CHARACTERISATION OF POLYMORPHISM WITHIN GENES ENCODING BOVINE Fc GAMMA RECEPTORS CD16, CD32 AND CD64

BY

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DECLARATION

I, William Ngachu Mwangi, hereby declare that this thesis is my original work and has not been presented for a degree in any other university.

15/5/2000

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DEDICATION

This thesis is dedicated to my parents Mr. and Mrs. Francis Mwangi.

CONTENTS

ABBREVIATIONS

DNA	Dioxyribonucleic acid
EC	Extracellular
ECF	East Coast Fever
EDTA	Ethylenediamino-tetraacetic acid
G-3-PDH	Glyceraldehyde-3-phosphate dehydrogenase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
hr	Hour
kb	Kilo base(s)
kDA	Kilo daltons
lt	Litre
mg	Milligram
Min	Minute
ml	Millilitre
mm	Millimetre
M	Molar
PBM^c	Peripheral Blood Mononuclear cells
PBL^s	Peripheral Blood Lymphocytes
Sec	Seconds
µ	Micron
μ_g	Microgram
ng	Nanogram
nm	Nanometres
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis

PBS	**Phosphate buffered saline**
RNA	**Ribonucleic acid**
Tris	**Tris (hydroxyl-methyl) aminomethane.**

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ABSTRACT

Fc gamma receptors (FcγR) are a group of transmembrane molecules that specifically bind immunoglobulins of the IgG isotypes via the Fc portion and mediate several immunological functions. These molecules have been extensively studied in human and mouse at both the nucleotide and amino acid levels. Allelic polymorphism within the human FcγR, CD32, significantly alters the affinity of the receptor for its ligand with important immunological consequences. So far, the study of bovine Fcγ receptors has concentrated on the *Bos taurus* sub-species of cattle. Using East African *B. indicus* cattle representing the extremes of reaction to antibody neutralising subunit vaccination and challenge, the study aimed to design genomic DNA-based methods to determine if the genes encoding the FcγR CD16, CD32 and CD64 are polymorphic.

Initially, peripheral blood lymphocytes from two *B. indicus* cattle (F100 and BR37) were selected for RNA extraction and subsequent cDNA synthesis. Polymerase chain reaction (PCR) primers were designed to amplify the extracellular encoding regions of the CD16, CD32 and CD64 genes. Fragments of 622 base pairs (bp), 601 bp and 778 bp were amplified, cloned and sequenced for CD16, CD32 and CD64, respectively. Sequence analysis revealed limited nucleotide and amino acid polymorphism at the three loci when compared to the published *B. taurus* sequences. These include a change of proline to threonine and proline to leucine for CD16, an insertion of an alanine for CD32 and a change of serine to proline for CD64. The extracellular distribution of these changes suggests a possible functional role associated with the observed polymorphism.

Design of DNA based methods to screen for polymorphism in large numbers of samples concentrated on CD32 and CD64. PCR primers were designed to amplify the exons encoding the extracellular regions of these molecules from 10 genomic DNA

samples. DNA samples were derived from cattle representing equal numbers of the extremes of reactions to challenge in cattle immunised with an *E. coli* recombinant form of p67, the major sporozoite surface protein of *Theileria parva*. The PCR products were analysed by single strand conformational polymorphism (SSCP). No differences were apparent among the 10 cattle analysed. However, positive controls differing at only two nucleotides could be successfully resolved using the SSCP technique, demonstrating that this method is a useful tool for analysis of allelic polymorphism within FcγR genes. Although only a limited sample, the failure to identify polymorphism within the exons encoding the extracellular regions of the 10 cattle CD32 and CD64 loci suggests that these genes have no influence on the outcome of the subunit vaccine trial.

CHAPTER ONE

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.0 East Coast Fever (ECF) and its control

1.1 *Theileria parva* the causative agent of ECF

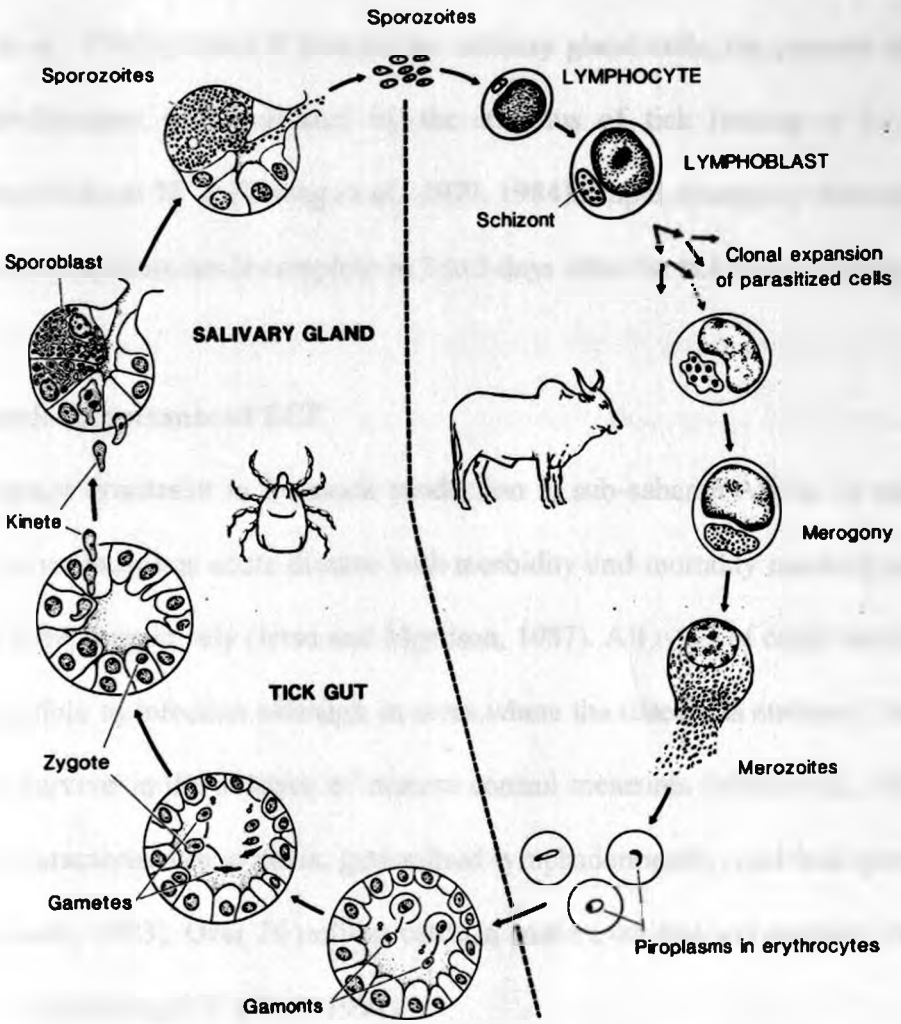
Theileria parva is an intracellular protozoan parasite that infects and transforms bovine lymphocytes, causing a severe lymphoproliferative disease known as East Coast fever (ECF) in eastern, central and southern Africa (Irvin and Morrison, 1987).

The life cycle of *T. parva* is characterised by two stages of development, one in the tick vector and the other in the bovine host. A diagrammatic representation of the life cycle is shown in Figure 1. The infective stage to the bovine host, the sporozoite, is injected with the saliva during tick feeding. The sporozoite enters rapidly into a subpopulation of host lymphocytes by a mechanism of endocytosis (Fawcett *et al.*, 1982a; 1984) and develop into macroschizonts. The host membrane surrounding the parasite is rapidly destroyed so that the parasite lies freely within the cytoplasm and close to the Golgi region of the host cell (Fawcett *et al.*, 1984; Stagg *et al.*, 1981). The infected cells are then induced to undergo blast-transformation and the parasite divides synchronously with the cell resulting in rapid expansion of the parasitized cell population (Irvin *et al.*, 1982).

After a period of growth and division in the proliferating host lymphoid cells, a proportion of macroschizonts develop to microschizonts that acquire a surface coat and induce lysis of the lymphocyte from which they are released into the circulation. The merozoites rapidly invade erythrocytes within which they develop into piroplasms. When

Figure 1. Life cycle of *Theileria parva*.

(Annual report of ILRAD, 1989)


Sporozoites
Sporozoites
LYMPHOCYTE
LYMPHOBLAST
Schizont
Clonal expansion
of parasitized cells
Sporoblast
SALIVARY GLAND
Kinete
Merogony
TICK GUT
Zygote
Merozoites
Gametes
Piroplasms in erythrocytes
Gamonts

infected erythrocytes are ingested by ticks during feeding piroplasms are released into gut lumen where they differentiate into male and female gametes (Schein *et al.*, 1977; Mehlhorn and Schein, 1984). The gametes fuse to form zygotes, which enter the lining epithelium of the tick gut and differentiate into motile kinetes. The kinetes then pass through the haemolymph and invade the E cells of type III acini of salivary glands (Fawcett *et al.*, 1982b). Once it invades the salivary gland cells, the parasite undergoes limited development until activated by the stimulus of tick feeding or by *in vitro* incubation of ticks at 37 ^{o}C (Young *et al.*, 1979; 1984). Rapid sporogony then occurs and development of sporozoites is complete in 3 to 5 days after the tick starts feeding.

1.2 Economic importance of ECF

ECF is a major constraint to livestock production in sub-saharan Africa. In susceptible cattle, *T. parva* causes an acute disease with morbidity and mortality reaching as high as 100% and 95%, respectively (Irvin and Morrison, 1987). All types of cattle breeds appear to be susceptible to infection although in areas where the disease is endemic, local Zebu cattle may survive in the absence of disease control measures (Moll *et al.*, 1986). The disease is characterised by pyrexia, generalised lymphadenopathy, and leukopenia (Irvin and Mwamachi, 1983). Over 20 million cattle in eastern, central and southern Africa are at a risk of contracting ECF (Gray, 1991).

1.3 Control strategies for ECF

1.3.1 Tick Control

One of the major methods of preventing transmission of ECF is by controlling the tick vector by application of acaricides through regular spraying and dipping of cattle. However, arsenic compounds, such as arsenic trioxide, used for this purpose have been discontinued due to their toxicity. A major problem associated with tick control is the emergence of resistant tick population. Some tick populations have been shown to be resistant to commonly used organophosphorous compounds such as Delnav DFF [Dioxathion] (Musisi, 1990; Chizyuka and Mulilo, 1990). The cost of acaricides, which are forbiddingly high for the ordinary farmer, and the frequent failure of farmers to adhere strictly to the instructions for use of a given acaricide, makes ECF control by this method impractical.

In an attempt to circumvent the disadvantages associated with the use of chemical control of ectoparasites such as ticks, immunological alternatives are being developed. An anti-tick vaccine, Tick-GARD™ for the control of *Boophilus microphis* is already commercially available (Pruett, 1999). The vaccine is based on a membrane-bound 89 kDa gut-associated antigen. Animals vaccinated using this antigen develop immunity that has apparent detrimental effects upon adult tick gut cells (Willadsen, 1997).

Restricted movement of cattle from affected areas into disease-free areas may also be used in the control of ECF. The effectiveness of this method, however, depends on many factors that may include the cultural practices of the people, cattle rustling as well as the economic and political pressures of the country at a particular time. The wide distribution of the tick vector and the presence of a carrier state in cattle or buffalo make this type of

ECF control very difficult.

1.3.2 Chemotherapy

Several therapeutic drugs effective against *T. parva* have been developed. The first effective anti-theilerial drug to be tested and used for treatment of ECF was chlorotetracycline given intravenously at a dose of 10 mg/kg body weight (reviewed by Dolan, 1981). Further testing of a wide range of compounds for antitheilerial activity has resulted in the use of menoctone and methotraxate (McHardy *et al.*, 1976; McHardy, 1978), parvaquone (McHardy *et al.*, 1983), and buparvaquone (Dhar *et al.*, 1986) as antitheilarial drugs. However, the cost of these drugs is very high for an ordinary farmer and the animals are also not protected against future infection with a different strain of the parasite.

1.4 Immunity to ECF

1.4.1 Immunisation by infection and treatment

It was observed that a mild infection with recovery and subsequent immunity could be induced in cattle by infecting the animal with sub-lethal dose of sporozoites (Cunningham *et al.*, 1974a). Further experimentation showed that inoculation of cattle with a lethal dose of sporozoites followed by four daily inoculations of oxytetracycline (5 mg/kg of body weight), produced a self-limiting infection with subsequent immunity (Radley *et al.*, 1975a). This method of immunisation was later refined using tick-derived stabilates and concurrent administration of a single dose of long-acting oxytetracycline or parvaquone at a dosage of 20 mg/kg (Radley *et al.*, 1975a, b and c; Radley *et al.*, 1979;

Dolan *et al.*, 1980; 1984). Animals immunised by this method were found to be immune to homologous challenge (Radley *et al.*, 1975a;). A major disadvantage of using this method of immunisation is the possible establishment of a carrier state in some animals which transmit the disease to susceptible non-immunised cattle (Dolan, 1986).

1.4.2 Immunisation by subunit vaccines

There is a need for improved vaccines that can circumvent the limitations of infection and treatment method outlined above. An aspect of current research at the International Livestock Research Institute (ILRI) is the development of a subunit vaccine against ECF. It has been demonstrated that monoclonal antibodies (mAbs) that recognise a 67-kDa stage specific surface antigen (p67) of *T. parva* sporozoite also neutralise sporozoite infectivity (Musoke *et al.*, 1984; Dobbelaere *et al.*, 1984). Further characterisation of p67 with mAbs has indicated that the B-cell epitopes on the antigen are conserved among the sporozoites of different parasite stocks (Musoke *et al.*, 1984; Dobbelaere *et al.*, 1984). Hence a recombinant vaccine, p67 (Musoke *et al.*, 1992) based on the major surface protein of the sporozoite has completed laboratory trials and moved to field trials. Laboratory based trials demonstrated protection against subsequent challenge in approximately 70% of animals. The cause for the 30% failure and 70% success has not yet been identified.

Current interest focuses on the identification of immunogenetic associations between polymorphic genes of cattle and the efficacy of the p67 vaccine. This requires the identification of polymorphic genes that are directly involved in controlling immune responses and the development of rapid methods to analyse this polymorphism in DNA

samples from large numbers of cattle.

Polymorphism has previously been identified in genes encoding the class I (Ellis *et al.*, 1992) and class II (Nishino *et al.*, 1995) major histocompatibility complex (MHC) antigens. These molecules are directly involved in the recognition of foreign antigens and therefore the generation of immune response in cattle. Fc gamma receptors (FcγRs) are a group of heterogeneous molecules that form a critical link between humoral and cellular immune pathways (Ravetch and Kinet, 1991). Genetic polymorphism has previously been described in human FcγRII (CD32) and linked to an altered immune response (Carlsson *et al.*, 1998). In addition there is evidence for polymorphism within genes encoding bovine FcγRIII (CD16) (Collins *et al.*, 1997). This project seeks to determine the extent of polymorphism in the genes encoding the FcγRs in African cattle populations.

1.5 Introduction to the Fc gamma receptors (FCγRs)

Fc gamma Receptors are a group of transmembrane, cell surface molecules that specifically bind homologous immunoglobulins via the Fc portion, and hence mediate biological functions by linking the humoral and cellular immune responses. FcRs exist for every antibody class: FcγRs bind IgG, Fcαrs bind IgA, Fcεrs bind IgE, Fcμrs bind IgM, and Fcδrs bind IgD. Three major groups of FcRs for IgG in human, murine and bovine have been described. Fcγ RI, FcγRII and FcγRIII. However, a novel class of mammalian FcγR (Fcγ2R) has been demonstrated in cattle (Zhang *et al.*, 1995).

1.6 Tissue distribution and functions of FcγRs

1.6.1 FcγR I (CD 64)

Human and mouse FcγRI is expressed on monocytes and macrophages (Perrusia *et al.*, 1983; Looney *et al.*, 1986) and induced on neutrophils by γ-interferon (Perrusia *et al.*, 1983). Expression can be enhanced on monocytes and various monocyte lines as much as 20-fold by culture in interferon-α (Guyre *et al.*, 1983). FcγRI has been designated CD64.

The presence of the bovine FcγRI has been demonstrated on bovine lymphocytes. From the nucleotide and amino acid sequence similarity with human and mouse FcγRs, this molecule has been identified as bovine FcγRI (Symons and Clarkson, 1992). However, there are no available data on the precise molecular weight, cellular distribution and biological function of this molecule in cattle.

FcγRI binds monomeric immunoglobulin with an affinity constant ranging from 10^{-8} to 10^{-9} M (Anderson and Abraham, 1980). Human FcγRI displays preferential binding for IgG_1 and IgG_3, while IgG_4 binds less readily and IgG_2 does not bind at all (Anderson and Abraham, 1980). Following the binding of the appropriate ligands (Fc region of the IgGs) FcγRI mediates several biological functions. These include cell activation and internalisation of the soluble and particulate immune complexes. It mediates antibody dependent cell mediated cytotoxicity (ADCC) by macrophages and monocytes and trigger superoxide production and secretion of inflammatory cytokines (Anderson and Looney, 1986). FcγRI also mediates endocytosis which has been demonstrated to enhance antigen presentation by MHC class II-positive cells (Gosselin *et al.*, 1992, Davies *et al.*, 1995).

This molecule has also been demonstrated to mediate phagocytosis (Anderson *et al.*,

1990) and this has been shown to depend on FcRγ chain (Davies *et al.*, 1995).

1.6.2 FcγRII (CD32)

The FcγRII molecules are the most widespread and expressed on virtually every FcγR-bearing cells except for natural killer (NK) cells. The number of copies varies greatly from 10^3 on platelets to more than 10^5 on murine macrophages. Unlike FcγRI the affinity of FcγRII for monomeric immunoglobulin is so low that binding cannot be measured (Cohen *et al.*, 1983). Nevertheless, the presence and function of FcγRII is readily demonstrated by the binding of immune complexes (Rosenfeld and Anderson, 1989). This molecule is also known as CD32.

A FcγR molecule has been described on the surface of bovine alveolar macrophages, which confer the capacity to bind IgG-sensitized erythrocytes on transfected COS cells. From comparisons with human and mouse sequences the molecule has been established to be bovine FcγRII (Zhang *et al.*, 1994). Studies carried out previously indicated that it is also expressed on cultured bovine monocytes and has a low affinity for bovine IgG_2 (McGuire *et al.*, 1979. Howard *et al.*, 1980). The specific tissue distribution and full biological function of this molecule in cattle has not been studied.

As with FcγRI, human FcγRII shows preferential specificity for the IgG subclasses IgG_1 and IgG_3, while they bind IgG_4 and IgG_2 less readily (Rosenfeld and Anderson, 1989).

FcγRIIA, a subset of FcγRII, has been demonstrated to mediate phagocytosis when expressed on mouse fibroblasts (Tuijnman *et al.*, 1992). This phagocytosis depends on calcium (Elberg *et al.*, 1995) and requires tyrosine kinase activity (Park and Schreiber, 1995).

FcγRIIB is involved in capping, endocytosis and phagocytosis when aggregated by multivalent ligands. It also triggers calcium responses and oxidative burst when aggregated on neutrophils (Van de Herik-oudijk *et al.*, 1994). Endocytosis via FcγRIIB enables MHC class-I positive cells to present IgG-complexed antigen with increased efficiency (Amigorena *et al.*, 1992).

1.6.3 FcγRIII (CD16)

Two distinct forms of human FcγRIII have been defined. The form found on neutrophils is anchored to the plasma-membrane by a glycosyl-phosphatidylinositol (GPI) moiety, whereas the form of the receptor expressed on NK cells, macrophages and cultured monocytes is a conventional transmembrane protein with a distinct cytoplasmic domain (Ravetch and Perrusia, 1989). This molecule is designated CD16.

The presence of bovine FcγRIII has been described and its expression ascertained in bovine $\gamma\delta$ T cells (Collins *et al.*, 1997). The presence of the membrane-anchored form of this receptor has not been demonstrated in cattle. This study was carried out by comparison with human FcγRIIIA cDNA sequence. No data are available on the specific function, affinity constants or the tissue distribution of this molecule in cattle.

FcγRIIIA has been demonstrated to trigger ADCC on IgG-coated cells by NK cells (Viveir *et al.*, 1992). It has also been demonstrated to induce apoptosis in NK cells previously exposed to interleukin-2 (IL-2). IL-2 induces an accumulation of c-myc transcripts in NK cells, and c-myc induction seems to be necessary for FcγRIIIA to trigger apoptosis (Azzoni *et al.*, 1995).

FcγRIIIA has also been shown to mediate endocytosis (Amigorena *et al.*, 1992). This

endocytosis enhances antigen presentation by MHC class-II positive cells.

This molecule is also involved in the mediation of phagocytosis, which depends on FcRγ chain (Davies *et al.*, 1995).

1.6.4 Bovine FcγR2

A novel class of mammalian FcγR has been demonstrated in cattle. COS-7 Cells expressing the receptor on the surface following transfection were shown to bind erythrocytes specifically sensitised with IgG2 but not IgG1. The study was performed by use of cattle alveolar macrophages suggesting that these are among the cells that express this FcγR *in vivo*. The evolution of this novel mammalian FcγR is believed to have been influenced by the truncated hinge of the bovine IgG2 molecule (Zhang *et al.*, 1995).

1.7 Genetic organisation of FCγRs

1.7.1 FcγRI (CD64)

cDNA clones for FcγRI receptor have been isolated and characterised in humans. Transcripts of 1.7 kilobases (kb) were detected by northern blot analysis in RNA prepared from monocytes (Allen and Seed, 1989).

Three genes for FcγRI (A, B & C) have been characterised. Each gene consists of six exons. The signal peptide is encoded by two exons, the extracellular region is encoded by three exons and a single exon encodes the transmembrane and cytoplasmic regions. The three genes differ in the third exon for the extracellular region, EC3 (Ernst *et al.*, 1992). Using somatic cell hybrids the genes for FcγRI as well as all other human FcRs genes were mapped to chromosome I (Osman *et al.*, 1992).

The three extracellular domain exons of bovine FcγRI have been cloned, mapped and sequenced (Symons and Clarkson, 1992). The study suggests a single copy gene for bovine FcγRI as has been proposed for the same molecule in mouse (Sears *et al.*, 1990).

