *Bos taurus* could also be recognised by CTL obtained from the zebu. Autologous immortalised skin fibroblasts were used as antigen presenting cells since the MHC class I restriction of the CTL had not been defined. With the exception of a component of the Muguga stimulated polyclonal CTL line from BY120, none of the CTL recognised any of the known antigens. This important result provided strong support for the hypothesis that CTL isolated from Zebu cattle immunised with the FAO1 cocktail would recognise a unique set of antigens.

The $CD8^+$ T cell epitope on antigen Tp2 recognised by BY120 was mapped to a 10mer peptide, Tp2 $_{138\text{-}147}$. This was determined by comparing the consensus sequence of

antigenic peptides. The consensus 10mer and derivative peptides need to be synthesised and tested in order to confirm the minimal length antigenic peptide. Nonetheless, the data generated has confirmed a fifth epitope on antigen Tp2. Previous work had identified 4 other epitopes on Tp2 ($Tp2_{27-37}$, $Tp2_{49-59}$, $Tp2_{96-104}$ and $Tp2_{98-106}$) that were restricted by 3 different BoLA class I alleles from *B. taurus* cattle (Graham *et al.*, 2007). Tp2 is a small secretory, highly polymorphic molecule with an unknown function (Graham *et al.*, 2006) and the presence of multiple CTL epitopes suggests that it is under diversifying pressure from the bovine immune system. Interestingly, Tp2 has been the least immunogenic of vaccine candidate antigens tested to date using DNA and viral vectors for immunisation (Graham and Mwangi, personal communication).

Despite not recognising any of the known CTL target antigens, immunoscreening of the *T. parva* Muguga cDNA library and selected genes failed to identify any new antigens. Previous immunoscreening with CTL from *B. taurus* and Boran cattle had been successful with eight antigens being identified after screening with CTL lines derived from 14 cattle (Graham *et al.*, 2006). The explanation as to why the immunoscreening was unsuccessful in the present study is most likely a technical issue. One possibility relates to the efficiency of transient transfection; the cDNA screened in the present study was a different batch from that used previously and perhaps was of an inferior quality. The poor/erratic transfection efficiencies may have been caused by transfection reagents as was evidenced by low transfection efficiencies when a known A10 expressing gene JSP 1 was transfected into skin fibroblasts and the expression of A10 was assessed. Characteristics of the immortalised skin fibroblasts may also have been a contributing

factor as COS 7 cells transfected by JSP 1 exhibited a good transfection. It appears less likely that the problem lay with the zebu CTL lines since in all assays they mounted significant IFN-γ responses to TpM and with the exception of BY124, the background responses were negligible. The response of BY126 CTL to cDNA pool B12 needs to be urgently followed up since this preliminary result may represent the identification of a new vaccine candidate antigen.

The results of this study have important implications for ECF vaccine development. There is now evidence that *T. parva* specific CTL can be generated from ITM immunised zebu cattle and can be shown to exhibit MHC class I restricted cytotoxic activity against schizont infected cells. This indicates that zebu cattle are capable of responding to *T. parva* challenge by mounting a high quality cell mediated immune response. Consequently it was imperative to identify *T. parva* specific CTL target antigens for inclusion into the designing of an all inclusive vaccine against ECF. Only CTL generated from cattle number BY120 was able to recognise a CTL target antigen previously identified using CTL generated from *Bos taurus* and Boran. This suggested that CTL generated from Zebu cattle may recognise a unique set of antigens.

5.2 CONCLUSIONS

This study has demonstrated the generation of *T. parva* specific CTL from zebu cattle immunised by ITM using the FAO1 cocktail. The CTL that were generated were shown to exhibit parasite specific and MHC class I restricted cytotoxic activity against schizont infected cells and express a $CD3^+$ $CD8^+$ phenotype. CTL from animal BY120 responded to antigen Tp2 and a new epitope was mapped. The other CTL did not recognise any of the previously identified vaccine candidate antigens. Screening the library for additional vaccine candidate antigens was largely unsuccessful except that CTL from BY126 responded to a cDNA library pool and this remains to be studied further to identify a potential target antigen.

5.3 RECOMMENDATIONS

Antigenic diversity between parasite isolates is a major characteristic of ECF with isolates falling into different cross immunity groups. This requires a search for vaccine candidate antigens that are conserved between parasite stocks and which are recognised by CTLs from animals of varying genetic background. Vaccination with the FAO1 cocktail induced CTL populations that recognised epitopes that were not conserved in the Muguga stock. Since the cDNA library and selected genes were all derived from the Muguga stock, it was not possible to screen for the antigen/epitope recognised by these CTL lines. It is therefore recommended that parasite material from different stocks be prepared, ideally for all three components of the FAO1 cocktail in order to identify all the

potential antigens and epitopes that are involved in protection. In order to overcome the poor transfection efficiencies in SF, it is recommended that an alternative method of cloning full length BOLA class I DNA from these zebu animals should be used in co-transfection of COS-cells (De Plaen *et al.*, 1997).

The identification of CTL target epitopes may have a direct effect on the development of the ECF subunit vaccine. Given that many parasite antigens are targeted by CTL from immune cattle and that many of these antigens may be polymorphic, immunisation with T cell epitopes may be a logical approach to constructing a sub-unit vaccine. Poly-epitope vaccines also have the advantage that only those epitopes contributing to the desired immune response are delivered and the success of the vaccine can be evaluated by looking for T cell responses against the epitopes used (Sbai *et al.*, 2001). However, polyepitope vaccines have a number of drawbacks; parasites may develop mechanisms to evade the immunity engendered, all the essential epitopes may not be represented in the vaccine.

Recombinant DNA and pox virus polyantigen/epitope vaccine constructs encoding malaria and TB antigens/epitopes have shown to be effective in inducing polyclonal cellular immune responses in animal models and clinical trials (Gilbert *et al.*, 1997; Schneider *et al.*, 1998; Amara *et al.*, 2001; Moorthy *et al.*, 2004; Webster *et al.*, 2005). Antigenic diversity between parasite isolates is a major characteristic of ECF with isolates falling into different cross immunity groups (Irvin *et al.*, 1983). A primary concern with T cell epitope based vaccines is therefore related to the number of T cell

epitopes that need to be included in a vaccine to confer protection across geographically distributed populations and one that will be effective against multiple *T. parva* strains hence they have a narrow range of protection as all the important epitopes may not be included in the vaccine. Parasites may also develop ways of evading the immunity induced by the vaccine. CTL epitopes identified to date may not be sufficient to protect the out-bred population in the region against *T. parva* strains hence there is need to continue the search for additional antigens and epitopes. There is need to expand the scope of the vaccine by identifying epitopes recognised by $CD4^+$ T cells because $CD4^+$ T cells are important in providing $CD8^+$ T cell help. Nevertheless ITM should continue being used as it has provided solid protection, but an attempt should continue being made to the Muguga cocktail to make it cross protect against other strains and to make it more affordable.

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