1.7.2 FcγRII (CD32)

cDNA clones have been described for both murine (Hogarth *et al.*, 1987) and human (Stuart *et al.*, 1989) FcγRII genes. The murine beta gene encodes the prototype molecules of this group. This gene encodes a low-affinity receptor that binds IgG1, IgG2a and IgG2b (Weinsshank *et al.*, 1988). Two distinct cDNAs have been isolated and characterised and are referred to as beta 1 and beta 2. These transcripts have been shown to be derived by alternative splicing of the cytoplasmic encoding exons (Qui *et al.*, 1990). A single gene on chromosome 1 encodes the murine beta molecule.

FcγRII in humans is encoded by a minimum of three genes (Qui *et al.*, 1990), however differential splicing result in six distinct transcripts (Brooks *et al.*, 1989). These genes are evolutionally related to the murine beta genes. Three distinct transcripts are derived from the FcγRIIb gene by alternative splicing of cytoplasmic encoding exons (b1 and b2), as in murine beta transcript, with an additional spliced form derived from alternative splicing of the exons encoding the signal sequence (b3). Three other transcripts have been identified in the FcγRII group of molecules (Brooks *et al.*, 1989). These transcripts known as IIa and IIa′, are derived from a minimum of two distinct genes. The overall intron-exon structure of the IIb/beta family genes is retained. IIa gives rise to transcripts of 1.8 and 2.5 kb as a result of alternative poly-adenylation while IIa′ is encoded by a distinct gene producing a 1.8 kb transcript.

The gene encoding the IIb has two exons for the signal peptide, two exons for the extracellular region, a single exon for the transmembrane region and two exons for the cytoplasmic region. The same general organisation is observed in the other two genes. Human FcγRII genes are linked to each other and to FcγRIII on chromosome 1 (Sammartino *et al.*, 1988).

cDNA clones for bovine FcγRII have been isolated and characterised. This cDNA was prepared from alveolar macrophages, since previous investigations indicated that they expressed high levels of FcγRs for IgG1 and IgG2 (Howard *et al.*, 1980). The clones had a bovine FcγRII insert of 1.5 kilobases (kb), which when subcloned and sequenced constituted a 1474 nucleotide fragment containing a single long open reading frame which extends 888 base pairs from the first initiation codon. Of these, 126 base pairs code for the signal peptide, 75 base pairs code for the transmenbrane region, 543 base pairs code for the extracellular region and 144 base pairs code for the cytoplasmic region. A search of the PIR database using the FASTA programme (Lipman and Pearson, 1985) revealed 74% and 68% similarities for the cattle sequence with human and mouse FcγRII sequences, respectively (Zhang *et al.*, 1994).

It has been predicted that more than one FcγRII gene is present in cattle, as in the other species (Zhang *et al.*, 1995). However, there has been no study so far on the precise structure of the gene or its specific mapping.

1.7.3 FcγRIII (CD16)

Murine FcγIIR$^{\alpha}$ and the human FcγRIII genes are now recognised as homologous molecules by virtue of their sequence homology, genomic organisation, cellular

distribution and shared function.

A single gene found on chromosome 1 encodes FcγRIIα (Qui *et al.*, 1990). A single transcript of 1.6 Kb has been identified for α. Two genes, referred to as III-1 and III-2 encode FcγRIII in humans (Ravetch and Perussia, 1989). They are almost identical genes, differing by 10 nucleotide substitutions in the coding regions. The intron-exon structure is identical to that of the murine α with each gene giving rise to a single transcript. III-1 is expressed exclusively in neutrophils, while III-2 is expressed in NK cells and macrophages (Ravetch and Perussia, 1989). The gene encoding the III-2 transcript has two exons for the signal peptide, two exons for the extracellular region and a single exon for the transmembrane and cytoplasmic region. As in the mouse, human FcγRIII genes are linked to FcγRII genes (Qui *et al.*, 1990).

A cDNA clone for bovine FcγRIII has been isolated and characterised in cattle. The cDNA was obtained from mesenteric lymph node cells. This full-length cDNA was found to be 1071 nucleotide long and contains a single long open reading frame extending 753 base pairs from the initiation codon.

The sequence shows a 67 % nucleotide similarity with mouse FcγRIII when compared using the GCG bestfit programme (Collins *et al.*, 1997).

1.7.4 Bovine FcγR

A cDNA clone isolated from a library made from bovine alveolar macrophage, was found to encode a FcR that bound complexed IgG1 but not IgG2. Sequence data show that this gene is distinct from the previously reported bovine FcγRI gene. The 59% and 55% sequence identities with human and mouse FcγRII sequences, respectively, implied this

gene encoded bovine FcγRII. The cDNA insert in the plasmid was found to be 1,582 nucleotides in length and contained a single open reading frame that extended 792 base pairs from the first in-frame initiation codon. The clone had a 5'–untranslated region of 122 nucleotides and 3'–untranslated region of 668 nucleotides terminating in a poly-A tract (Zhang *et al.*, 1995).

1.8 Protein structures of FcγRs

1.8.1 FcγRI (CD64)

The relative molecular mass (Mr) of murine FcγRI has been reported to be 67 kDa (Lane *et al.*, 1980). Human FcγRI is a glycoprotein of approximately 72 kDa (Cohen *et al.*, 1983).The human molecule has 375 amino acid residues. It has a short hydrophobic signal peptide, a 21 amino acid hydrophobic membrane-spanning domain and a short highly charged cytoplasmic domain. The extracellular portion contains six potential N-linked glycosylation sites and six cysteine residues distributed among the three Ig-like domains (Allen and Seed, 1988). This molecule may be associated with the kinases Hck and Lyn, which can be co-immunoprecipitated with it from cell lysates (Wang *et al.*, 1994).

From the DNA structure of the three sequenced extracellular domains of the bovine FcγRI, the protein structure has been predicted. This region is made up of 267 amino acids. There are potential intra-chain disulphide bonds that occur between cysteine residues 29-71, 110-154 and 198-246. The derived bovine protein sequence has been aligned with that of human (Allen and Seed., 1989) and mouse (Sears *et al.*, 1990). The cysteine residues involved in intrachain disulphide bonds are coincident in all these

species.

Amino acid residues of FcγRs from the three species have a similarity order of 60, 58 and 58% for the first, second and third extracellular domains, respectively. All these domains can be assigned to a subset of the immunoglobulin superfamily (Williams and Barclay, 1988). In the second extracellular domain of bovine FcγRI, as in human and mouse the first residue aspartate of this motif is replaced by histidine. Arginine replaces glycine in this motif in the third domain of bovine FcγRI.

The bovine extracellular domains of the FcγRI have 5 potential N-linked glycosylation sites. Two sites, at positions 4 and 145 are conserved in all three species. A site at position 24 is also present in mouse but not in human FcγRI. Two sites at residues 195 and 219 in the third domain are present only in the bovine receptor, while human and mouse molecules have a single site in third domain at position 226. The bovine sequences maintain the pattern of homology between domains 1 and 2 of FcγRI and their counterpart domains in FcγRII and FcγRIII receptors (Sears *et al.*, 1990). Domains 1 and 2 are homologous within all FcγRs. Domain 3 is unique to FcγRI.

1.8.2 FcγRII (CD32)

The molecular mass of human FcγRII is 40 kDa and murine FcγRII ranges between 40-60 kDa. Human FcγRIIB2 molecule has 317 amino acid residues. The signal peptide is made up of 36 amino acid residues. The extracellular region has 182 amino acid residues, which include the four cysteines that are predicted to form the two Ig-like domains. A hydrophobic core of 23 amino acid residues form the transmembrane region. A 76 amino acid region forms the extracellular domains (Brooks *et al.*, 1989). The cytoplasmic

portion of B1 isoform of FcγRII associates with the tyrosine phosphatase SHP-1 via a phosphorylated YXXL motif (Scharenberg and Kinet, 1996).

Based on the nucleotide sequence the cattle molecule has been predicted to be a 296 amino acid residue polypeptide encoded by 888 base pairs. It has 42 amino acid residues for signal peptide; 181 amino acid residues for the extracellular domain; 25 amino acid residues for the transmembrane region and 48 amino acid residues for the cytoplasmic region. Cysteine residues at the positions 70, 112, 151 and 195 are predicted to be involved in the formation of the disulphide bonds of the two Ig-superfamily (IgSF) domains. Five potential N-liked glycosylation sites occur along the molecule (positions 79, 86, 105, 179, 186), which all fall within the Ig domains.

Comparisons between the amino acid sequences of the molecule from cattle, human and mouse FcγRII molecules, respectively showed that there was 62%-59% identity over the extracellular region, 35%-32% identity over the transmembrane region, and 55%-50% identity over the cytoplasmic region. High interspecies sequence similarity along the four cysteine residues in the extracellular region suggests that the cattle molecules also have two extracellular IgSF domains (Zhang *et al.*, 1994).

1.8.3 FcγRIII (CD16)

The molecular weight of the transmembrane form of human FcγRIII is 50-80 kDa (Lanier *et al.*, 1988). It is made up of 254 amino acid residues. The signal peptide has 17 amino acid residues, 20 amino acid residues make up the transmembrane region and 25 amino acid residues are in the cytoplasmic region. The extracellular region has 192 amino acid residues which includes the four cysteine residues, forming the two Ig-like domains

(Ravetch and Perussia, 1989). On mast cells, FcγRIII is non-covalently associated with the β chain of the FcεRI (Kurosaki *et al.*, 1992).

From the nucleotide sequence, the bovine FcγRIII has been predicted to have 250 amino acid residues. The signal peptide is made up of 16 amino acid residues, the extracellular region is made up 187 amino acid residues, the transmembrane and cytoplasmic regions are made up of 47 amino acid residues.

The cysteine residues at positions 47, 89, 128 and 172 on the extracellular domain are predicted to demarcate the two Ig-SF domain boundaries. There are 3 potential N-linked glycosylation sites, (asparagine 56, 63, and 180) in the extracellular region of the molecule. The presence of phenylalanine at position 203, as in the human FcγRIIIA (Lanier *et al.*, 1989; Kurosaki and Ravetch., 1989), by analogy with studies in human indicates that the cattle molecule is not GPI-anchored and that cattle FcγRIII are conventional transmembrane proteins. It is not known whether a cattle FcγRIII GPI-linked form exists (Collins *et al.*, 1997).

1.8.4 Bovine FcγR2

This is a 264-residue polypeptide molecule. It has a 19 amino acid residue signal peptide a 211-amino acid residue extracellular domain, which includes three potential sites for N-linked glycosylation and several conserved residues that are the hallmark of Ig-superfamily-like sequences. A hydrophobic stretch of 19 amino acid residues is evident, forming a presumptive transmembrane domain followed by a short cytoplasmic tail of 15 amino acids. The molecular weight of the molecule would be 35.8 kDa to 38.8 kDa, assuming that each of the three potential N-linked glycosylation sites bound a

carbohydrate moiety of 3,000 to 4,000 Dalton. These residues included four cysteine residues which are assumed to form the two intra-chain disulphide bonds that hold together each of two Ig folds.

A search of the PIR database using the FASTA programme revealed greatest similarity with human Fc$^{\alpha}$R with a percentage of 41% identical amino acids (Zhang *et al.*, 1995).

1.9 Importance of this study

Fc$^{\gamma}$R are a group of cell-surface molecules that specifically bind homologous immunoglobulins via the Fc portion, and hence mediate biological functions by linking the humoral and cellular immune responses. Evidence from the studies carried out so far suggests that these molecules play a critical role in the recognition of foreign antigens and therefore the generation of immune response. The study of Fc$^{\gamma}$Rs is important in understanding of linkage between the cellular and humoral immune mechanisms and may be utilised in the design of more efficient subunit vaccines.

ECF is currently controlled by immersing cattle or spraying them with acaricides to kill the tick vector and break the life cycle of the parasite, *Theileria parva*. This control strategy has its limitations. The high cost of acaricides makes them unaffordable by many farmers. In addition, dipping of cattle in community-based cattle dips may need to be done as frequently as twice a week to be effective. This leads to land degradation due to soil erosion caused by cattle movement, pollution of the environment with toxic substances and it may lead to tick resistance to acaricides. Killing of ticks by acaricides prevents exposure of cattle to *T. parva*. Such cattle do not develop immunity to the

parasite and hence have no protection if acaricides treatment is interrupted or stopped. Treatment of ECF is achieved using drugs such as buparvaquone. The use of chemotheurapeutic agents in treatment of ECF is expensive and unaffordable by most farmers in developing countries. There is therefore a need to devise a less expensive, yet effective way of controlling ECF.

An infection and treatment method of vaccination is currently available (Irvin and Mwamachi, 1983). This involves inoculation of a live sporozoite stabilate into the cattle with simultaneous treatment using a long-acting tetracycline. There are a number of disadvantages associated with this method. Firstly, it introduces live strains of the parasite where such strains may not exist. The parasites may be ingested by ticks during feeding and may be transmitted to other animals in the area. Secondly, the protection achieved through this method is strain-specific. An animal needs to be immunised against all strain of *T. parva* to be effectively vaccinated. The vaccine stabilate needs to be stored in liquid nitrogen to remain viable. This makes its transportation to rural areas in developing countries unrealistic due to the unavailability of liquid nitrogen and poor infrastructure. These limitations highlight the necessity of alternative subunit vaccine against *T. parva*. Development of a subunit vaccine involves identification of immunogenetic components of the parasite, preferably those common to all parasite strains. The gene coding for the parasite component is isolated and expressed in recombinant form. Immunisation of animals with the recombinant protein stimulates an immunological response. In this way, a subunit vaccine (p67) against ECF has been developed based on a major surface protein of *T. parva* sporozoites.

However, the outcome of vaccination may be influenced by the polymorphic nature of

molecules involved directly or indirectly in various aspects of immune response. In humans, it has been demonstrated that the presence of the HLA class II allele DPB1*0501z in ethnic Thais correlates with an enhanced vaccine-induced antibody response to a malaria sporozoite antigen (Stephens *et al.*, 1995). Similarly it has been observed in H-2 disparate mouse strains, that both humoral and cellular immune response to various malaria target antigens (including CS protein) correlate with H-2 phenotype (Good *et al.*, 1988). Polymorphism has been identified in human FcγRIIa. This has been shown to occur in two co-dominantly expressed allelic forms (R131 and H131). The change arises from a single nucleotide difference at position 494 which is either guanine (G) or adenine (A) giving rise arginine(R) or histidine (H), respectively as the amino acid residue 131 (Manger *et al.*, 1998). This polymorphism has been further demonstrated to be functionally relevant, as cells expressing IIa-H131 interact much more effectively with complexed IgG2 and IgG3 than do cells with IIa-R131. This phenomenon has been linked to variability in immune complex handling, *in vivo*, and therefore related to disease pathogenesis in systemic lupus erythematosus (Manger *et al.*, 1998), Heparin-induced thrombocytopenia (Carlsson *et al.*, 1998) and myasthenia gravis (Raknes *et al.*, 1998).

Based on the evidence above, it can be deduced that a single recombinant parasite protein like p67 may not adequately protect all individuals within outbred populations. This has prompted study aimed at characterising MHC gene polymorphism in cattle under immunisation trial (Ballingall *et al.*, 1997). In addition, the antibodies that play a role in sporozoite neutralisation following p67 vaccination will be recognised by other effector molecules on the surface of immune cells. Hence, polymorphism within the FcγRs may

affect the outcome of vaccination and influence design of more effective vaccines.

Field trials since this project was carried out using p67 have not demonstrated a high efficacy as observed under laboratory conditions (unpublished data). This is an indication that a single antigen may not be sustainable under field conditions, hence a search for schizont antigens that induce protective immune responses continues. However the work carried out in this project would be of importance in the study of immunogenetic association in other vaccine trials.

This project sought to determine whether there is polymorphism within the CD16, CD32 and CD64 molecules in cattle and to design rapid PCR based methods to analyse this polymorphism in genomic DNA samples from animals previously vaccinated and challenged.

1.10 Aims and objectives

The aim of the project was to identify polymorphism in the genes encoding bovine FcγRs (CD16/CD32/CD64) and to design a rapid PCR-based method to analyse this polymorphism in bovine genomic DNA samples.

1.10.1 Specific objectives

1. To prepare complementary DNA (cDNA) from the peripheral blood leukocytes (PBLs) or peripheral blood mononuclear cells (PBMCs) of *Bos indicus*, cattle.

2. To amplify the CD16, CD32 and CD64 genes from the cDNA preparations using the polymerase chain reaction (PCR).

3. To clone and sequence the amplified genes for the identification of genetic polymorphism.

4. To design rapid PCR based methods to analyse polymorphism in genomic DNA.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Experimental animals

All the cattle used in this project were provided by the International Livestock Research Institute (ILRI), Nairobi, Kenya. They were all Kenyan Boran cattle, which are classified as African Zebu, *Bos indicus* subspecies. The two animals chosen for initial cDNA analysis were BR37 and F100. Of the two animals, BR37 was a non-reactor following p67 vaccine trials, while F100 was not involved in this trial.

2.2 Cell culture and molecular biology reagents

Ficoll paque used in the isolation of PBMCs was obtained from Pharmacia (Pharmacia Biotech, Uppsala, Sweden). Alsevers solution and RPMI 1640 were prepared at ILRI Nairobi, Kenya. Most of the molecular biology reagents such as *Taq* DNA polymerase, RT-PCR kit, F-*mol* kit, T4-DNA ligase, restriction enzymes, deoxyribonucleotide triphosphates (dNTPs) were obtained from Promega (Promega, Madson, USA). The RNA extraction kit was obtained from Biotex (Biotex Laboratories, Houston, Texas). γ^{33}P ATP was obtained from Amersham (Amersham Radiochemicals, Amersham, UK).

2.3 Collection of blood samples

Peripheral blood mononuclear cells (PBMCs) were prepared from blood obtained from the jugular vein of an animal using a 60 ml syringe containing 30 ml of Alsever's solution, Appendix A.1.1 (0.55 g/l citric acid, 20.5 g/l dextrose, 4.2 g/l NaCl, 8.0 g/l sodium citrate) (Godderris *et al*., 1989) as an anticoagulant.

Alternatively, peripheral blood lymphocytes (PBLs) were prepared from jugular blood collected in 10 ml vacutainer tubes (Becton Dickson vacutainer systems, Europe) containing a small volume of ethylenediaminetetraacetic acid (EDTA) as an anticoagulant.

2.4 Preparation of PBMCs by density gradient centrifugation

PBMCs were prepared by Ficoll-paque gradient centrifugation in a modification of the method described by Peper *et al.*, (1968). In this procedure, 30 ml of the uncoagulated blood collected in Alsevers solution (Appendex A.1.1) was layered onto 18 ml of Ficoll/sodium diatrizoate solution (Pharmacia Fine Chemicals, Uppsala, Sweden) in 50 ml falcon tubes and centrifuged at 890 x g for 30 mins at 20 ^{o}C. The upper layer of plasma was drawn off using a clean Pasteur pipette, leaving the lymphocyte layer undisturbed at the interface. Using a clean Pasteur pipette the lymphocyte layer was harvested and transferred to a clean Falcon tube. Three volumes of Alsevers solution were added and the cells suspended by gently drawing them in and out of a Pasteur pipette. This was centrifuged at 500 x g for 10 min at 20 ^{o}C in the same centrifuge. The supernatant was removed and the lymphocytes resuspended in three volumes of Alsever's solution. The mixture was centrifuged at 220 x g for 10 min at 20 ^{o}C, as a final wash and resuspended in 20 ml Alsever's solution. The resuspended cells were stored in ice for RNA extraction.

2.5 Preparation of PBLs by red cell lysis using Tris-Ammonium Chloride

To 1 ml of whole blood in EDTA as anticoagulant, 9 ml of Tris-Ammonium Chloride (Appendix A.1.2) was added and kept in ice for 2 min to allow lysis of red blood cells. The mixture was centrifuged at 11000 rpm for 5 min at 4 °C, using a Sorvall RC-3B centrifuge (H6000A rotor). The supernatant was poured off and the pellet resuspended in 10 ml PBS buffer (Appendix A.1.3) and centrifuged under the same conditions. The supernatant was discarded and the pellet (PBLs) kept in ice for RNA extraction.

2.6 Isolation of DNA from fresh blood

DNA was isolated from fresh cattle blood using salt extraction method, modified from a procedure described by Kupiec *et al*,. (1987).

In this procedure 10 ml of fresh blood was mixed with 45 ml lysis buffer in a 50 ml Falcon tube (Becton Dickson Labware, USA) and left for 10 min on ice. The mixture was centrifuged at 1400 x g for 10 minutes at 4 °C and the resulting pellet resuspended in 9 ml of solution B (Appendix A.2.2). This step was performed three times after which 0.5 ml proteinase-K/10% SDS solution (Appendix A.2.3) was added followed by incubation at 50 °C overnight. A 2.7 ml volume saturated NaCl (Appendix A.2.4) was added and after thorough mixing the mixture was centrifuged at 2000 x g for 10 min at 4 °C. The supernatant containing the DNA was transferred to a clean Falcon tube and two volumes of cold absolute ethanol added. The DNA was then removed by a pipette into another Falcon tube and washed with 80% ethanol by spinning at 1400 x g for 10 min at 4 °C. The DNA pellet was finally air dried and resuspended in 10 ml sterile reagent water.

2.7 Preparation of total RNA from PBLs or PBMCs.

The total RNA extraction was carried out using previously described methods (Stronman *et al.*, 1977 and Chirgwin *et al.*, 1979). A volume of the cell suspension containing 10^7 cells was transferred to a sterilin tube and the cells pelleted by centrifugation (220 x g, 10 min, 4 °C). After discarding the supernatant, the cells were lysed by addition of 2 ml of RNAzol ™ B (4M guanidium isothiocyanate, 25 mM sodium citrate pH 7.0 and 0.1M 2-mercaptoethanol), transferred to a 1.5 ml eppendorf tube and the RNA solubilised by passing the lysate a few times through the pipette. Chloroform (0.2 ml) was added and the tightly covered eppendorf tubes shaken for 15 seconds then left in ice for 5 min. RNA was then extracted by centrifugation (10,000 rpm for 15 min at, 4 °C), in an eppendorf minifuge (Tomy, Seiko Co., Tokyo, Japan). The aqueous upper phase was transferred to a fresh eppendorf tube and an equal volume of isopropanol added. The sample was stored at 4 °C for 15 min then centrifuged at 12000 rpm for 15 min at 4 °C using the same centrifuge. This step precipitates RNA as white-yellow pellet at the bottom of tube. The supernatant was aspirated and the RNA pellet washed with 500 µl of 75% ethanol by vortexing and subsequent centrifugation (12,000 rpm, 8 min at 4 °C). The pellet was then dried under vacuum for 10 min and resuspended in 20µl RNAse-free water and stored at -20 °C.

RNA concentration was estimated by measuring optical density (OD) at a wavelength of 260 nm assuming 1 unit represents a concentration of 40 µg/ml of RNA (Sambrook *et al.*, 1989).

2.8 cDNA Synthesis (reverse transcription system)

cDNA was synthesised by reverse transcription of total RNA using avian myeloblastic virus reverse transcriptase (Promega, Madison, USA), as described by Bailer *et al.*, (1993) and the Promega reverse transcription system protocol (Promega) according to the manufacturers instructions. Reverse transcription of 1 µg of RNA was carried out in a 20 µl reaction containing 10 mM of deoxyribonucleotide triphosphate (dNTPs), 1x reaction buffer (appendix A.2.5), 25 mM $MgCl_2$, 15 units AMV reverse transcriptase, 20 units of rRNAsin ribonuclease inhibitor and 0.5 µg oligo-dT primer.

Table 1: Complementary DNA synthesis reaction mix.

Reagents	Quantity in µl
$MgCl_2$	4
10 x RT buffer	2
dNTP mix	2
15 u AMV RT Enzyme	0.6
rRNAsin	0.5
Oligo-dT primer	2.5
RNA (1 µg)	X
water	Y
Total	20

Legend: X and Y vary depending on the concentration of RNA.

The mixture was incubated at 42 °C for 1 hour then heated to 99 °C for 5 min to inactivate the enzyme and prevent it from binding to the cDNA, which would interfere with its subsequent use. After a further 5 min incubation at 4 °C the cDNA was then stored at –20 °C.

2.9 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is an efficient procedure for enzymatic amplification of specific target nucleic acid sequence *in vitro* in the presence of appropriate nucleotide primers (Saiki *et al.*, 1988). The PCR involves a cycle of three temperature-controlled steps. In the first step, target double stranded DNA is denatured into single strands by heating (93-94 °C). Synthetic complementary oligonucleotides are annealed to the denatured DNA strand in the second step and finally the primers are extended in the 5′-3′ direction using heat stable *Thermus aquaticus* (*Taq*) DNA polymerase. This leads to accumulation of the specific segments of DNA.

Because the primer extension product synthesised in one cycle can serve as template in the next, the number of target DNA templates approximately doubles with each cycle. Initially, the klenow fragment of *E. coli* DNA polymerase was used to extend the annealed primers (Mullis and Faloona, 1987; Saiki *et al.*, 1985). However, since the enzyme was inactivated by the high temperatures necessary to separate the two strands of DNA, fresh enzyme had to be added during each cycle. However, with the discovery of *Taq* polymerase, the problem has been overcome. The successful amplification of nucleotide sequences by PCR is highly empirical because for any given pair of oligonucleotide primers, an optimal set of conditions (enzymes, primers, Mg^{++}

concentration as well as the temperature cycle profile) has to be established. There is, therefore, no single set of conditions that will be optimal for all possible reactions.

The high temperatures tolerated by *Taq* polymerase (75 ^{o}C) allows for the use of higher temperatures for primer annealing and extension, thereby increasing the overall stringency of the reaction and minimising extension of primers that were mismatched with the template (Chien *et al.*, 1976). In addition to this, the use of *Taq* polymerase, allows amplification of much longer fragments (to 1 kilobase, albeit with reduced efficiency) than does the klenow enzyme (<400 base pairs). Contamination of the sample reaction with material from the exogenous sources is a potential problem, since PCR has the capacity to amplify any DNA template. Careful laboratory handling and carrying out only a minimal number of PCR cycles required for analysis also minimises the chance that a rare contaminating template will be amplified. A negative control, without any template DNA, is necessary to detect potential contamination.

2.10 Design of primers for amplification of bovine CD16, CD32 AND CD64

As the three Fc gamma receptors genes had been previously sequenced in European taurine (*B. taurus*), their sequences from the data base were used in design of primers for the PCR amplification. The primers were designed to give a PCR amplification of the region encoding the extracellular part of the molecules.

A full length CD16 cDNA had been previously sequenced (Collins *et al.*, 1997). Using this sequence a forward primer was designed from the 3′ end of the region encoding the signal peptide and a reverse primer from the 5′ end of the region encoding the transmembrane region (Figure 2). This was to make sure that the entire extracellular

domain encoding region was amplified (Appendix C a).

From published sequenced for bovine CD32 (Zhang *et al.*, 1995), primers were again designed to amplify the region encoding the extracellular part of the molecule by designing the forward primer at the 3′ end of the signal peptide encoding region and the reverse primer on the 5′ end of the transmembrane encoding region (Figure 3, Appendix C b).

For CD64 only the exons encoding the three extracellular domains had been sequenced in bovine genomic DNA (Symons and Clarkson, 1992). Hence from these sequences the forward primer was designed at the 5′ end of exon I for the extracellular encoding region while the reverse primer was designed at the 3′ end of exon III for the extracellular encoding region (Figure 4, Appendix Cc). In addition, two pairs of primers were designed to amplify the two exons encoding the extracellular region of CD32 (Appendix D, a) and in a similar way another two pairs of primers were designed to amplify the second and third exons encoding the extracellular region of CD64 (Appendix D, b).

The primers designed had a GC content of 52-66%, a GC clamp and were not self-complementary. These primers were then synthesised by the oligonucleotide synthesis unit at ILRI as described below.

2.10.1 Oligonucleotide synthesis

Oligonucleotides were synthesised using the automated Applied Biosystems DNA synthesizer model 381A (ABI, Foster City, CA, USA). The procedure uses solid phase synthesis chemistry in which the growing DNA chain remains covalently attached to an

Figure 2

Bovine CD16 sequence showing PCR primer positions. Arrows represent the prin
with the arrow showing the direction of amplification. The primers are labelled as
and 065. Primer 064 represent the forward primer while 065 represent the reverse

064

```
        10          20          30          40          50          60
AGGCGCACTC TAACACACAG CATGTGGCAA CTGCTACCAC CGGCAGCTCT GCCTGTTCTA
        70          80          90         100         110         120
GTTTCAGCTG ACACGCAAAC TGCAGATCCC TCAAAGGCTG TGGTGCTCCT AGACCCTCAG
       130         140         150         160         170         180
TGGAACCACG TGCTCACGAA TGACCGTGTG ACTCTGAAGT GCCAGGGAGA CTACCCTGTT
       190         200         210         220         230         240
GAAGACAATT CCACAAAGTG GTGGCACAAT GGGACTCTCA TCTCAAGCCA GACCCCCAGC
       250         260         270         280         290         300
TACTTCATCG CAGATGTCAA GGTTCAGGAC AGTGGCGAGT ACAAGTGTCA GACAGGCCTC
       310         320         330         340         350         360
TCTGCACCCA GTGACCCGGT GAAGCTAGAA GTCCACGTAG GCTGGCTATT GCTCCAGGTC
       370         380         390         400         410         420
GCTCAACGGG TGGTAAATGT GGGAAAGCCC ATTCGGCTGA AGTGTCACAG CTGGAAGAAA
       430         440         450         460         470         480
ACTCCTGTAG CAAAGGTCCA GTATTTCCGG AATGGCAGAG GCAAGAAGTA TTCTCATGGG
       490         500         510         520         530         540
AATTCTGACT TCCACATTCC AGAAGCAAAA CTTGAACACA GTGGTTCCTA CTTCTGCAGG
       550         560         570         580         590         600
GGCATTATCG GGTCTAAAAA CGAGTCTTCA GAGTCTGTGC AGATCACTGT TCAAGCTCCA
       610         620         630         640         650         660
GAAACTTTAC AAACTGTCTC GTCATTCTTT CCACCTTGGC ACCAGATCAC CTTCTGTCTG
                                                065
       670         680         690         700         710         720
GTGATGGGAG TCCTGTTTGC AGTGGACACG GGACTGTATT TTTCTGTACG GAGACACCTT
       730         740         750         760         770         780
CAAAGCTCAG AGGAATGGAG GGATGGCAAA GTCACATGGA GCAAGGGCCC TTAGGACAAA
       790         800         810         820         830         840
TGGAGGCATC TCTGGGAGCA GCAGGAAAAT TACACGTCAG ACTCTAGGGA TCTGAGGATC
       850         860         870         880         890         900
CTTCACCCAA CTCTGTTTCT TCTAGTCTCC TATGGAAAGA AAGTGCCAAC CGTGAAGGAC
       910         920         930         940         950         960
CAGTAAATAT CTGCATCACC AGAAATAGAA GTCTCAGAGC TACGTGGCTG CTTTCTGTAT
       970         980         990        1000        1010        1020
TCCAACCATT CTGTCCCAAG AGAACAACCA TACAGACTCT CAAAGATCAG CCCTGATGTA
      1030        1040        1050        1060        1070        1080
TGAATATTGT GCTAAAATAA ATGGATATGT AAAGAAAAAA AAACTTTCCT G
```

Figure 3

Bovine CD32 sequence showing PCR primer positions. Arrows represent the primers with the arrow showing the direction of amplification. The primers are labelled as 066 and 067. Primer 066 represent the forward primer while 067 represent the reverse primer.

```
        10          20          30          40          50          60
ATGGGGATCC CCTCATTCCT AGCCTTCCCT GCTGCCAGGA GGAACCGAGC TCACTGCACG
                                          O66
        70          80          90         100         110         120
CCCTGGCATC CTTGGGGCCA CATGCTACTG TGGACAGCTC TGCTCTTCCT GGCTCCTGTT
       130         140         150         160         170         180
TCTGGGAAAC CTGATCTCCC AAAAGCTGTG GTGACCATCC AGCCTGCGTG GATCAATGTG
       190         200         210         220         230         240
CTCAGGGAGG ATCACGTGAC GCTGACGTGC CAGGGGACCA GCTTCTCTGC AGGCAACCTC
       250         260         270         280         290         300
ACCACATGGT TCCATAACGG GAGCTCCATC CACACCCAGA AGCAGCCCAG CTACAGCTTT
       310         320         330         340         350         360
AGGGCCGGCA GCAACGACAG TGGGTCCTAC AGGTGCCAGA GGGAGCAGAC CAGCCTCAGC
       370         380         390         400         410         420
GACCCTGTGC ATCTGGATGT GATTTCCGAC TGGCTGTTGC TCCAGACCCC CAGCCTCGTG
       430         440         450         460         470         480
TTCCAGGAAG GGGAGCCCAT CATGCTGAGG TGCCACAGCT GGAGAAACCA GCCTCTGAAT
       490         500         510         520         530         540
AAGATCACAT TCTACCAGGA TAGGAAATCC AAGATATTTT CCTATCAGCG CACCAACTTC
       550         560         570         580         590         600
TCTATCCCAC GCGCCAACCT CAGTCACAGC GGCCAGTACC ACTGCACAGC GTTTATCGGG
       610         620         630         640         650         660
AAGATGCTAC ACTCGTCACA ACCAGTGAAC ATCACTGTCC AAGAGTCCAG CTCGAGCGGC
       670         680         690         700         710         720
CCCTCATCGA TGACAGCTGT GGCTATAGGC ACCTGTTTTG CTGCAGTGGC TATTGTTGCG
                      067
       730         740         750         760         770         780
GCCATAATAA CCTGGTTCCG CCTCAGGAGA AAGCCAATCT CAGCCGGTCT CACTGATGCT
       790         800         810         820         830         840
GAAAATGATG CTGCCAGAAC TGAGGCTGAG AACACAGTCA CCTATTCACT CCTCTCGCAC
       850         860         870         880         890         900
CCGGATGTTG CAGAGGAAGA CTCAGAGTCC GATTACCAGA AACGCCTTTA G.........
```

Figure 4

Bovine CD64 sequence showing PCR primer positions. Arrows represent the primers with the arrow showing the direction of amplification. The primers are labelled as 068 and 069. 068 represent the forward primer while 069 represent the reverse primer.

```
                 068
        10         20         30         40         50         60
ACAAAGCCAG TGATCACCTT GAAGCCTCCG TGGGTCAGTG TATTCCAAGA AGAAAATGTA
        70         80         90        100        110        120
ACCTTATTGT GTGAGGGGCC CCACCGGCCT GGGGACACTG CTACACAGTG GTTTCTCAAC
       130        140        150        160        170        180
GGCACAGCCA TCAAGACCCT GGCCCCCAGA TACAGTATTA ACAGTGCTAC ATTCGATGAC
       190        200        210        220        230        240
AGTGGTGAAT ACAAGTGCCA GACAGGCCTC TCAATGCTAA GTGACCCAGT ACAGCTAGAA
       250        260        270        280        290        300
ATCCACAGTG aTTGGCTACT ACTCCAGGTC ACTAGCAGAG TCTTCACAGA AGGGGACCCT
       310        320        330        340        350        360
CTGGCCTTGA GGTGTCATGC ATGGAAGAAT ATGCCGGTGT ACAAAATGCT TTTCTACAAA
       370        380        390        400        410        420
GATGGCAAGC CCTTTAGGTT TTCTAGTCAG GATTCTGAAT TCACCATTCT GCAAACCAAC
       430        440        450        460        470        480
TTGNGTCACA ATGGCATCTA TCACTGCTCG GGCGAGAGAA GGCGTCGCTA CACATCGGCA
       490        500        510        520        530        540
GGAGTATCTA TCACTATAAA AGAGCTATTT CCAGCCCCAG TGCTGAGAAC ATCCTTCTCA
       550        560        570        580        590        600
TCCCCTCACC AAGAGGGGAA TCTGGTCAAC CTGAGCTGTG AAACAAAGTT GCCCTCAGAG
       610        620        630        640        650        660
AAGCCTGGTC AGCAGCTTTA CTTCTCCTTC TATGTGGGAA ACAAGACCCT AATAAGCAGG
       670        680        690        700        710        720
ACCACATCCT CTGAATACCA GACATTCATT GCTAAAAAAG AAGACCGTAG GCTATACTGG
       730        740        750        760        770        780
TGTGAAGCTG CCACAGGAGA TGGGAATCTT ATCAAGCGCA GCCCTGAGCT GGAGCTTCCG
       790                                                  069
GTGCTTG...
```

insoluble controlled glass pore (CGP) matrix to which the first 3′ base (protected) of the desired oligonucleotide is already attached.

The first step in oligonucleotide synthesis is the removal of acid-labile, dimethoxytrityl protecting group on the 5′-hydroxyl group of the matrix –bound nucleoside. Treatment with the protic acid, trichloroacetic acid (TCA), will deprotect or detritylate the 5′ end. This will yield a reactive 5′-hydroxyl group which can react with a phosphoramidite during the coupling reaction.

The next step involves coupling in which phosphoramidites, which are chemically modified nucleosides, are used as the building blocks for synthesising the DNA. Two types of phosphoramidites are available; methyl or β-cyanoethyl. According to the sequence one more of the phosphoramidites and tetrazole are simultaneously delivered to the column.

When the synthesis is complete, the product and the truncated capped failed sequences (still attached to the column) exist as phosphate-protected, base-protected phosphotriesters. Complete deprotection is necessary to produce the biologically active DNA. In addition, the nucleotide must be cleaved from the matrix. These steps are performed manually after removal of the column from the instrument. When synthesising with β-cyanolethyl phosphoramidites, the β-cyanolethyl protecting groups are removed by treatment with concentrated ammonium hydroxide.

Following removal of the methyl group, the DNA remains covalently attached to the matrix. The diester oligonucleotides are then cleaved from the support by four 30 min treatment with concentrated ammonium hydroxide.

The protecting groups on the exocyclic amines of A, G, and C must also be removed.

This is done by placing the vial of DNA in ammonia at 55 °C for 8 to 15 hrs. Base deprotection is an ammonialysis reaction. After completing deprotection, the ammonium hydroxide-DNA solution is cooled to prevent losses from bubbling and the ammonia removed under vacuum. The fourteen oligonucleotides designed and synthesized are shown below:

CD16

Oligonucleotide 064: 5′(TAC CAC CGG CAG CTC TGC) 3′

Oligonucleotide 065: 5′(CAG AAG GTG ATC TGG TGC) 3′

CD32

Oligonucleotide 066: 5′(AGC TCT GCT CTT CCT GGC) 3′

Oligonucleotide 067: 5′ (GGT GCC TAT AGC CAC AGC) 3′

CD64

Oligonuleotide 068: 5′(CAG TGA TCA CCT TGA AGC) 3′

Oligonucleotide 069: 5′(AGC ACC GGA AGC TCC ACC) 3′

Genomic DNA primers

CD32 (exon 1 for extracellular region)

Oligonucleotide 114: 5′ (AAG CTG TGG TGA CCA TCC) 3′

Oligonucleotide 115: 5′ (GAA ATC ACA TCC AGA TGC) 3′

CD32 (exon 2 for extracellular region)

Oligonucleotide 116: 5′ (CTG GCT GTT GCT CCA GAC) 3′

Oligonucleotide 117: 5′ (TGT TCA CTG GTT GTG ACG) 3′

CD64 (exon 2 for extracellular region)

Oligonucleotide 118: 5′ (CAT ATT TTC CAG ATT GGC) 3′

Oligonucleotide 119: 5′ (AAC TAA TAG CTC TAT GAC) 3′

CD64 (exon 3 for extracellular region)

Oligonucleotide 120: 5′ (TTC AGA GCT ATT TCC AGC) 3′

Oligonucleotide 121: 5′ (CCT ACT TAC CAA GCA CCG) 3′

2.10.2 Preparation of primers for PCR reaction

The primers were precipitated from ammonium hydroxide by addition of 0.1 v/v sodium acetate and 2.5 v/v cold absolute ethanol and left at –20 °C for 1 hr, after which they were centrifuged (5 min at 15000 rpm, 4 °C) using an eppendorf minifuge. The pellet was then washed with 75% ethanol by spinning and then vacuum dried and resuspeded in 100 µl sterile reagent water. The primers concentration was estimated by measuring OD at 260 nm assuming that OD_{260} 1 was equivalent to a concentration of 20 µg/ml of single strand DNA (Sambrook *et al.*, 1989). A dilution of 100 ng/µl was made as working stock for each primer while the concentrated primer stocks were stored at –20 °C.

2.11 PCR amplification of bovine CD16, CD32 and CD64.

PCR amplification was carried out using a PTC-100 programmable thermocycler (MJ. Research, Inc., Watertown, USA). Each PCR reaction mix contained the four dNTPs (0.2 mM each) in 1x *Taq* polymerase buffer made in 10x stock (Appendix A.2.6), 2.0 mM $MgCl_2$, 1 unit *Taq* polymerase (Promega), 100 ng of appropriate forward and reverse primers and 10 ng of the template in a 50 µl reaction. The temperature cycles for the

amplification of the three genes was optimised as follows.

CD16

94 °C 1 min denaturation

50 °C 1 min annealing

72 °C 1 min extension

Programmed to run for 30 cycles.

CD 32

94 °C 1 min denaturation

55 °C 1 min annealing

72 °C 1 min extension

Programmed to run for 30 cycles.

CD 64

94 °C 1 min denaturation

50 °C 1.5 min annealing

72 °C 1.5 min extension

Programmed to run for 35 cycles.

For each reaction there was an initial denaturation step for 4 min (94 °C) and a final extension for 6 minutes (72 °C).

2.12 Electrophoresis of DNA

2.12.1 Polyacralamide gels

Polyacrylamide gels (8%) were used for resolving PCR fragments. The gels were made by mixing 26.6 mls of a 30% polyacrylamide stock solution with 10 ml TBE 10x buffer

(Appendix A.3.4), 800µl of 10% ammonium persulphate (APS), 40µl of TEMED and 63 ml double distilled water to give a final volume of 100 ml. The tube contents were then stirred to mix. The 30% polyacrylamide stock solution was made by mixing 29g acrylamide and 1g Bisacrylamide (Serva, Boehringer Ingelheim Bioproducts) in double distilled water to give a final volume of 100 ml, then filtered through 0.45 micron pore size, The mixture was poured into a gel-casting chamber (Hoefer Scientific Instruments, San Fransisco, CA) with appropriate plates after which combs were inserted and the gels left for about 45 minutes to set. A single gel was mounted onto an electrophoresis tank (Mighty Small Tank, Hoefer Scientific Instruments). Samples (5 µl) was mixed with 3 µl of loading buffer (Appendix A.3.2) then loaded onto the gel using Hamilton syringe (Hamilton, Reno, Nevada) before electrophoresis. The resolution was carried out at 150V, 33AMP for one hour. The negative controls and DNA size markers (ϕx174 DNA/*Hinf* 1 markers) were also run in separate wells along side the samples. The ϕx174 DNA markers were made by digesting ϕx174 DNA to completion with *Hinf* 1. The DNA fragments resolved in the gel were visualised by staining with ethidium bromide. The gels were then photographed in UV transiluminator.

2.12.2 Agarose gels

Agarose gels (1%) in TAE (Appendix A.3.1) were used for the separation of PCR products and restriction digested DNA. The gels were made by boiling the agarose in 1 x TAE (Appendix A.3.1) containing 0.1µg/ml ethidium bromide. The melted agarose was allowed to cool (50-55 °C) and poured into a sealed gel former of IBI model H4 horizontal electrophoresis apparatus. The combs were then inserted and the gels allowed

to set. The gel was mounted into a MPH gel electrophoresis tank (International Biotechnologies Inc., New Haven, Connecticut) containing 1x TAE. 3 µl of 6x loading buffer (Appendix A.3.2) was added to the 9 µl of samples before loading. The negative controls were treated the same and a DNA marker included. Electrophoresis was carried out at 150V, 33 AMP using a power pack (Pharmacia, Uppsala, Sweden). The gels were photographed in an UV transiluminator.

2.13 Cloning PCR products

2.13.1 Purification of PCR products for cloning

The PCR products were purified, as described below using the GENECLEAN II kit (BIO 101 Inc), Strul, (1985). The kit contains a specially formulated silica matrix called GLASSMILK that binds single and double stranded DNA without binding DNA contaminants. For each PCR reaction the lower aqueous phase was transferred to a fresh 1.5 ml eppendorf tube. The DNA fragments were precipitated overnight in 0.1 v/v 3M sodium acetate and 2.5 v/v cold absolute ethanol at –20 °C. This was centrifuged at maximum speed using an eppendorf minifuge for 10 min followed by washing using 70% ethanol. The DNA pellet was dried in a vacuum after discarding the supernatant and resuspended in 10 µl of sterile reagent water and samples separated in 1% agarose gel as described above (section 2.2.10.2). For each PCR product the band was excised using a sterile blade and put into a 1.5 ml eppendorf tube.

The weight of the fragment was taken and three volumes of 6M sodium iodide stock solution added and incubated for 5 min at 55 °C to dissolve the agarose. 5 µl of GLASSMILK suspension was added and incubated on for five minutes ice with gentle

shaking after every two minutes. The GLASSMILK/DNA complex was pelleted by spinning at maximum speed for 5 min in an eppendorf minifuge.

The supernatant was then removed and set aside. The pellet obtained was washed three times with 400 µl of the NEWWASH (Appendix A.4.1). Each washing step involved resuspending the pellet in NEWWASH followed by spinning at maximum for 2 min using an eppendorf minifuge.

DNA was then eluted from the GLASSMILK using 20 µl sterile reagent water by incubating for 5 min at 55 °C followed by centrifugation at maximum using an eppendorf minifuge. The supernatant (DNA solution) was carefully aspirated and put in a 0.5 ml eppendorf tube then stored at 4 °C.

2.13.2 Cloning PCR products into pGEM-T Easy vector.

The PCR products purified as described above were ligated into the pGEM-T Easy vector (Promega, Madson, USA). The pGEM-T vector systems provides a convenient method for the cloning of PCR products. The vectors are prepared by cutting the Promega's pGEM-5Zf(+) and pGEM-T Easy vectors with *Eco*RV and adding a 3′ terminal thymidine to both ends. These single 3′-T overhangs at the insertion sites greatly improves the efficiency of ligation of a PCR product into the plasmids by preventing recirculalization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases. These polymerases often add a single deoxyadenosine in a template independent fashion, to the 3′ end of the amplified fragments (Clark, 1988). The high copy number pGEM-T and pGEM-T Easy vectors contain T7 & SP6 RNA polymerase promoters flanking a multiple cloning site (MCS)

within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by colour screening on indicator plates.

The pGEM-T Easy vector MCS is flanked by recognition sites for the restriction enzyme *Eco*R1, *Bst*Z1 and *Not*1, thus providing three single-enzyme digestions for release of the insert (Appendix E).

2.13.3 Ligation reaction

To set the ligation reaction the following procedure was used. pGEM-T Easy vector tube was briefly centrifuged to collect the contents at the bottom of the tube. To 0.5 ml eppendorf tube known to have low DNA-binding capacity the following was added: 1 µl of T4 DNA ligase 10x buffer (Appendix A.2.7); 5µl of the purified resuspended PCR products (approx. 25 ng); 1 µl T4 DNA ligase (3 weiss units/µl) and the volume was made up to 10 µl with 2 µl double distilled water. The contents were mixed and the reaction incubated overnight at 4 °C.

2.13.4 Preparation of electrocompetent bacterial cells (*E. coli*)

The electrocompetent cells were prepared using a procedure described by Hanahan (1983) and modified by Sambrook *et al.*, (1989). Starting with a frozen glycerol stock, JM109, *E. coli* cells were streaked aseptically on 2XYT plates (without antibiotic) and grown overnight at 37 °C. A single colony was picked and aseptically inoculated into a 50 ml. LB medium (10g Bacto-Tryptone, 5g Bacto-Yeast Extract, 5g NaCl per litre with the pH adjusted to 7.0 with NaOH) and grown overnight. One litre of LB broth without

antibiotic was inoculated with the 50 ml of overnight culture. The medium was aliquoted into seven pre-chilled culture (1 litre) flasks and grown at 37 °C with agitation to an OD_{550} of 0.5-0.6, the cells at this OD are in the mid-log phase. The culture was transferred into a chilled 1 litre centrifuge bottle and placed on ice then kept in cold room (at 4 °C) for 30 min. This was followed by centrifugation at 4500 x g in a low speed centrifuge with swinging bucket rotor at 0 °C for 30 min.

The supernatant was carefully aspirated and the pellet resuspended in prechilled 10% sterile glycerol equal to the original culture volume (1 litre). The resuspended cells were centrifuged again for 15 min at 2700 x g at 0 °C using the same centrifuge. The supernatant was carefully aspirated using a vacuum suction pump and a pre-chilled 25 ml pipette. The pellet was resuspended in 10% glycerol (1Litre) and centrifuged under the same conditions.

The washing step was repeated again after which the pellet was resuspended in pre-chilled 10% glycerol (40ml) in the bottle then transferred into pre-chilled falcon tubes (50ml). The cells were centrifuged for 13 min, at 3600 x g at 0 °C in the same centrifuge. The supernatant was poured off and cells resuspended in 10% glycerol (2 ml) by vortexing. This was aliquoted (80 µl) into sterile microfuge tubes that had been pre-chilled in liquid nitrogen, and snap frozen quickly in liquid nitrogen. They were stored in a freezer at –80 °C until use.

2.13.5 Transformation of bacteria

The bacteria cells were transformed by electroporation using a GENE PULSER 11 (BioRad) as procedure described by Sambrook *et al* (1989). The ligation products were precipitated using 0.1 v/v sodium acetate, and 2.5 v/v absolute ethanol for 20 min followed by washing with 70% ethanol. The precipitated plasmid DNA was resuspended in 5 µl of double distilled water and used for transformation of the 80 µl of the bacterial cells in a chilled 0.1 cm electroporation cuvette (BioRad).

The transformed bacterial cells were transferred to 1 ml LB-broth in a sterilin tube and incubated for 1 minute at 37 ^{0}C with shaking. The cells were then plated on NZYCM media plates (Appendix B.2) containing 50 µg ampicillin/ml , 50 µl of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 50 µl of 2% isopropyl-β-D-thio-galactopyranoside (IPTG). The plates were incubated in an inverted position overnight.

2.13.6 Identification of positive clones

Positive recombinant clones were identified by colour selection (white colonies for recombinant clone and blue colonies for non-recombinant clones). 24 white colonies were picked for each transformation and streaked on a master plate with 2XYT medium (Appendix B.1) which was grown overnight in an inverted position at 37 ^{o}C. For each colony after streaking the plate the remaining bacterial cells were resuspended in 100 µl of double distilled water and boiled for 5 minutes in PCR machine. This served as the source of the template for the PCR reaction.

A PCR reaction was performed to identify the white colonies with right insert in 20 µl reaction. The reaction contained 0.2 mM of each dNTPs (Promega, Madson, USA), 1x

Taq polymerase buffer (Promega), 1.5 mM MgCl, 1 unit *Taq* DNA polymerase (Promega) and 50 ng of appropriate primers. The PCR reaction was performed using the conditions applied initially for each gene fragment and the resulting products resolved in 1% agarose gels.

The positive clones were identified as those with an amplified fragment the size of the initially cloned gene region.

For each gene three clones were chosen at random for subsequent sequencing.

This clones were grown overnight at 37 °C with shaking in 3 ml of 2XYT media (Appendix B.1) with 1 µl Amplicilin by transferring some bacteria from the master plate to the medium in a sterilin tube using a sterile tip.

2.13.7 Harvesting plasmid DNA

1.5 ml of the cells grown overnight were utilised in isolation of plasmid DNA using the alkaline lysis method. The cells (1.5 ml) were pelleted by centrifugation for 15 minutes at top speed in a microcentrifuge. They were then resuspended in 200 µl of cell resuspension solution (Appendix A.5.1). To this mixture 200 µl of the cell lysis solution (Appendix A.5.2) was added and mixed by gently inverting the tube several times until the cell suspension cleared. 200 µl of neutralisation solution (Appendix A.5.3) was added and mixed by inverting the tube several times. This mixture was centrifuged at top speed in a microcentrifuge for 5 min. The cleared supernatant was decanted to a new eppendorf tube and 1 ml of the wizard™ miniprep DNA purification resin was added and mixed by inverting the tube. For each miniprep one wizard ™ minicolumn was prepared. The plunger was removed from a 2 ml disposable syringe and the syringe barrel attached to

the luer-lock extension of each minicolumn. The resin/DNA mix was added into the syringe barrel, the plunger inserted and slurry pushed into the minicolumn with the syringe plunger.

The syringe was then detached from the minicolumn and the plunger removed from the syringe. The syringe barrel was reattached to the minicolumn and 2 ml of column wash solution pippeted into the barrel. The syringe plunger was inserted and the column wash solution pushed through the minicolumn . The minicolumn was transferred to a 1.5 ml eppendorf tube and centrifuged for 2 min to dry the resin. The minicolumn was transferred to a 1.5 ml eppendorf tube and 50 µl of water applied into the minicolumn. This was centrifuged at top speed for 20 seconds in a microcentrifuge to elute the plasmid DNA which was stored at –20 °C.

2.14 DNA Sequencing

Sequencing was done by the dideoxynucleotide chain termination method described by Sanger *et al,*. (1977). This method involves the synthesis of a DNA strand by DNA polymerase *in vitro* using a single-stranded DNA template. Synthesis is initiated at only one site where an oligonucleotide primer anneals to the template and the synthesis reaction is terminated by the incorporation of a nucleotide analog that does not support continued DNA elongation. These analogues are the 2′-3′-dideoxynucleotide -5′-triphosphate (ddNTP's) which lack the 3′hydroxyl group necessary for DNA chain elongation. When proper mixtures of dNTPS and one of the ddNTP is used, the catalysed polymerisation will be terminated whenever the ddNTP is incorporated thereby generating a sequencing ladder. Since the incorporation of the ddNTPs will assume a

normal distribution, representative strands differing by single nucleotides should be found in the reaction mixtures. The fragments are separated according to size by high resolution denaturing polyacrylamide gel electrophoresis.

2.14.1 Labelling reaction (oligo end labelling)

The radioisotope used in primer labelling was gamma ^{33}P adenosine triphosphate ($\gamma\ ^{33}P$ ATP) (Amersham Radiochemicals). The reaction contained 1 µl of 10x T4 polynucleotide kinase (PNK) buffer (appendix A.6.1) and 5 units of the enzyme PNK (Promega). Sterile water was added to a volume of 10 µl. The mix was incubated in a thermocycler (MJ Research Inc., Watertown, USA) programmed at 37 °C for 30 min followed by heating at 92 °C for 2 min to inactivate the kinase and then soaked at 4 °C until use.

2.14.2 Sequencing reaction

The sequencing reaction was done using the procedure described in *fmol* DNA sequencing system technical manual (Promega, Madson, USA). This was carried out by adding 2 µl of the appropriate ddNTP in separate wells of a thermowell plate placed on ice. In each separate tube for the different sample to be sequenced 5 µl of the template plasmid DNA was added, 5 µl of 5x sequencing buffer (Appendix A.6.2), 1.5 µl of the labelled primer, 1 µl of *Taq* DNA polymerase, sequencing grade (In 50% glycerol, 100mM KCl, 20mM Tris-HCl (pH 8) at 25 °C, 1mM EDTA, 1mM DTT, 0.5% Tween 20 and 0.5% NP40) and the volume was made up to 16 µl by addition of 4.5 µl of double distilled water.

After mixing 4 µl of this mixture was added to each ddNTP and one drop of mineral oil added on top. The reaction was then incubated in a thermol cycler programmed with initial pre-heating at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C (30 min) and annealing/extension at 70 °C for 30 min and final lowering of temperature to 4 °C.

The reaction was stopped by addition of 3 µl sequencing stop solution (Appendix A.6.3).

2.14.3 Sequencing gels.

2.14.3.1 Preparation and running of gels

Six percent acrylamide gel was made by mixing 42g of ultra-pure urea, 10 ml of 10 x TBE (Appendix A.3.4) and 15 ml of 40% acrylamide (38 g acrylamide and 2 g bis-acrylamide) and made to 100 ml with sterile water. The solution was warmed to dissolve the urea and filter-sterilised using a filter with a pore size of 0.45 micros.

Polymerisation of 100 ml of this solution was initiated by the addition of 600 µl of freshly prepared 10% ammonium persulphate and 100 µl of TEMED. The gels were cast between two sequencing glass plates separated by 0.2 mm spacers. Before casting, the smaller plate was siliconized by wiping it with Sigma cote (Chlorinated organopolysiloxane in haptane) which eases the detachment of the gel from the plate.

Gels were run with a Pharmacia Model ECPS 3000/150 power supply set at 65 W for each gel. Both buffer chambers were filled with 1x TBE buffer. Samples were heated at 75 °C for 2 min immediately before loading 3.5 µl of each sample was loaded into adjacent wells. The gels were run for 3 hours (short run) and 6 hours (long run).

After completion of electrophoresis, the gels were fixed in 10% acetic acid and 10%

methanol for 15 min to wash out the urea, which is hygroscopic agent and may cause the film to stick to the gel. The gel was dried at 80 °C for one hour then exposed to X-ray film for 12–48 hrs at room temperature.

2.14.4 Analysis of sequences

Sequencing gel autoradiographs were read using a sonic digitizer (Science Accessories Corporation, Southport, USA) attached to an IBM PS/2 Model 70 computer equipped with a 486 microprocessor employing a READGEL programme. Comparative analysis of the sequences was done using the DNASIS programme (Hitachi Software Engineering, San Bruno, CA, USA). Comparison of the sequences obtained with those in the database was carried out using the Basic Local Alignment Search Tool (BLAST), (Altschul *et al.*, 1990).

2.15 PCR-single strand conformation polymorphism (PCR-SSCP)

This procedure was carried out in a modification of the method described by Orita *et al.*, (1989) and Hayashi (1992).

This was carried out by amplifying the original fragments by PCR. Five µl PCR products were mixed with 3 µl formamide load mix (95% formamide, 20mM EDTA, 0.05% bromophenol blue & 0.05 % xylene cyanol), stored at 94 °C for 3 min to denature the DNA using a PCR machine then cooled down immediately in wet ice. After 5 min the samples were short centrifuged to mix, then kept in ice. These products were resolved in 10% acrylamide gels by using 1x TBE buffer at 4 °C, 4 µl per well at 350V, 50mA and 30W.

2.15.1 Silver staining

After running for 45 min the gels were stained by silver staining as described by Ainsworth *et al.*, (1991) as follows. Fixation was carried out for 30 min in a fixing solution made by mixing 25 ml of fixing solution, 5x (benzene sulphonic acid; 3.0% w/v in 24% v/v ethanol) with 100 ml 24% ethanol. Silver impregnation was carried out for 30 min by incubating the gels in a solution made by mixing the staining solution, 5x (silver nitrate; 1.0% w/v, benzene sulphonic acid; 0.35% w/v) in 100 double distilled water. The gels were washed with double distilled water for 1 min then developed for 5 min in a developing solution (Appendix A.7.2). The reaction was stopped and gels preserved by the addition of 125 ml of stopping and preserving solution (Appendix A.7.3)

CHAPTER THREE

EXPERIMENTAL RESULTS

3.1 Genetic analysis of CD16

Initial genetic analysis of the FcγRs in African zebu cattle was performed using two Kenyan boran animals (F100 and BR37) to identify whether allelic polymorphism is present.

This analysis used RT-PCR on cDNA obtained from PBMC followed by cloning and nucleotide sequence analysis to identify allelic polymorphism.

3.1.1 Amplification of the extracellular domain of Bovine CD16 by RT-PCR

Bovine CD16 was amplified from cDNA using forward and reverse oligonucleotide primers designed within the signal peptide and transmembrane encoding regions respectively. Glyceraldehyde-3-phophate dehydrogenase (G3PDH) primers were used as a positive control for the quality of the cDNA. Using the primers designed for CD16, 622 bp fragments were obtained from the two experimental animals, BR37 and F100 (Figure 5). This DNA fragment coded for the entire extracellular region of bovine CD16.

3.1.2 Cloning of CD16 fragments into the pGEM-T Easy vector

The 622 bp fragments obtained from the two animals (F100 and BR37) were purified from a 1% agarose gel using the gene clean method described in section 2.13.1. Purified fragments were cloned into pGEM-T easy vector as described in section 2.13.2.

Twelve recombinant clones were identified by initial blue and white colour selection followed by PCR colony screening using the primers 064 and 065 (fig 6). After the

screening, five and nine colonies gave PCR fragments of the expected size (622 bp) for F100 and BR37, respectively. The colonies without the expected PCR fragment may be as a result of ligation of other minor PCR fragments.

Three independent clones were selected at random from each animal and digested with the DNA restriction enzyme *Eco*R1. As shown in Figure 7, two fragments (448 bp and 174 bp) were released. This indicates that the 622 bp inserts has an *Eco*R1 restriction site. Identical results were obtained for BR37.

3.1.3 Nucleotide sequence analysis of the 622 bp boCD16 fragment

The nucleotide sequence of the 622 bp fragments from both animals in three independent clones was determined as described in section 2.14. The full-length nucleotide sequence presented in Figure 8 were derived using forward and reverse sequencing primers located in the cloning vector. The sequencing was carried for the three clones as a control for possible errors that could be introduced in the sequence by *Taq* polymerase during DNA synthesis.

A BLAST search of the nucleotide and protein database (Gen Bank) revealed that the sequence obtained was bovine CD16 and closely related to that of human, mouse and rat. The alignment of the consensus sequences from the two animals with that published *B. taurus* sequence is shown in Figure 9. The sequence in the analysed region is identical for the published sequence and BR37. However, F100 displayed two nucleotide base substitutions. A change of C to A at position 201 and a change of C to T at position 598 relative to the first base of the forward primer. In addition, the sequencing confirmed the presence of the *Eco*R1 restriction site within the cloned fragment as predicted from the

EcoRI restriction digest.

3.1.4 Predicted amino acid sequences of bovine CD16

Using the DNASIS programme, the amino acid sequences of bovine CD16 was predicted for the published sequence, which was identical to BR37. The two nucleotide base substitutions in the F100 sequence translated to amino acid differences at positions 67 and 199 of the protein representing a substitution of proline by threonine and proline by leucine respectively (Fig 10).

Figure 5. PCR amplification of bovine CD16 from complementary DNA

An ethidium bromide stained, 8% polyacrylamide gel showing the PCR products obtained using CD16 primers 064 and 065. M, DNA size markers (ϕx174 digested with *Hinf* 1). Lane 1, amplification using F100 cDNA; Lane 2, amplification using BR37 cDNA; Lane 3, negative control (no cDNA) for the reaction.

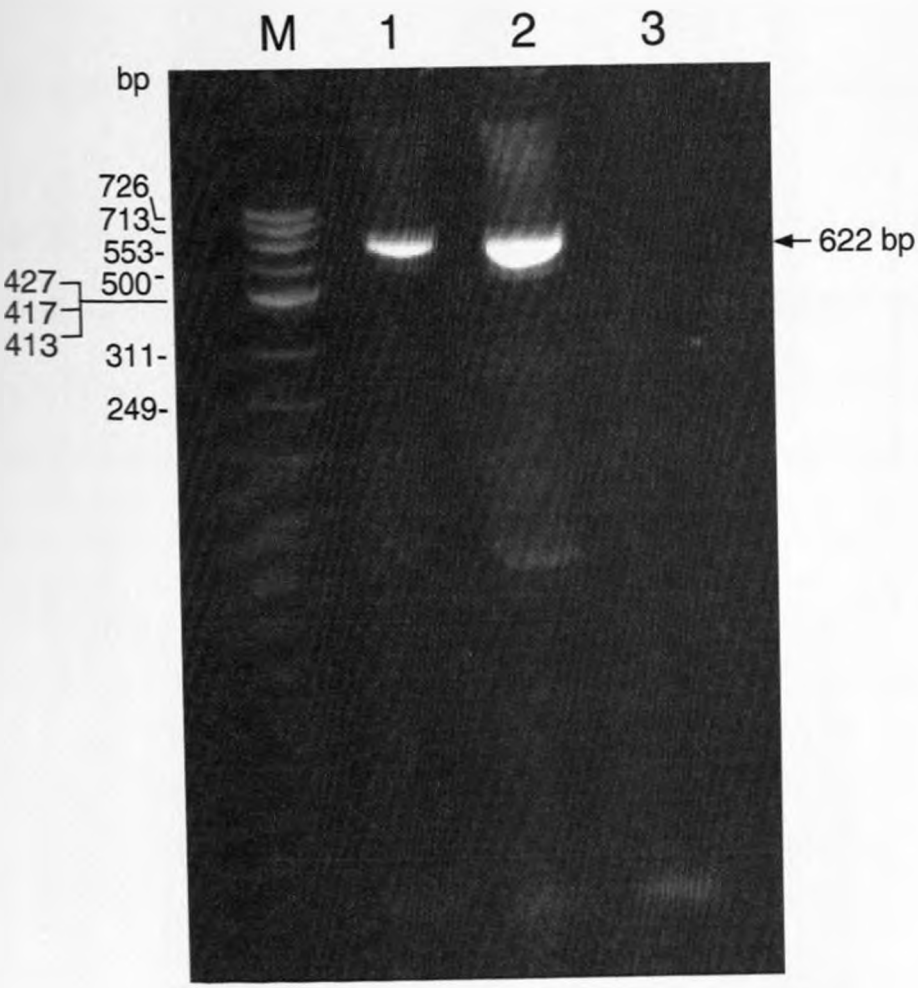
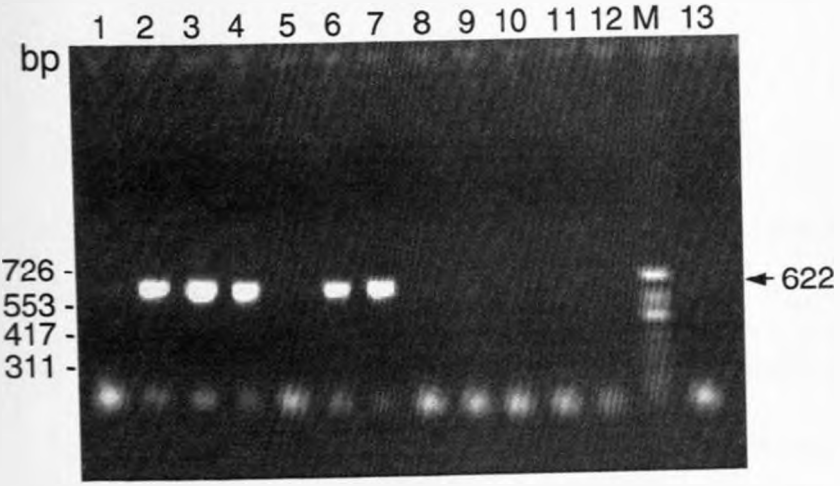

M
1
2
3
bp
726
713
553-
500-
427
417
413
311-
249-
622 bp

Figure 6. Identification of recombinant clones containing the 622 bp inserts

Ethidium bromide stained 1% agarose gel showing PCR screening of individual bacterial colonies to identify clones with an insert. Figure 3.2a shows the results from F100 while Figure 3.2b shows the results from BR37. M. DNA size markers (ϕx174 digested with *Hinf* 1). Each lane represents an individual bacteria colony. Lane 13 in both figures shows the negative control (without bacteria colony) for each screen. The absence of bands in some lanes indicates that the intended PCR insert was not ligated into the cloning vector in those particular colonies.

a


1 2 3 4 5 6 7 8 9 10 11 12 M 13
bp
726
553
417
311
622

b

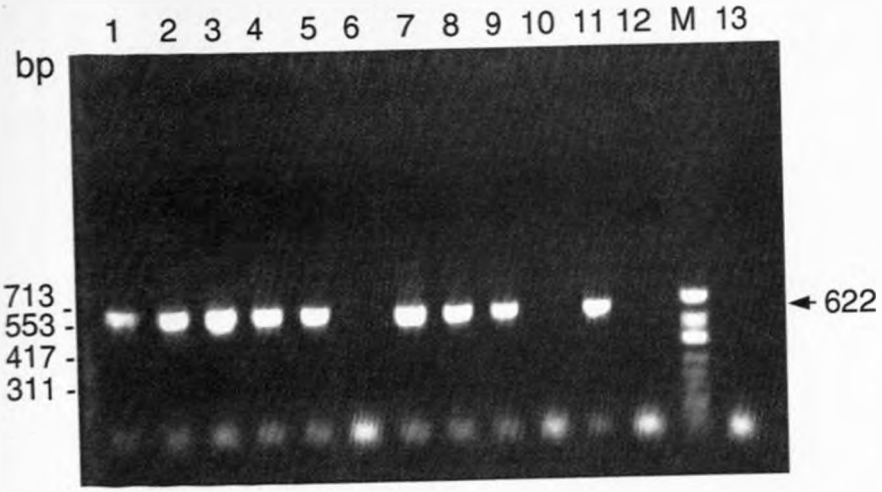

1 2 3 4 5 6 7 8 9 10 11 12 M 13
bp
713
553
417
311
622

Figure 7. *Eco*R1 digestion of F100 (CD16) recombinant pGEM-T Easy clones

An ethidium bromide stained 1% agarose gel showing the results of *Eco*R1 digestion of three recombinant clones from F100 containing the 622 bp insert: Lanes 1, 2 and 3 show the linearised plasmid DNA (3000 bp) and the released DNA inserts. M1, lambda DNA size markers (lambda DNA digested to completion with *Hin*d III); M2, ϕx 174 DNA size markers (ϕx 174 DNA digested with *Hin*f 1).

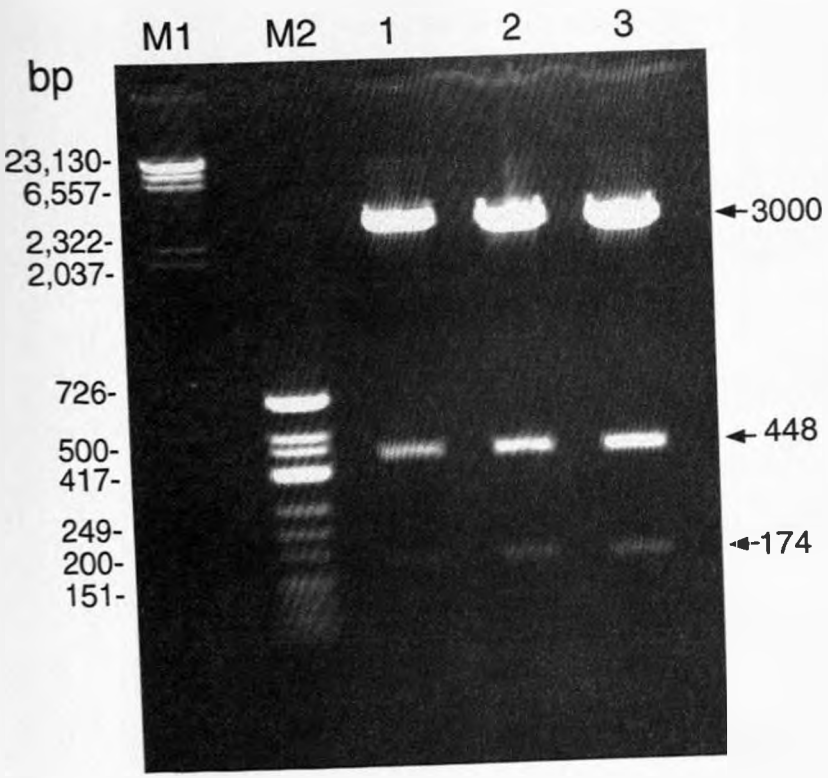

M1
M2
1
2
3
bp
23,130-
6,557-
2,322-
2,037-
726-
500-
417-
249-
200-
151-
3000
448
174

Figure 8. Alignment of the CD16 nucleotide sequences obtained from F100 and BR37

Figure showing the alignment of nucleotide sequences obtained from F100 and BR37. A total of six nucleotide sequences are shown, three sequences from each animal. The identity of each clone is shown on the left (B, BR37; F, F100). The shaded sites indicate nucleotide differences between the two animals. The primer sequences are shown in lower case. The arrow at position 213 of the nucleotide sequence indicate a possible PCR or sequencing error.

```
                    10         20         30         40         50
CD16BC1      1 taccaccggc agctctgcCT GTTCTAGTTT CAGCTGACAC GCAAACTGCA
CD16BC2      1 taccaccggc agctctgcCT GTTCTAGTTT CAGCTGACAC GCAAACTGCA
CD16BC3      1 taccaccggc agctctgcCT GTTCTAGTTT CAGCTGACAC GCAAACTGCA
CD16FC1      1 taccaccggc agctctgcCT GTTCTAGTTT CAGCTGACAC GCAAACTGCA
CD16FC2      1 taccaccggc agctctgcCT GTTCTAGTTT CAGCTGACAC GCAAACTGCA
CD16FC3      1 taccaccggc agctctgcCT GTTCTAGTTT CAGCTGACAC GCAAACTGCA
                    60         70         80         90        100
CD16BC1     51 GATCCCTCAA AGGCTGTGGT GCTCCTAGAC CCTCAGTGGA ACCACGTGCT
CD16BC2     51 GATCCCTCAA AGGCTGTGGT GCTCCTAGAC CCTCAGTGGA ACCACGTGCT
CD16BC3     51 GATCCCTCAA AGGCTGTGGT GCTCCTAGAC CCTCAGTGGA ACCACGTGCT
CD16FC1     51 GATCCCTCAA AGGCTGTGGT GCTCCTAGAC CCTCAGTGGA ACCACGTGCT
CD16FC2     51 GATCCCTCAA AGGCTGTGGT GCTCCTAGAC CCTCAGTGGA ACCACGTGCT
CD16FC3     51 GATCCCTCAA AGGCTGTGGT GCTCCTAGAC CCTCAGTGGA ACCACGTGCT
                   110        120        130        140        150
CD16BC1    101 CACGAATGAC CGTGTGACTC TGAAGTGCCA GGGAGACTAC CCTGTTGAAG
CD16BC2    101 CACGAATGAC CGTGTGACTC TGAAGTGCCA GGGAGACTAC CCTGTTGAAG
CD16BC3    101 CACGAATGAC CGTGTGACTC TGAAGTGCCA GGGAGACTAC CCTGTTGAAG
CD16FC1    101 CACGAATGAC CGTGTGACTC TGAAGTGCCA GGGAGACTAC CCTGTTGAAG
CD16FC2    101 CACGAATGAC CGTGTGACTC TGAAGTGCCA GGGAGACTAC CCTGTTGAAG
CD16FC3    101 CACGAATGAC CGTGTGACTC TGAAGTGCCA GGGAGACTAC CCTGTTGAAG
                   160        170        180        190        200
CD16BC1    151 ACAATTCCAC AAAGTGGTGG CACAATGGGA CTCTCATCTC AAGCCAGACC
CD16BC2    151 ACAATTCCAC AAAGTGGTGG CACAATGGGA CTCTCATCTC AAGCCAGACC
CD16BC3    151 ACAATTCCAC AAAGTGGTGG CACAATGGGA CTCTCATCTC AAGCCAGACC
CD16FC1    151 ACAATTCCAC AAAGTGGTGG CACAATGGGA CTCTCATCTC AAGCCAGACC
CD16FC2    151 ACAATTCCAC AAAGTGGTGG CACAATGGGA CTCTCATCTC AAGCCAGACC
CD16FC3    151 ACAATTCCAC AAAGTGGTGG CACAATGGGA CTCTCATCTC AAGCCAGACC

                   210        220        230        240        250
CD16BC1    201 ▉CCAGCTACT TCATCGCAGA TGTCAAGGTT CAGGACAGTG GCGAGTACAA
CD16BC2    201 ▉CCAGCTACT TCATCGCAGA TGTCAAGGTT CAGGACAGTG GCGAGTACAA
CD16BC3    201 ▉CCAGCTACT TCATCGCAGA TGTCAAGGTT CAGGACAGTG GCGAGTACAA
CD16FC1    201 ▉CCAGCTACT TCATCGCAGA TGTCAAGGTT CAGGACAGTG GCGAGTACAA
CD16FC2    201 ▉CCAGCTACT TCATCGCAGA TGTCAAGGTT CAGGACAGTG GCGAGTACAA
CD16FC3    201 ▉CCAGCTACT TCTTCGCAGA TGTCAAGGTT CAGGACAGTG GCGAGTACAA
                   260        270        280        290        300
CD16BC1    251 GTGTCAGACA GGCCTCTCTG CACCCAGTGA CCCGGTGAAG CTAGAAGTCC
CD16BC2    251 GTGTCAGACA GGCCTCTCTG CACCCAGTGA CCCGGTGAAG CTAGAAGTCC
CD16BC3    251 GTGTCAGACA GGCCTCTCTG CACCCAGTGA CCCGGTGAAG CTAGAAGTCC
CD16FC1    251 GTGTCAGACA GGCCTCTCTG CACCCAGTGA CCCGGTGAAG CTAGAAGTCC
CD16FC2    251 GTGTCAGACA GGCCTCTCTG CACCCAGTGA CCCGGTGAAG CTAGAAGTCC
CD16FC3    251 GTGTCAGACA GGCCTCTCTG CACCCAGTGA CCCGGTGAAG CTAGAAGTCC
                   310        320        330        340        350
CD16BC1    301 ACGTAGGCTG GCTATTGCTC CAGGTCGCTC AACGGGTGGT AAATGTGGGA
CD16BC2    301 ACGTAGGCTG GCTATTGCTC CAGGTCGCTC AACGGGTGGT AAATGTGGGA
CD16BC3    301 ACGTAGGCTG GCTATTGCTC CAGGTCGCTC AACGGGTGGT AAATGTGGGA
CD16FC1    301 ACGTAGGCTG GCTATTGCTC CAGGTCGCTC AACGGGTGGT AAATGTGGGA
CD16FC2    301 ACGTAGGCTG GCTATTGCTC CAGGTCGCTC AACGGGTGGT AAATGTGGGA
CD16FC3    301 ACGTAGGCTG GCTATTGCTC CAGGTCGCTC AACGGGTGGT AAATGTGGGA
                   360        370        380        390        400
CD16BC1    351 AAGCCCATTC GGCTGAAGTG TCACAGCTGG AAGAAAACTC CTGTAGCAAA
CD16BC2    351 AAGCCCATTC GGCTGAAGTG TCACAGCTGG AAGAAAACTC CTGTAGCAAA
CD16BC3    351 AAGCCCATTC GGCTGAAGTG TCACAGCTGG AAGAAAACTC CTGTAGCAAA
CD16FC1    351 AAGCCCATTC GGCTGAAGTG TCACAGCTGG AAGAAAACTC CTGTAGCAAA
CD16FC2    351 AAGCCCATTC GGCTGAAGTG TCACAGCTGG AAGAAAACTC CTGTAGCAAA
CD16FC3    351 AAGCCCATTC GGCTGAAGTG TCACAGCTGG AAGAAAACTC CTGTAGCAAA
                   410        420        430        440        450
CD16BC1    401 GGTCCAGTAT TTCCGGAATG GCAGAGGCAA GAAGTATTCT CATGGGAATT
CD16BC2    401 GGTCCAGTAT TTCCGGAATG GCAGAGGCAA GAAGTATTCT CATGGGAATT
CD16BC3    401 GGTCCAGTAT TTCCGGAATG GCAGAGGCAA GAAGTATTCT CATGGGAATT
CD16FC1    401 GGTCCAGTAT TTCCGGAATG GCAGAGGCAA GAAGTATTCT CATGGGAATT
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CD16FC2      401 GGTCCAGTAT TTCCGGAATG GCAGAGGCAA GAAGTATTCT CATGGGAATT
CD16FC3      401 GGTCCAGTAT TTCCGGAATG GCAGAGGCAA GAAGTATTCT CATGGGAATT
                        460        470        480        490        500
CD16BC1      451 CTGACTTCCA CATTCCAGAA GCAAAACTTG AACACAGTGG TTCCTACTTC
CD16BC2      451 CTGACTTCCA CATTCCAGAA GCAAAACTTG AACACAGTGG TTCCTACTTC
CD16BC3      451 CTGACTTCCA CATTCCAGAA GCAAAACTTG AACACAGTGG TTCCTACTTC
CD16FC1      451 CTGACTTCCA CATTCCAGAA GCAAAACTTG AACACAGTGG TTCCTACTTC
CD16FC2      451 CTGACTTCCA CATTCCAGAA GCAAAACTTG AACACAGTGG TTCCTACTTC
CD16FC3      451 CTGACTTCCA CATTCCAGAA GCAAAACTTG AACACAGTGG TTCCTACTTC
                        510        520        530        540        550
CD16BC1      501 TGCAGGGGCA TTATCGGGTC TAAAAACGAG TCTTCAGAGT CTGTGCAGAT
CD16BC2      501 TGCAGGGGCA TTATCGGGTC TAAAAACGAG TCTTCAGAGT CTGTGCAGAT
CD16BC3      501 TGCAGGGGCA TTATCGGGTC TAAAAACGAG TCTTCAGAGT CTGTGCAGAT
CD16FC1      501 TGCAGGGGCA TTATCGGGTC TAAAAACGAG TCTTCAGAGT CTGTGCAGAT
CD16FC2      501 TGCAGGGGCA TTATCGGGTC TAAAAACGAG TCTTCAGAGT CTGTGCAGAT
CD16FC3      501 TGCAGGGGCA TTATCGGGTC TAAAAACGAG TCTTCAGAGT CTGTGCAGAT
                        560        570        580        590        600
CD16BC1      551 CACTGTTCAA GCTCCAGAAA CTTTACAAAC TGTCTCGTCA TTCTTTCTAC
CD16BC2      551 CACTGTTCAA GCTCCAGAAA CTTTACAAAC TGTCTCGTCA TTCTTTCTAC
CD16BC3      551 CACTGTTCAA GCTCCAGAAA CTTTACAAAC TGTCTCGTCA TTCTTTCTAC
CD16FC1      551 CACTGTTCAA GCTCCAGAAA CTTTACAAAC TGTCTCGTCA TTCTTTCTAC
CD16FC2      551 CACTGTTCAA GCTCCAGAAA CTTTACAAAC TGTCTCGTCA TTCTTTCTAC
CD16FC3      551 CACTGTTCAA GCTCCAGAAA CTTTACAAAC TGTCTCGTCA TTCTTTCTAC
                        610        620
CD16BC1      601 CTTGgcacca gatcaccttc tg
CD16BC2      601 CTTGgcacca gatcaccttc tg
CD16BC3      601 CTTGgcacca gatcaccttc tg
CD16FC1      601 CTTGgcacca gatcaccttc tg
CD16FC2      601 CTTGgcacca gatcaccttc tg
CD16FC3      601 CTTGgcacca gatcaccttc tg
```

Figure 9. Alignment of the consensus nucleotide sequences obtained from F100 and BR37 with the published bovine CD16

Figure showing the alignment of the consensus sequences obtained from F100 and BR37 with that of the published bovine CD16 from *B. taurus*. The identity of each clone is shown on the left (BT, *B. taurus*). The shaded sites indicate nucleotide differences between the sequences.

```
          10         20         30         40         50
  1 taccaccggc agctctgcCT GTTCTAGTTT CAGCTGACAC GCAAACTGCA
  1 taccaccggc agctctgcCT GTTCTAGTTT CAGCTGACAC GCAAACTGCA
  1 taccaccggc agctctgcCT GTTCTAGTTT CAGCTGACAC GCAAACTGCA
          60         70         80         90        100
 51 GATCCCTCAA AGGCTGTGGT GCTCCTAGAC CCTCAGTGGA ACCACGTGCT
 51 GATCCCTCAA AGGCTGTGGT GCTCCTAGAC CCTCAGTGGA ACCACGTGCT
 51 GATCCCTCAA AGGCTGTGGT GCTCCTAGAC CCTCAGTGGA ACCACGTGCT
         110        120        130        140        150
101 CACGAATGAC CGTGTGACTC TGAAGTGCCA GGGAGACTAC CCTGTTGAAG
101 CACGAATGAC CGTGTGACTC TGAAGTGCCA GGGAGACTAC CCTGTTGAAG
101 CACGAATGAC CGTGTGACTC TGAAGTGCCA GGGAGACTAC CCTGTTGAAG
         160        170        180        190        200
151 ACAATTCCAC AAAGTGGTGG CACAATGGGA CTCTCATCTC AAGCCAGACC
151 ACAATTCCAC AAAGTGGTGG CACAATGGGA CTCTCATCTC AAGCCAGACC
151 ACAATTCCAC AAAGTGGTGG CACAATGGGA CTCTCATCTC AAGCCAGACC
         210        220        230        240        250
201 GCCAGCTACT TCATCGCAGA TGTCAAGGTT CAGGACAGTG GCGAGTACAA
201 GCCAGCTACT TCATCGCAGA TGTCAAGGTT CAGGACAGTG GCGAGTACAA
201 GCCAGCTACT TCATCGCAGA TGTCAAGGTT CAGGACAGTG GCGAGTACAA
         260        270        280        290        300
251 GTGTCAGACA GGCCTCTCTG CACCCAGTGA CCCGGTGAAG CTAGAAGTCC
251 GTGTCAGACA GGCCTCTCTG CACCCAGTGA CCCGGTGAAG CTAGAAGTCC
251 GTGTCAGACA GGCCTCTCTG CACCCAGTGA CCCGGTGAAG CTAGAAGTCC
         310        320        330        340        350
301 ACGTAGGCTG GCTATTGCTC CAGGTCGCTC AACGGGTGGT AAATGTGGGA
301 ACGTAGGCTG GCTATTGCTC CAGGTCGCTC AACGGGTGGT AAATGTGGGA
301 ACGTAGGCTG GCTATTGCTC CAGGTCGCTC AACGGGTGGT AAATGTGGGA
         360        370        380        390        400
351 AAGCCCATTC GGCTGAAGTG TCACAGCTGG AAGAAAACTC CTGTAGCAAA
351 AAGCCCATTC GGCTGAAGTG TCACAGCTGG AAGAAAACTC CTGTAGCAAA
351 AAGCCCATTC GGCTGAAGTG TCACAGCTGG AAGAAAACTC CTGTAGCAAA
         410        420        430        440        450
401 GGTCCAGTAT TTCCGGAATG GCAGAGGCAA GAAGTATTCT CATGGGAATT
401 GGTCCAGTAT TTCCGGAATG GCAGAGGCAA GAAGTATTCT CATGGGAATT
401 GGTCCAGTAT TTCCGGAATG GCAGAGGCAA GAAGTATTCT CATGGGAATT
         460        470        480        490        500
451 CTGACTTCCA CATTCCAGAA GCAAAACTTG AACACAGTGG TTCCTACTTC
451 CTGACTTCCA CATTCCAGAA GCAAAACTTG AACACAGTGG TTCCTACTTC
451 CTGACTTCCA CATTCCAGAA GCAAAACTTG AACACAGTGG TTCCTACTTC
         510        520        530        540        550
501 TGCAGGGGCA TTATCGGGTC TAAAAACGAG TCTTCAGAGT CTGTGCAGAT
501 TGCAGGGGCA TTATCGGGTC TAAAAACGAG TCTTCAGAGT CTGTGCAGAT
501 TGCAGGGGCA TTATCGGGTC TAAAAACGAG TCTTCAGAGT CTGTGCAGAT
         560        570        580        590        600
551 CACTGTTCAA GCTCCAGAAA CTTTACAAAC TGTCTCGTCA TTCTTTCCAC
551 CACTGTTCAA GCTCCAGAAA CTTTACAAAC TGTCTCGTCA TTCTTTCCAC
551 CACTGTTCAA GCTCCAGAAA CTTTACAAAC TGTCTCGTCA TTCTTTCCAC
         610        620
601 CTTGgcacca gatcaccttc tg
601 CTTGgcacca gatcaccttc tg
601 CTTGgcacca gatcaccttc tg
```

Figure 10. Alignment of amino acid sequences obtained from BR37, F100 and the published sequence

Alignment of amino acid sequences from BR37, published *B. taurus* sequence and that obtained from F100. The identity of the sequences is shown on the left (BTP, *B. taurus* amino acid sequence; FP, F100 amino acid sequence; BRP, BR37 amino acid sequence). The shaded sites show the substituted amino acid residues.

```
                    10         20         30         40         50
I16BTP    1 PPAALPVLVS ADTQTADPSK AVVLLDPQWN HVLTNDRVTL KCQGDYPVED
I16BRP    1 PPAALPVLVS ADTQTADPSK AVVLLDPQWN HVLTNDRVTL KCQGDYPVED
I16FP     1 PPAALPVLVS ADTQTADPSK AVVLLDPQWN HVLTNDRVTL KCQGDYPVED
                   60         70         80         90        100
I16BTP   51 NSTKWWHNGT LISSQTRSYF IADVKVQDSG EYKCQTGLSA PSDPVKLEVH
I16BRP   51 NSTKWWHNGT LISSQTRSYF IADVKVQDSG EYKCQTGLSA PSDPVKLEVH
I16FP    51 NSTKWWHNGT LISSQTRSYF IADVKVQDSG EYKCQTGLSA PSDPVKLEVH
                  110        120        130        140        150
I16BTP  101 VGWLLLQVAQ RVVNVGKPIR LKCHSWKKTP VAKVQYFRNG RGKKYSHGNS
I16BRP  101 VGWLLLQVAQ RVVNVGKPIR LKCHSWKKTP VAKVQYFRNG RGKKYSHGNS
I16FP   101 VGWLLLQVAQ RVVNVGKPIR LKCHSWKKTP VAKVQYFRNG RGKKYSHGNS
                  160        170        180        190        200
I16BTP  151 DFHIPEAKLE HSGSYFCRGI IGSKNESSES VQITVQAPET LQTVSSFPSP
I16BRP  151 DFHIPEAKLE HSGSYFCRGI IGSKNESSES VQITVQAPET LQTVSSFPSP
I16FP   151 DFHIPEAKLE HSGSYFCRGI IGSKNESSES VQITVQAPET LQTVSSFPSP
                  210
I16BTP  201 WHQITFCL..
I16BRP  201 WHQITFCL..
I16FP   201 WHQITFCL..
```

2Genetic analysis of bovine CD32

21 Amplification of extracellular domain of Bovine CD32 by RT-PCR

ovine CD32 was amplified from cDNA using forward and reverse oligonucleotide mers designed within the signal peptide and transmembrane encoding regions spectively. G3PDH primers were used as a positive control of the quality of the cDNA. ing the primers designed for CD32, 601 bp fragments were obtained from the two perimental animals, BR37 and F100 (Figure 11).

22 Cloning of CD32 PCR fragments into pGEM-T Easy vector

he 601 base pairs PCR products obtained from the two animals (F100 and BR37) were rified from a 1% agarose gel using the gene clean method described in section 2.13.1. rified fragments were cloned into pGEM-T Easy vector as described in section 2.13.2. welve recombinant clones were identified by initial blue and white colour selection llowed by PCR colony screening using the primers 066 and 067. Seven colonies for ch animal (BR37 and F100) gave the expected 601 bp PCR fragment (Fig 12).

hree independent clones were selected at random from each animal and digested with he DNA restriction enzyme *Eco*R1. As shown in Figure 13, a fragment of the expected ize (601 bp) was released from the vector.

23 Nucleotide sequence analysis of the 601 bp bovine CD32 fragment

he nucleotide sequence of the cloned 601 bp fragments from both animals in three dependent clones was determined as described in section 2.14. The full-length ucleotide sequences presented in Figure 14 were derived using forward and reverse

sequencing primers located within the cloning vector. The sequencing was carried for all the three clones as control for errors in the sequence which could be introduced by *Taq* polymerase during DNA synthesis.

A BLAST search of the nucleotide and protein database (Gen Bank) revealed that the sequence obtained was bovine CD32 and closely related to that of human, mouse and rat. The alignment of the consensus sequences from the two animals with the published one for *B. taurus* is shown in Figure 15. One clone from BR37 is identical to the published sequence while all the other five clones (two from BR37 and three from F100) show an insertion of three nucleotide bases. This insertion (CAG) occurs after nucleotide thirty-eight from the first base of the forward primer.

3.1.4 Predicted amino acid sequences of bovine CD32

Using the DNASIS programme, the amino acid sequence of bovine CD32 was predicted for both the published sequence which was identical to one of the BR37 clones. The other two clones from BR37 were identical to the three clones from F100 showing the three nucleotide base insertion. From this translation it is observed that the three-nucleotide base insertion represents an additional codon for alanine as shown in Figure 16.

Figure 11. PCR amplification of bovine CD32 from complementary DNA

An ethidium bromide stained, 8% polyacrylamide gel showing the fragments amplified using CD32 primers 066 and 067. M, DNA size markers (ϕx174 digested with *Hinf* 1). Lane 1, amplification using F100 cDNA; lane 2, amplification using BR37 cDNA; lane 3, negative control (without cDNA) for the reaction.

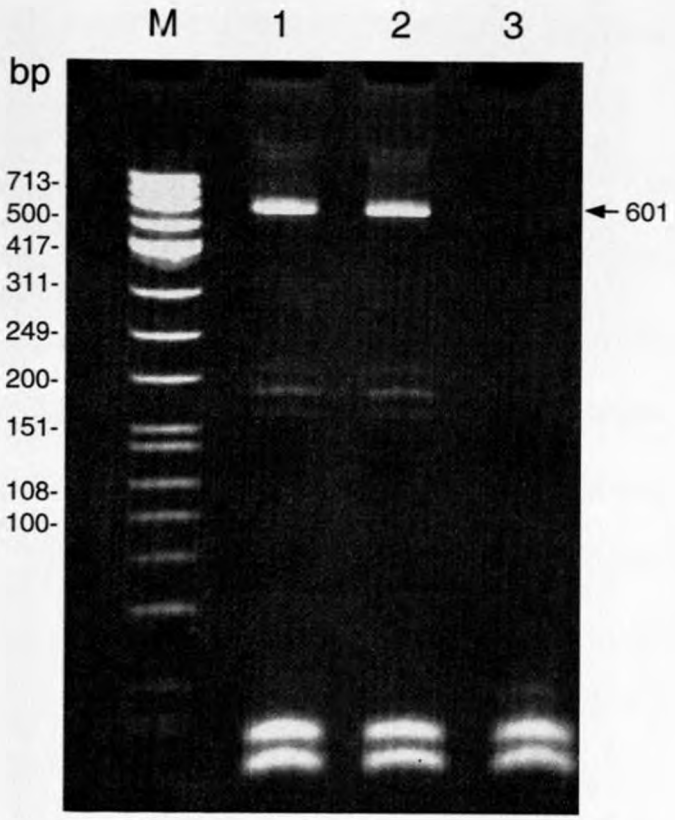
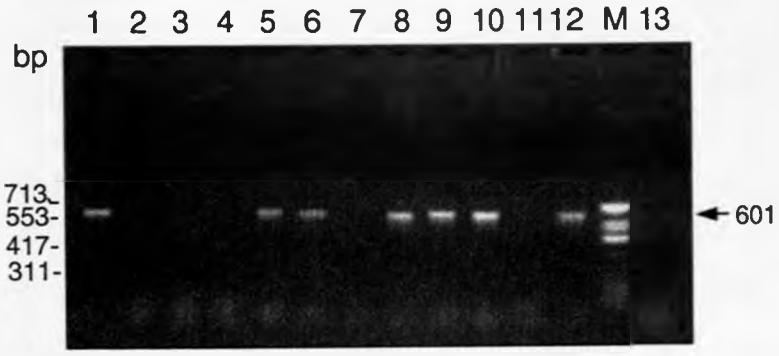

M
1
2
3
bp
713-
500-
417-
311-
249-
200-
151-
108-
100-
601

Figure 12. Identification of recombinant clones containing the 601 bp inserts

Ethidium bromide stained 1% agarose gel showing PCR screening of individual bacterial colonies to identify clones with an insert. Figure 3.9a shows the result from F100 while Figure 3.9b shows the results from BR37. M, DNA size markers (ϕx174 DNA digested with *Hinf* 1). Each lane represents an individual colony. Lane 13 in both figures shows the negative control (without bacterial colony) for each screen. The absence of bands in some lanes indicates that the intended PCR insert was not ligated into the cloning vectors in the particular colonies.

a


1 2 3 4 5 6 7 8 9 10 11 12 M 13
bp
713
553
417
311
601

b

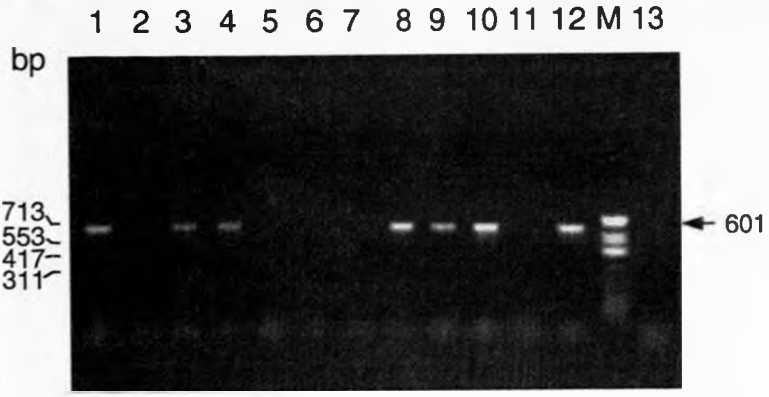

1 2 3 4 5 6 7 8 9 10 11 12 M 13
bp
713
553
417
311
601

Figure 13. *Eco*R1 digestion of F100 (CD32) recombinant pGEM-T Easy clones

An ethidium bromide stained 1% agarose gel showing the results of *Eco*R1 digestion of three recombinant clones from F100 containing the 601 bp insert. Lanes 1, 2 and 3 shows the linearised plasmid DNA (3000 bp) and the released DNA insert. M1, Lambda DNA size marker (Lambda DNA digested to completion with *Hin*d III); M2, ϕx174 DNA size marker (ϕx174 DNA digested with *Hin*f 1).

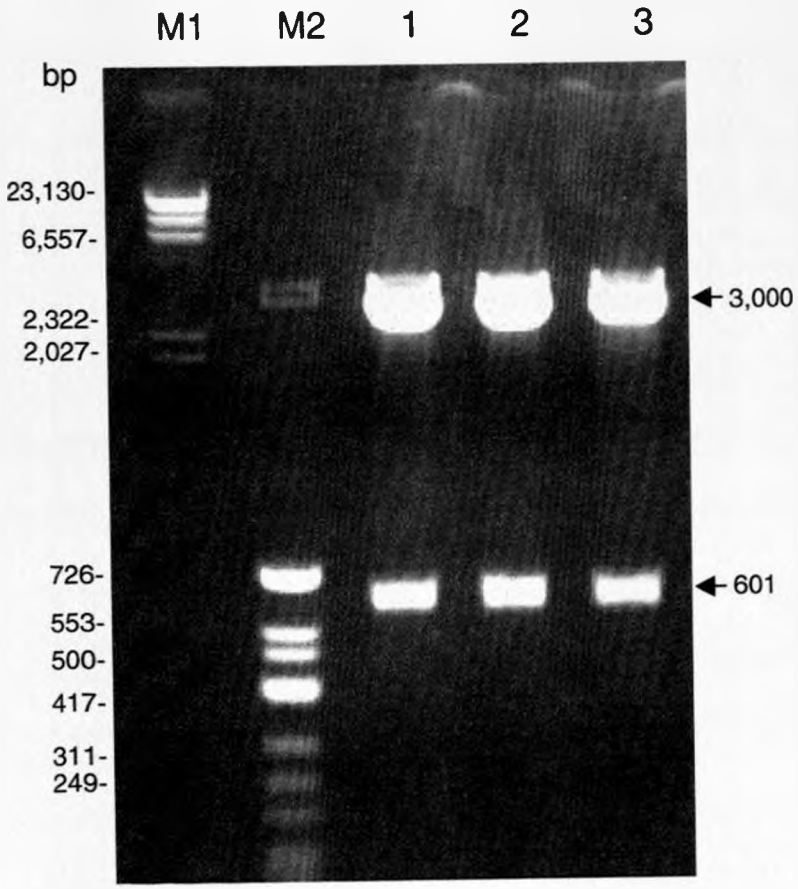

M1
M2
1
2
3
bp
23,130-
6,557-
2,322-
2,027-
726-
553-
500-
417-
311-
249-
3,000
601

Figure 14. Alignment of the CD32 nucleotide sequences obtained from F100 and BR37

Figure showing the alignment of nucleotide sequences obtained from F100 and BR37. A total of six nucleotide sequences are shown, three sequences from each animal. The identity of each clone is shown on the left (B, BR37; F, F100). The shaded sites indicate nucleotide differences between the two animals. The primer sequences are shown in lower case. The arrow at position 180 of the nucleotide sequence indicate a possible PCR error.

```
                    10         20         30         40         50
CD32BC1   1 agctctgctc ttcctggcTC CTGTTTCTGG GAAACCTGCA GATCTCCCAA
CD32BC2   1 agctctgctc ttcctggcTC CTGTTTCTGG GAAACCTG--- ATCTCCCAA
CD32BC3   1 agctctgctc ttcctggcTC CTGTTTCTGG GAAACCTGCA GATCTCCCAA
CD32FC1   1 agctctgctc ttcctggcTC CTGTTTCTGG GAAACCTGCA GATCTCCCAA
CD32FC2   1 agctctgctc ttcctggcTC CTGTTTCTGG GAAACCTGCA GATCTCCCAA
CD32FC3   1 agctctgctc ttcctggcTC CTGTTTCTGG GAAACCTGCA GATCTCCCAA
                    60         70         80         90        100
CD32BC1   1 AAGCTGTGGT GACCATCCAG CCTGCGTGGA TCAATGTGCT CAGGGAGGAT
CD32BC2  51 AAGCTGTGGT GACCATCCAG CCTGCGTGGA TCAATGTGCT CAGGGAGGAT
CD32BC3  51 AAGCTGTGGT GACCATCCAG CCTGCGTGGA TCAATGTGCT CAGGGAGGAT
CD32FC1  51 AAGCTGTGGT GACCATCCAG CCTGCGTGGA TCAATGTGCT CAGGGAGGAT
CD32FC2  51 AAGCTGTGGT GACCATCCAG CCTGCGTGGA TCAATGTGCT CAGGGAGGAT
CD32FC3  51 AAGCTGTGGT GACCATCCAG CCTGCGTGGA TCAATGTGCT CAGGGAGGAT
                   110        120        130        140        150
CD32BC1 101 CACGTGACGC TGACGTGCCA GGGGACCAGC TTCTCTGCAG GCAACCTCAC
CD32BC2 101 CACGTGACGC TGACGTGCCA GGGGACCAGC TTCTCTGCAG GCAACCTCAC
CD32BC3 101 CACGTGACGC TGACGTGCCA GGGGACCAGC TTCTCTGCAG GCAACCTCAC
CD32FC1 101 CACGTGACGC TGACGTGCCA GGGGACCAGC TTCTCTGCAG GCAACCTCAC
CD32FC2 101 CACGTGACGC TGACGTGCCA GGGGACCAGC TTCTCTGCAG GCAACCTCAC
CD32FC3 101 CACGTGACGC TGACGTGCCA GGGGACCAGC TTCTCTGCAG GCAACCTCAC
                   160        170        180        190        200
CD32BC1 151 CACATGGTTC CATAACGGGA GCTCCATCCC CACCCAGAAG CAGCCCAGCT
CD32BC2 151 CACATGGTTC CATAACGGGA GCTCCATCCA CACCCAGAAG CAGCCCAGCT
CD32BC3 151 CACATGGTTC CATAACGGGA GCTCCATCCA CACCCAGAAG CAGCCCAGCT
CD32FC1 151 CACATGGTTC CATAACGGGA GCTCCATCCA CACCCAGAAG CAGCCCAGCT
CD32FC2 151 CACATGGTTC CATAACGGGA GCTCCATCCA CACCCAGAAG CAGCCCAGCT
CD32FC3 151 CACATGGTTC CATAACGGGA GCTCCATCCA CACCCAGAAG CAGCCCAGCT
                   210        220        230        240        250
CD32BC1 201 ACAGCTTTAG GGCCGGCAGC AACGACAGTG GGTCCTACAG GTGCCAGAGG
CD32BC2 201 ACAGCTTTAG GGCCGGCAGC AACGACAGTG GGTCCTACAG GTGCCAGAGG
CD32BC3 201 ACAGCTTTAG GGCCGGCAGC AACGACAGTG GGTCCTACAG GTGCCAGAGG
CD32FC1 201 ACAGCTTTAG GGCCGGCAGC AACGACAGTG GGTCCTACAG GTGCCAGAGG
CD32FC2 201 ACAGCTTTAG GGCCGGCAGC AACGACAGTG GGTCCTACAG GTGCCAGAGG
CD32FC3 201 ACAGCTTTAG GGCCGGCAGC AACGACAGTG GGTCCTACAG GTGCCAGAGG
                   260        270        280        290        300
CD32BC1 251 GAGCAGACCA GCCTCAGCGA CCCTGTGCAT CTGGATGTGA TTTCCGACTG
CD32BC2 251 GAGCAGACCA GCCTCAGCGA CCCTGTGCAT CTGGATGTGA TTTCCGACTG
CD32BC3 251 GAGCAGACCA GCCTCAGCGA CCCTGTGCAT CTGGATGTGA TTTCCGACTG
CD32FC1 251 GAGCAGACCA GCCTCAGCGA CCCTGTGCAT CTGGATGTGA TTTCCGACTG
CD32FC2 251 GAGCAGACCA GCCTCAGCGA CCCTGTGCAT CTGGATGTGA TTTCCGACTG
CD32FC3 251 GAGCAGACCA GCCTCAGCGA CCCTGTGCAT CTGGATGTGA TTTCCGACTG
                   310        320        330        340        350
CD32BC1 301 GCTGTTGCTC CAGACCCCCA GCCTCGTGTT CCAGGAAGGG GAGCCCATCA
CD32BC2 301 GCTGTTGCTC CAGACCCCCA GCCTCGTGTT CCAGGAAGGG GAGCCCATCA
CD32BC3 301 GCTGTTGCTC CAGACCCCCA GCCTCGTGTT CCAGGAAGGG GAGCCCATCA
CD32FC1 301 GCTGTTGCTC CAGACCCCCA GCCTCGTGTT CCAGGAAGGG GAGCCCATCA
CD32FC2 301 GCTGTTGCTC CAGACCCCCA GCCTCGTGTT CCAGGAAGGG GAGCCCATCA
CD32FC3 301 GCTGTTGCTC CAGACCCCCA GCCTCGTGTT CCAGGAAGGG GAGCCCATCA
                   360        370        380        390        400
CD32BC1 351 TGCTGAGGTG CCACAGCTGG AGAAACCAGC CTCTGAATAA GATCACATTC
CD32BC2 351 TGCTGAGGTG CCACAGCTGG AGAAACCAGC CTCTGAATAA GATCACATTC
CD32BC3 351 TGCTGAGGTG CCACAGCTGG AGAAACCAGC CTCTGAATAA GATCACATTC
CD32FC1 351 TGCTGAGGTG CCACAGCTGG AGAAACCAGC CTCTGAATAA GATCACATTC
CD32FC2 351 TGCTGAGGTG CCACAGCTGG AGAAACCAGC CTCTGAATAA GATCACATTC
CD32FC3 351 TGCTGAGGTG CCACAGCTGG AGAAACCAGC CTCTGAATAA GATCACATTC
                   410        420        430        440        450
CD32BC1 401 TACCAGGATA GGAAATCCAA GATATTTTCC TATCAGCGCA CCAACTTCTC
CD32BC2 401 TACCAGGATA GGAAATCCAA GATATTTTCC TATCAGCGCA CCAACTTCTC
CD32BC3 401 TACCAGGATA GGAAATCCAA GATATTTTCC TATCAGCGCA CCAACTTCTC
CD32FC1 401 TACCAGGATA GGAAATCCAA GATATTTTCC TATCAGCGCA CCAACTTCTC
CD32FC2 401 TACCAGGATA GGAAATCCAA GATATTTTCC TATCAGCGCA CCAACTTCTC
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```
CD32FC3 401 TACCAGGATA GGAAATCCAA GATATTTTCC TATCAGCGCA CCAACTTCTC
                  460        470        480        490        500
CD32BC1 451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
CD32BC2 451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
CD32BC3 451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
CD32FC1 451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
CD32FC2 451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
CD32FC3 451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
                  510        520        530        540        550
CD32BC1 501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
CD32BC2 501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
CD32BC3 501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
CD32FC1 501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
CD32FC2 501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
CD32FC3 501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
              560        570        580        590        600
CD32BC1 551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAgctgtgg ctataggcac
CD32BC2 551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAgctgtgg ctataggcac
CD32BC3 551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAgctgtgg ctataggcac
CD32FC1 551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAgctgtgg ctataggcac
CD32FC2 551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAgctgtgg ctataggcac
CD32FC3 551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAgctgtgg ctataggcac

CD32BC1 601 c
CD32BC2 601 c
CD32BC3 601 c
CD32FC1 601 c
CD32FC2 601 c
CD32FC3 601 c
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CD32FC3 401 TACCAGGATA GGAAATCCAA GATATTTTCC TATCAGCGCA CCAACTTCTC
                  460        470        480        490        500
CD32BC1 451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
CD32BC2 451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
CD32BC3 451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
CD32FC1 451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
CD32FC2 451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
CD32FC3 451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
                  510        520        530        540        550
CD32BC1 501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
CD32BC2 501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
CD32BC3 501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
CD32FC1 501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
CD32FC2 501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
CD32FC3 501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
              560        570        580        590        600
CD32BC1 551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAgctgtgg ctataggcac
CD32BC2 551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAgctgtgg ctataggcac
CD32BC3 551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAgctgtgg ctataggcac
CD32FC1 551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAgctgtgg ctataggcac
CD32FC2 551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAgctgtgg ctataggcac
CD32FC3 551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAgctgtgg ctataggcac

CD32BC1 601 c
CD32BC2 601 c
CD32BC3 601 c
CD32FC1 601 c
CD32FC2 601 c
CD32FC3 601 c
```

Figure 15. Alignment of the consensus nucleotide sequences obtained from F100 and BR37 with the published bovine CD32

Figure showing the alignment of the consensus sequences obtained from F100 and BR37 with that of the published bovine CD32 from *B. taurus*. The identity of each clone is shown on the left (BT, *B. taurus*). The shaded sites indicate nucleotide differences between the sequences.

```
                        10         20         30         40         50
CD32BT            1 AGCTCTGCTC TTCCTGGCTC CTGTTTCTGG GAAACCTG-- -ATCTCCCAA
CD32BR37.1        1 AGCTCTGCTC TTCCTGGCTC CTGTTTCTGG GAAACCTGCA GATCTCCCAA
CD32BR37.2        1 AGCTCTGCTC TTCCTGGCTC CTGTTTCTGG GAAACCTG-- -ATCTCCCAA
CD32F100          1 AGCTCTGCTC TTCCTGGCTC CTGTTTCTGG GAAACCTGCA GATCTCCCAA
                        60         70         80         90        100
CD32BT           51 AAGCTGTGGT GACCATCCAG CCTGCGTGGA TCAATGTGCT CAGGGAGGAT
CD32BR37.1       51 AAGCTGTGGT GACCATCCAG CCTGCGTGGA TCAATGTGCT CAGGGAGGAT
CD32BR37.2       51 AAGCTGTGGT GACCATCCAG CCTGCGTGGA TCAATGTGCT CAGGGAGGAT
CD32F100         51 AAGCTGTGGT GACCATCCAG CCTGCGTGGA TCAATGTGCT CAGGGAGGAT
                       110        120        130        140        150
CD32BT          101 CACGTGACGC TGACGTGCCA GGGGACCAGC TTCTCTGCAG GCAACCTCAC
CD32BR37.1      101 CACGTGACGC TGACGTGCCA GGGGACCAGC TTCTCTGCAG GCAACCTCAC
CD32BR37.2      101 CACGTGACGC TGACGTGCCA GGGGACCAGC TTCTCTGCAG GCAACCTCAC
CD32F100        101 CACGTGACGC TGACGTGCCA GGGGACCAGC TTCTCTGCAG GCAACCTCAC
                       160        170        180        190        200
CD32BT          151 CACATGGTTC CATAACGGGA GCTCCATCCA CACCCAGAAG CAGCCCAGCT
CD32BR37.1      151 CACATGGTTC CATAACGGGA GCTCCATCCA CACCCAGAAG CAGCCCAGCT
CD32BR37.2      151 CACATGGTTC CATAACGGGA GCTCCATCCA CACCCAGAAG CAGCCCAGCT
CD32F100        151 CACATGGTTC CATAACGGGA GCTCCATCCA CACCCAGAAG CAGCCCAGCT
                       210        220        230        240        250
CD32BT          201 ACAGCTTTAG GGCCGGCAGC AACGACAGTG GGTCCTACAG GTGCCAGAGG
CD32BR37.1      201 ACAGCTTTAG GGCCGGCAGC AACGACAGTG GGTCCTACAG GTGCCAGAGG
CD32BR37.2      201 ACAGCTTTAG GGCCGGCAGC AACGACAGTG GGTCCTACAG GTGCCAGAGG
CD32F100        201 ACAGCTTTAG GGCCGGCAGC AACGACAGTG GGTCCTACAG GTGCCAGAGG
                       260        270        280        290        300
CD32BT          251 GAGCAGACCA GCCTCAGCGA CCCTGTGCAT CTGGATGTGA TTTCCGACTG
CD32BR37.1      251 GAGCAGACCA GCCTCAGCGA CCCTGTGCAT CTGGATGTGA TTTCCGACTG
CD32BR37.2      251 GAGCAGACCA GCCTCAGCGA CCCTGTGCAT CTGGATGTGA TTTCCGACTG
CD32F100        251 GAGCAGACCA GCCTCAGCGA CCCTGTGCAT CTGGATGTGA TTTCCGACTG
                       310        320        330        340        350
CD32BT          301 GCTGTTGCTC CAGACCCCCA GCCTCGTGTT CCAGGAAGGG GAGCCCATCA
CD32BR37.1      301 GCTGTTGCTC CAGACCCCCA GCCTCGTGTT CCAGGAAGGG GAGCCCATCA
CD32BR37.2      301 GCTGTTGCTC CAGACCCCCA GCCTCGTGTT CCAGGAAGGG GAGCCCATCA
CD32F100        301 GCTGTTGCTC CAGACCCCCA GCCTCGTGTT CCAGGAAGGG GAGCCCATCA
                       360        370        380        390        400
CD32BT          351 TGCTGAGGTG CCACAGCTGG AGAAACCAGC CTCTGAATAA GATCACATTC
CD32BR37.1      351 TGCTGAGGTG CCACAGCTGG AGAAACCAGC CTCTGAATAA GATCACATTC
CD32BR37.2      351 TGCTGAGGTG CCACAGCTGG AGAAACCAGC CTCTGAATAA GATCACATTC
CD32F100        351 TGCTGAGGTG CCACAGCTGG AGAAACCAGC CTCTGAATAA GATCACATTC
                       410        420        430        440        450
CD32BT          401 TACCAGGATA GGAAATCCAA GATATTTTCC TATCAGCGCA CCAACTTCTC
CD32BR37.1      401 TACCAGGATA GGAAATCCAA GATATTTTCC TATCAGCGCA CCAACTTCTC
CD32BR37.2      401 TACCAGGATA GGAAATCCAA GATATTTTCC TATCAGCGCA CCAACTTCTC
CD32F100        401 TACCAGGATA GGAAATCCAA GATATTTTCC TATCAGCGCA CCAACTTCTC
                       460        470        480        490        500
CD32BT          451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
CD32BR37.1      451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
CD32BR37.2      451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
CD32F100        451 TATCCCACGC GCCAACCTCA GTCACAGCGG CCAGTACCAC TGCACAGCGT
                       510        520        530        540        550
CD32BT          501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
CD32BR37.1      501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
CD32BR37.2      501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
CD32F100        501 TTATCGGGAA GATGCTACAC TCGTCACAAC CAGTGAACAT CACTGTCCAA
                       560        570        580        590        600
CD32BT          551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAGCTGTGG CTATAGGCAC
CD32BR37.1      551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAGCTGTGG CTATAGGCAC
CD32BR37.2      551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAGCTGTGG CTATAGGCAC
CD32F100        551 GAGTCCAGCT CGAGCGGCCC CTCATCGATG ACAGCTGTGG CTATAGGCAC

CD32BT          601 C
```

CD32BR37.1 601 C
CD32BR37.2 601 C
CD32F100 601 C

Figure 16. Alignment of CD32 amino acid sequences obtained from BR37, F100 and the published sequence

Alignment of amino acid sequences from BR37, the published *B. taurus* sequence and that obtained from F100. The identity of the sequences is shown on the left (BT, *B. taurus*; BR, BR37 and F, F100). The shaded site shows the additional amino acid residue.

```
                          10         20         30         40         50
CD32BTP         1 MLLWTALLFL APVSGKP-DL PKAVVTIQPA WINVLREDHV TLTCQGTSFS
CD32BR37.1P     1 MLLWTALLFL APVSGKP-DL PKAVVTIQPA WINVLREDHV TLTCQGTSFS
CD32BR37.2P     1 MLLWTALLFL APVSGKP-DL PKAVVTIQPA WINVLREDHV TLTCQGTSFS
CD32FP          1 MLLWTALLFL APVSGKP-DL PKAVVTIQPA WINVLREDHV TLTCQGTSFS
                          60         70         80         90        100
CD32BTP        51 AGNLTTWFHN GSSIHTQKQP SYSFRAGSND SGSYRCQREQ TSLSDPVHLD
CD32BR37.1P    51 AGNLTTWFHN GSSIHTQKQP SYSFRAGSND SGSYRCQREQ TSLSDPVHLD
CD32BR37.2P    51 AGNLTTWFHN GSSIHTQKQP SYSFRAGSND SGSYRCQREQ TSLSDPVHLD
CD32FP         51 AGNLTTWFHN GSSIHTQKQP SYSFRAGSND SGSYRCQREQ TSLSDPVHLD
                         110        120        130        140        150
CD32BTP       101 VISDWLLLQT PSLVFQEGEP IMLRCHSWRN QPLNKITFYQ DRKSKIFSYQ
CD32BR37.1P   101 VISDWLLLQT PSLVFQEGEP IMLRCHSWRN QPLNKITFYQ DRKSKIFSYQ
CD32BR37.2P   101 VISDWLLLQT PSLVFQEGEP IMLRCHSWRN QPLNKITFYQ DRKSKIFSYQ
CD32FP        101 VISDWLLLQT PSLVFQEGEP IMLRCHSWRN QPLNKITFYQ DRKSKIFSYQ
                         160        170        180        190        200
CD32BTP       151 RTNFSIPRAN LSHSGQYHCT AFIGKMLHSS QPVNITVQES SSSGPSSMTA
CD32BR37.1P   151 RTNFSIPRAN LSHSGQYHCT AFIGKMLHSS QPVNITVQES SSSGPSSMTA
CD32BR37.2P   151 RTNFSIPRAN LSHSGQYHCT AFIGKMLHSS QPVNITVQES SSSGPSSMTA
CD32FP        151 RTNFSIPRAN LSHSGQYHCT AFIGKMLHSS QPVNITVQES SSSGPSSMTA

CD32BTP       201 VAIGT
CD32BR37.1P   201 VAIGT
CD32BR37.2P   201 VAIGT
CD32FP        201 VAIGT
```

3.3 Genetic analysis of CD64

3.3.1 Amplification of extracellular domain of Bovine CD64 by RT-PCR

Bovine CD64 was amplified from cDNA prepared from monocytes using forward (068) and a reverse (069) oligonucleotide primers designed within the signal peptide and transmembrane encoding regions respectively. G3PDH primers were used as a positive control for the quality of the cDNA. Using the primers designed for CD64, 778 bp fragments were obtained from the two experimental animals, Figure 17.

3.3.2 Cloning of CD64 fragments into pGEM-T Easy vector

The 778 bp PCR fragment obtained from F100 was purified from a 1% agarose gel using the gene clean method as described in section 2.13.1. The purified fragment was cloned into pGEM-T Easy vector as described in section 2.13.2. Due to technical difficulties and time constraints the fragment obtained from BR37 was not cloned.

Twelve recombinant clones were identified by initial blue and white colour selection followed by PCR colony screening using the primers 068 and 069. Seven colonies gave a PCR the expected 778 bp PCR fragment. Figure 18 show the results of this PCR screening.

Three independent clones were selected at random and digested with the DNA restriction enzyme *Eco*R1. As shown in Figure 19, a fragment of 389 bp was released from the vector. This indicates that the fragment of the expected size (778 bp) has an *Eco*R1 restriction site at the middle hence resulting into two equal fragments of 389 bp.

3.3.3 Nucleotide sequence analysis of the 778 base pairs bovine CD64 fragment

The nucleotide sequence of the cloned 778 base pairs fragment from F100 in the three independent clones was determined as described in section 2.14. The full-length nucleotide sequences presented in Figure 20 were derived using forward and reverse sequencing primers located in the cloning vector. The sequencing was carried out for all three clones as a control for sequence errors that could be introduced by *Taq* polymerase during DNA synthesis.

A BLAST search of the nucleotide and protein database (Gen Bank) revealed that the sequence obtained was bovine CD64 and closely related to that of human, mouse and rat. Following alignment with the published sequence (Figure 21), three nucleotide base substitutions were observed. These were a change of T to C, G to A and C to T at positions 480, 548 and 564 respectively relative to the first base of the forward primer. An unidentified base in the published genomic DNA sequence at position 417 was also identified as A. In addition, this sequence analysis confirms the presence of the *Eco*R1 restriction site within the cloned fragment as predicted from the restriction digests.

3.1.4 Predicted amino acid sequences of bovine CD64

Using the DNASIS programmes, the amino acid sequences of bovine CD64 was predicted from the published sequence and the F100 nucleotide sequence. The three nucleotide substitutions resulted in a single amino acid substitution at position 160 (serine to proline). The other two nucleotide base substitutions did not change the amino acids encoded by the affected codon. The alignment of the translated sequences are shown in Figure 22.

Figure 17. PCR amplification of bovine CD64 from complementary DNA

An ethidium bromide stained, 8% polyacrylamide gel showing the 778 bp fragments amplified using CD64 primers 068 and 069. M, DNA size markers (ϕx 174 digested with *Hin*f 1). Lane 1, amplification from F100 cDNA; lane 2, negative control for the reaction: lane 3, amplification from BR37 cDNA; lane 4, negative control for the reaction.

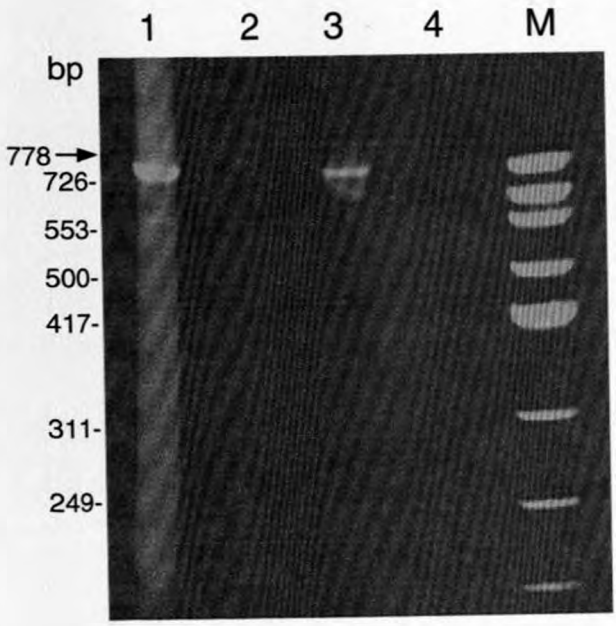

1
2
3
4
M
bp
778
726-
553-
500-
417-
311-
249-

Figure 18. Identification of recombinant clones containing the 778 bp inserts

Ethidium bromide stained 1% agarose gel showing the results of PCR screening of individual bacterial colonies to identify clones with an insert. M, DNA size markers (ϕx174 DNA digested with *Hinf* 1). Lane 13 shows a negative control for the reaction. Each lane represents an individual bacteria colony. Lane 13 shows the negative control (without bacterial colony) for each screen. The absence of bands in some lanes indicates that the intended PCR insert was not ligated into the cloned vectors for those particular colonies.

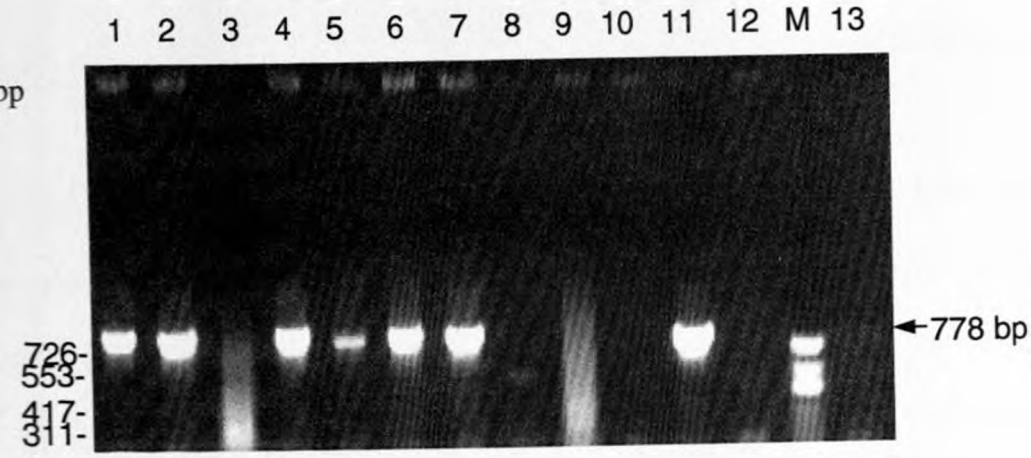

1 2 3 4 5 6 7 8 9 10 11 12 M 13
726-
553-
417-
311-
778 bp

Figure 19. *Eco*R1 digestion of F100 (CD64) recombinant pGEM-T Easy clones

An ethidium bromide stained 1% agarose gel showing the results of *Eco*R1 digestion of three recombinant clones containing the 778 bp insert: Lanes 1, 2 and 3 show the linearised plasmid DNA (3.0 kb) and the released DNA insert 389 bp. M1, Lambda DNA size marker (Lambda DNA digested to completion with *Hin*d III); M2, ϕx174 DNA size marker (ϕx174 DNA digested with *Hin*f 1).

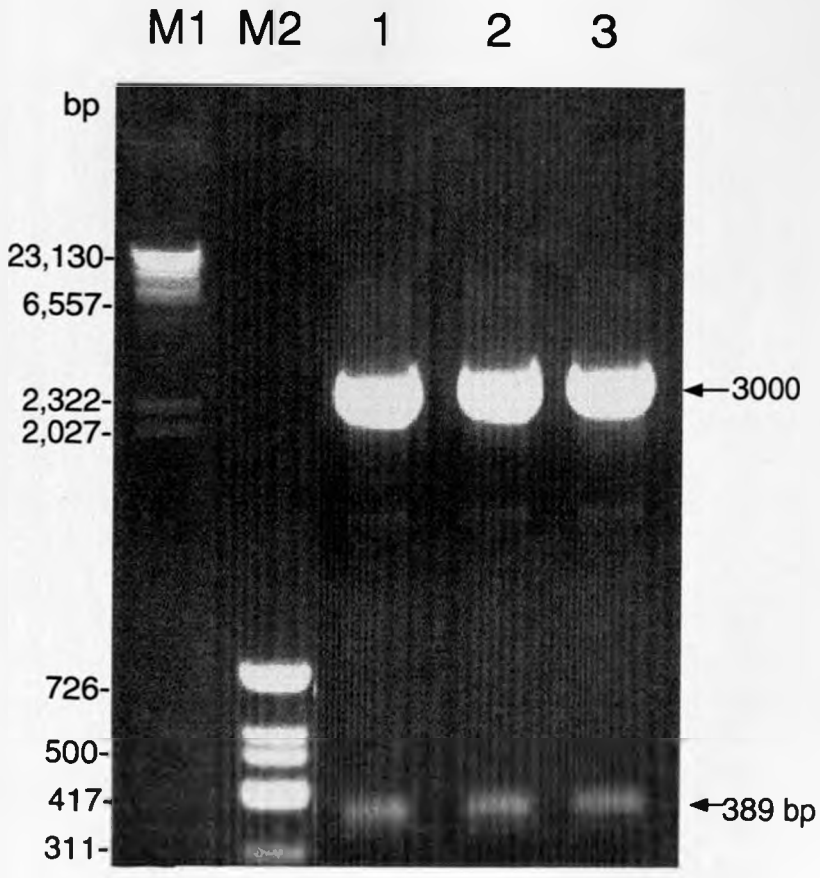

M1
M2
1
2
3
bp
23,130-
6,557-
2,322-
2,027-
726-
500-
417-
311-
3000
389 bp

Figure 20. Alignment of the CD64 nucleotide sequences obtained from F100

Figure showing the alignment of nucleotide sequences obtained from F100. A total of three nucleotide sequences are shown. The identity of each clone is shown on the left (F for F100). The primer sequences are shown in lower case.

```
          10         20         30         40         50
  1 cagtgatcac cttgaagcCT CCGTGGGTCA GTGTATTCCA AGAAGAAAAT
  1 cagtgatcac cttgaagcCT CCGTGGGTCA GTGTATTCCA AGAAGAAAAT
  1 cagtgatcac cttgaagcCT CCGTGGGTCA GTGTATTCCA AGAAGAAAAT
      60         70         80         90        100
 51 GTAACCTTAT TGTGTGAGGG GCCCCACCGG CCTGGGGACA CTGCTACACA
 51 GTAACCTTAT TGTGTGAGGG GCCCCACCGG CCTGGGGACA CTGCTACACA
 51 GTAACCTTAT TGTGTGAGGG GCCCCACCGG CCTGGGGACA CTGCTACACA
          110        120        130        140        150
101 GTGGTTTCTC AACGGCACAG CCATCAAGAC CCTGGCCCCC AGATACAGTA
101 GTGGTTTCTC AACGGCACAG CCATCAAGAC CCTGGCCCCC AGATACAGTA
101 GTGGTTTCTC AACGGCACAG CCATCAAGAC CCTGGCCCCC AGATACAGTA
          160        170        180        190        200
151 TTAACAGTGC TACATTCGAT GACAGTGGTG AATACAAGTG CCAGACAGGC
151 TTAACAGTGC TACATTCGAT GACAGTGGTG AATACAAGTG CCAGACAGGC
151 TTAACAGTGC TACATTCGAT GACAGTGGTG AATACAAGTG CCAGACAGGC
          210        220        230        240        250
201 CTCTCAATGC TAAGTGACCC AGTACAGCTA GAAATCCACA GTGATTGGCT
201 CTCTCAATGC TAAGTGACCC AGTACAGCTA GAAATCCACA GTGATTGGCT
201 CTCTCAATGC TAAGTGACCC AGTACAGCTA GAAATCCACA GTGATTGGCT
          260        270        280        290        300
251 ACTACTCCAG GTCACTAGCA GAGTCTTCAC AGAAGGGGAC CCTCTGGCCT
251 ACTACTCCAG GTCACTAGCA GAGTCTTCAC AGAAGGGGAC CCTCTGGCCT
251 ACTACTCCAG GTCACTAGCA GAGTCTTCAC AGAAGGGGAC CCTCTGGCCT
          310        320        330        340        350
301 TGAGGTGTCA TGCATGGAAG AATATGCCGG TGTACAAAAT GCTTTTCTAC
301 TGAGGTGTCA TGCATGGAAG AATATGCCGG TGTACAAAAT GCTTTTCTAC
301 TGAGGTGTCA TGCATGGAAG AATATGCCGG TGTACAAAAT GCTTTTCTAC
          360        370        380        390        400
351 AAAGATGGCA AGCCCTTTAG GTTTTCTAGT CAGGATTCTG AATTCACCAT
351 AAAGATGGCA AGCCCTTTAG GTTTTCTAGT CAGGATTCTG AATTCACCAT
351 AAAGATGGCA AGCCCTTTAG GTTTTCTAGT CAGGATTCTG AATTCACCAT
          410        420        430        440        450
401 TCTGCAAACC AACTTGAGTC ACAATGGCAT CTATCACTGC TCGGGCGAGA
401 TCTGCAAACC AACTTGAGTC ACAATGGCAT CTATCACTGC TCGGGCGAGA
401 TCTGCAAACC AACTTGAGTC ACAATGGCAT CTATCACTGC TCGGGCGAGA
          460        470        480        490        500
451 GAAGGCGTCG CTACACATCG GCAGGAGTAC CTATCACTAT AAAAGAGCTA
451 GAAGGCGTCG CTACACATCG GCAGGAGTAC CTATCACTAT AAAAGAGCTA
451 GAAGGCGTCG CTACACATCG GCAGGAGTAC CTATCACTAT AAAAGAGCTA
          510        520        530        540        550
501 TTTCCAGCCC CAGTGCTGAG AACATCCTTC TCATCCCCTC ACCAAGAAGG
501 TTTCCAGCCC CAGTGCTGAG AACATCCTTC TCATCCCCTC ACCAAGAAGG
501 TTTCCAGCCC CAGTGCTGAG AACATCCTTC TCATCCCCTC ACCAAGAAGG
          560        570        580        590        600
551 GAATCTGGTC AACTTGAGCT GTGAAACAAA GTTGCCCTCA GAGAAGCCTG
551 GAATCTGGTC AACTTGAGCT GTGAAACAAA GTTGCCCTCA GAGAAGCCTG
551 GAATCTGGTC AACTTGAGCT GTGAAACAAA GTTGCCCTCA GAGAAGCCTG
          610        620        630        640        650
601 GTCAGCAGCT TTACTTCTCC TTCTATGTGG GAAACAAGAC CCTAATAAGC
601 GTCAGCAGCT TTACTTCTCC TTCTATGTGG GAAACAAGAC CCTAATAAGC
601 GTCAGCAGCT TTACTTCTCC TTCTATGTGG GAAACAAGAC CCTAATAAGC
          660        670        680        690        700
651 AGGACCACAT CCTCTGAATA CCAGACATTC ATTGCTAAAA AAGAAGACCG
651 AGGACCACAT CCTCTGAATA CCAGACATTC ATTGCTAAAA AAGAAGACCG
651 AGGACCACAT CCTCTGAATA CCAGACATTC ATTGCTAAAA AAGAAGACCG
          710        720        730        740        750
701 TAGGCTATAC TGGTGTGAAG CTGCCACAGG AGATGGGAAT CTTATCAAGC
701 TAGGCTATAC TGGTGTGAAG CTGCCACAGG AGATGGGAAT CTTATCAAGC
701 TAGGCTATAC TGGTGTGAAG CTGCCACAGG AGATGGGAAT CTTATCAAGC
```

```
                      760        770        780
CD64FC1    751 GCAGCCCTGA gctggagctt ccggtgct..
CD64FC2    751 GCAGCCCTGA gctggagctt ccggtgct..
CD64FC3    751 GCAGCCCTGA gctggagctt ccggtgct..
```

Figure 21. Alignment of the consensus nucleotide sequences obtained from F100 and the published bovine CD64

Figure showing the alignment of the consensus sequences obtained from F100 with that of the published bovine CD64 from *B. taurus*. The identity of each clone is shown on the left. The shaded sites indicate nucleotide differences between the sequences. F100 nucleotide sequence shows three nucleotide substitutions when aligned to the published sequence.

```
                    10          20          30          40          50
CD64BT       1 CAGTGATCAC CTTGAAGCCT CCGTGGGTCA GTGTATTCCA AGAAGAAAAT
CD64F100     1 CAGTGATCAC CTTGAAGCCT CCGTGGGTCA GTGTATTCCA AGAAGAAAAT
                    60          70          80          90          100
CD64BT      51 GTAACCTTAT TGTGTGAGGG GCCCCACCGG CCTGGGGACA CTGCTACACA
CD64F100    51 GTAACCTTAT TGTGTGAGGG GCCCCACCGG CCTGGGGACA CTGCTACACA
                   110         120         130         140         150
CD64BT     101 GTGGTTTCTC AACGGCACAG CCATCAAGAC CCTGGCCCCC AGATACAGTA
CD64F100   101 GTGGTTTCTC AACGGCACAG CCATCAAGAC CCTGGCCCCC AGATACAGTA
                   160         170         180         190         200
CD64BT     151 TTAACAGTGC TACATTCGAT GACAGTGGTG AATACAAGTG CCAGACAGGC
CD64F100   151 TTAACAGTGC TACATTCGAT GACAGTGGTG AATACAAGTG CCAGACAGGC
                   210         220         230         240         250
CD64BT     201 CTCTCAATGC TAAGTGACCC AGTACAGCTA GAAATCCACA GTGATTGGCT
CD64F100   201 CTCTCAATGC TAAGTGACCC AGTACAGCTA GAAATCCACA GTGATTGGCT
                   260         270         280         290         300
CD64BT     251 ACTACTCCAG GTCACTAGCA GAGTCTTCAC AGAAGGGGAC CCTCTGGCCT
CD64F100   251 ACTACTCCAG GTCACTAGCA GAGTCTTCAC AGAAGGGGAC CCTCTGGCCT
                   310         320         330         340         350
CD64BT     301 TGAGGTGTCA TGCATGGAAG AATATGCCGG TGTACAAAAT GCTTTTCTAC
CD64F100   301 TGAGGTGTCA TGCATGGAAG AATATGCCGG TGTACAAAAT GCTTTTCTAC
                   360         370         380         390         400
CD64BT     351 AAAGATGGCA AGCCCTTTAG GTTTTCTAGT CAGGATTCTG AATTCACCAT
CD64F100   351 AAAGATGGCA AGCCCTTTAG GTTTTCTAGT CAGGATTCTG AATTCACCAT
                   410         420         430         440         450
CD64BT     401 TCTGCAAACC AACTTG[ ]GTC ACAATGGCAT CTATCACTGC TCGGGCGAGA
CD64F100   401 TCTGCAAACC AACTTG[ ]GTC ACAATGGCAT CTATCACTGC TCGGGCGAGA
                   460         470         480         490         500
CD64BT     451 GAAGGCGTCG CTACACATCG GCAGGAGTA[ ] CTATCACTAT AAAAGAGCTA
CD64F100   451 GAAGGCGTCG CTACACATCG GCAGGAGTA[ ] CTATCACTAT AAAAGAGCTA
                   510         520         530         540         550
CD64BT     501 TTTCCAGCCC CAGTGCTGAG AACATCCTTC TCATCCCCTC ACCAAGA[ ]GG
CD64F100   501 TTTCCAGCCC CAGTGCTGAG AACATCCTTC TCATCCCCTC ACCAAGA[ ]GG
                   560         570         580         590         600
CD64BT     551 GAATCTGGTC AAC[ ]TGAGCT GTGAAACAAA GTTGCCCTCA GAGAAGCCTG
CD64F100   551 GAATCTGGTC AAC[ ]TGAGCT GTGAAACAAA GTTGCCCTCA GAGAAGCCTG
                   610         620         630         640         650
CD64BT     601 GTCAGCAGCT TTACTTCTCC TTCTATGTGG GAAACAAGAC CCTAATAAGC
CD64F100   601 GTCAGCAGCT TTACTTCTCC TTCTATGTGG GAAACAAGAC CCTAATAAGC
                   660         670         680         690         700
CD64BT     651 AGGACCACAT CCTCTGAATA CCAGACATTC ATTGCTAAAA AAGAAGACCG
CD64F100   651 AGGACCACAT CCTCTGAATA CCAGACATTC ATTGCTAAAA AAGAAGACCG
                   710         720         730         740         750
CD64BT     701 TAGGCTATAC TGGTGTGAAG CTGCCACAGG AGATGGGAAT CTTATCAAGC
CD64F100   701 TAGGCTATAC TGGTGTGAAG CTGCCACAGG AGATGGGAAT CTTATCAAGC
                   760         770         780
CD64BT     751 GCAGCCCTGA GCTGGAGCTT CCGGTGCT..
CD64F100   751 GCAGCCCTGA GCTGGAGCTT CCGGTGCT..
```

Figure 22. Alignment of amino acid sequences obtained from bovine CD64 published sequence and F100

Alignment of amino acid sequences from the consensus sequence from F100 and the published *B. Taurus* sequence. The identity of the sequences is shown on the left (BTP, *B. taurus* amino acid sequence; FP, F100 amino acid sequence). The shaded site shows the substituted amino acid residue.

```
          10         20         30         40         50
  1 VITLKPPWVS VFQEENVTLL CEGPHRPGDT ATQWFLNGTA IKTLAPRYSI
  1 VITLKPPWVS VFQEENVTLL CEGPHRPGDT ATQWFLNGTA IKTLAPRYSI
          60         70         80         90        100
 51 NSATFDDSGE YKCQTGLSML SDPVQLEIHS DWLLLQVTSR VFTEGDPLAL
 51 NSATFDDSGE YKCQTGLSML SDPVQLEIHS DWLLLQVTSR VFTEGDPLAL
         110        120        130        140        150
101 RCHAWKNMPV YKMLFYKDGK PFRFSSQDSE FTILQTNL▮H NGIYHCSGER
101 RCHAWKNMPV YKMLFYKDGK PFRFSSQDSE FTILQTNL▮H NGIYHCSGER
         160        170        180        190        200
151 RRRYTSAGV▮ ITIKELFPAP VLRTSFSSPH QEGNLVNLSC ETKLPSEKPG
151 RRRYTSAGV▮ ITIKELFPAP VLRTSFSSPH QEGNLVNLSC ETKLPSEKPG
         210        220        230        240        250
201 QQLYFSFYVG NKTLISRTTS SEYQTFIAKK EDRRLYWCEA ATGDGNLIKR
201 QQLYFSFYVG NKTLISRTTS SEYQTFIAKK EDRRLYWCEA ATGDGNLIKR
         260
251 SPELELPVL.
251 SPELELPVL.
```

3.4 Analysis of polymorphism in bovine FcγRs using genomic DNA

Single strand conformational polymorphism (SSCP) is an ideal method for detection of single-base substitutions in DNA fragments (Orita *et al.*, 1989). However, before the method was adapted for genomic DNA analysis a trial was performed on CD32 using the cDNA PCR products obtained from the two animals (BR37 and F100). The amplification of this gene was carried out using the initial primers 066 and 067. The same primers were used to amplify the fragments from the three clones sequenced for each animal for CD32. The products amplified directly from the cDNA and a mixture of the fragments obtained from the three clones were resolved by SSCP for both animals. As shown Figure 23, a difference in resolution of single strands was observed in both the cDNA product and cloned fragments for BR37 compared to F100. This indicates that the insertion observed after sequencing this gene could be successfully detected by SSCP.

Two sets of primers were also designed to amplify the second and third exons encoding the extracellular domain of CD64. The primers for CD64 were designed within introns as the genomic DNA sequence for this gene has been published. This was an advantage as the whole exon could be amplified from the genomic DNA.

Primers were also designed to amplify the two exons encoding the extracellular region of CD32. Using the results for CD64 where the actual size of the exons was known, it was possible to locate the exons for CD32 by estimating the size of the exons which was approximately 0.25 kb. However, it was noted that the codon insertion constituted the first three nucleotide bases of the first exon for the extracellular domain of CD32. This could only be detected if a short sequence within the intron was available to facilitate design of a forward primer to include the region in the amplification. An initial trial to

amplify the intron was not successful. The primers designed for the gene were hence to try and detect other polymorphic sites within the exons. Appendix D shows a diagrammatic representation of positions of primers designed to amplify the two exons encoding the extracellular region of CD32 and the second and third exons encoding for the same region in CD64. As positive control for the SSCP analysis, second exon for extracellular domain of CD32 was amplified from genomic DNA obtained from sheep (*Ovis aeris*) and African buffalo (*Syncerus caffer*) The fragments amplified from sheep and buffalo were sequenced to identify nucleotide differences with cattle sequence.

Eleven nucleotide base substitutions were observed between the sheep and cattle sequence. On the other hand, only two nucleotide base changes were found between the cattle and buffalo sequence. The alignment of these sequences are shown in Figure 24.

Ten cattle gDNA samples were used as a template for PCR amplification of the exons of interest and analysed by SSCP. Five of these were from severe reactors and five from none reactors from p67 vaccine trials. After the SSCP analysis no difference were observed in the cattle samples. However, notable differences were identified in the migration of the buffalo and sheep samples (positive controls). To further verify the observed differences, mixtures of the two controls and the cattle samples were also included in the analyses. The mixtures gave different banding patterns to the single samples showing differences in resolution of single DNA strands, equal in size, but with minor nucleotide base substitutions by SSCP. Figures 25 and 26 show the results of SSCP analysis for the two exons for CD32. Identical results were obtained for the CD64.

Figure 23. SSCP analysis of the 601 bp, CD32 fragments from BR37 and F100

Figure showing the SSCP analysis of PCR products in a 10% polyacrylamide gel. Lane 1, PCR product from F100 cDNA. Lane 2, mixture of the PCR products from three identical clone of F100. Lane 3, the PCR product from BR37 cDNA and Lane 4, mixture of the PCR products from the three clones of BR37. The arrows points to the different bands. one band clearly visible for F100 in both lanes and two clearly visible bands for BR37 in the other two lanes.

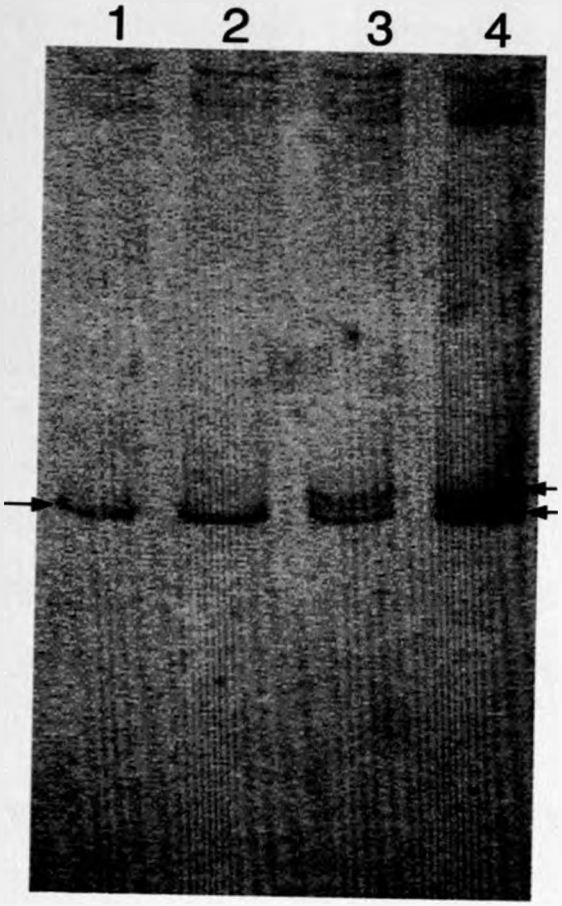

1
2
3
4

Figure 24. Alignment of cattle, African buffalo and sheep nucleotide sequences for the second exon encoding extracellular region of CD32

Figure showing the alignment of nucleotide sequences for second exon encoding for extracellular domain of CD32 from cattle, buffalo and sheep. The identity of each sequence is shown on the left (BOCD32, bovine sequence; BuCD32, African buffalo sequence; SCD32, sheep sequence). The shaded sites indicate the nucleotide differences. The primer sequences are shown in lower case.

Figure 25. SSCP analysis of the second exon for extracellular (EC) region of bovine CD32

Non-denaturing polyacrylamide gel of SSCP analysis of the second exon encoding for extracellular region of bovine CD32. The first 10 lanes show analysis of cattle genomic DNA samples (1-5 from severe reactors and 6 to 10 from non-reactors in p67 vaccine trials). Lane 11, African buffalo genomic DNA sample; lane 12 is sheep sample. Lane 13 and 14, mixtures of PCR products of African buffalo and cattle, sheep and cattle respectively. There are no differences in the cattle samples. However, the differences in the African buffalo and sheep samples are clearly resolved using this method. The arrows point to differences in banding pattern.

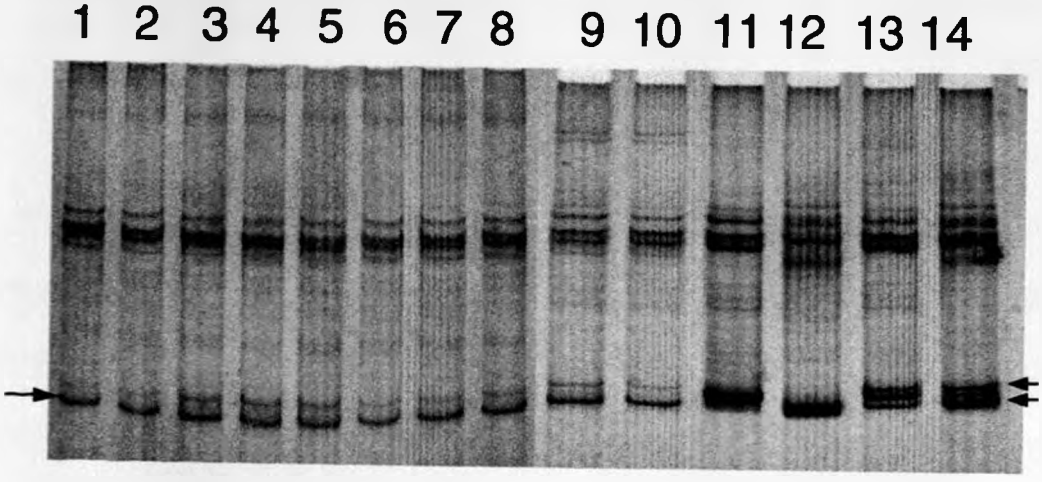
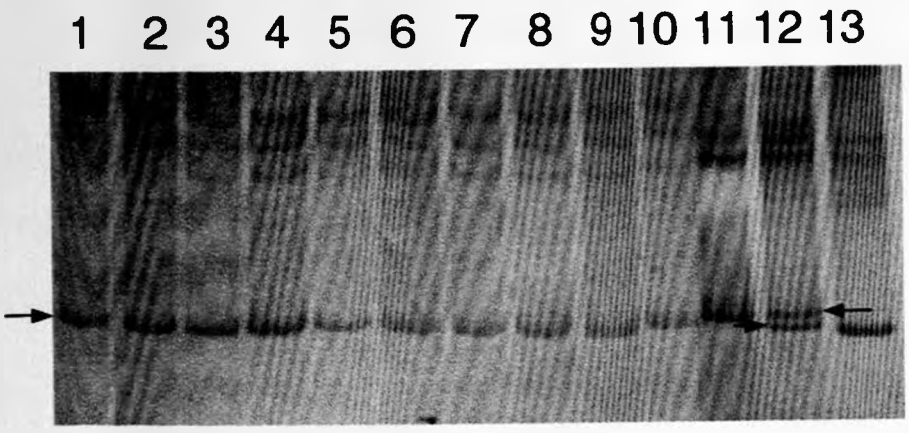

1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 26. SSCP analysis of the first exon for extracellular (EC) region of bovine CD32

Non-denaturing polyacrylamide SSCP analysis of the first exon encoding for EC region of bovine CD32. Lanes 1-10, genomic DNA from cattle vaccinated with p67 and subsequently challenged as a template in the PCR reaction (1-5 from severe reactors and 6-10 from non-reactors in p67 vaccine trials). Lane 11, African buffalo genomic DNA sample; lane 12, a mixture of PCR products from African buffalo and cattle genomic DNA samples. Lane 13 used sheep genomic DNA as PCR template. The arrows point to the differences in banding patterns.


1 2 3 4 5 6 7 8 9 10 11 12 13

CHAPTER FOUR

DISCUSSION

4.1 Polymorphism within the bovine FcγRIII (CD16) gene

In this study we report limited allelic polymorphism within the extracellular encoding domain of bovine FcγRIII (CD16) in a *B. indicus* Kenyan Boran when compared to the published *B. taurus* sequences. No nucleotide variation was observed in one animal (BR37) but two polymorphic nucleotides at positions 201 and 598 are described in the sequences obtained from the second animal (F100). These substitutions change a cytosine (C) to an adenine (A) and a cytosine (C) to a thiamine (T) respectively. Following translation both nucleotide substitutions were found to result in amino acid substitutions (non-synonymous substitutions). In the first instance a change of codon 67 (CCC) from proline to (ACC) threonine occurs within the first immunoglobulin-superfamily (Ig-SF) domain. An alignment of the translated protein with that of human FcγRIII suggest that this position is occupied by serine in human. The second nucleotide substitution results in a change of codon 199 (CCA) from proline to (CTA) leucine. Alignment of the amino acid sequences of bovine and human FcγRIII indicates that this position is also occupied by proline in the human sequence.

Further analysis of the amino acid sequence reveals conservation of the cysteine residues involved in the formation of the two Ig-SF domains. (Cysteine-47, -87, -128, -173) as well as the three potential N-glycosylation sites (Asparangine-56, -63 and -180) in the extracellular region of the molecule between human and cattle sequences. This is an indication that the general conformation of these protein structures is maintained between the different species.

As observed by Collins *et al.*, (1997) the study confirmed the presence of phenylalanine at position 203, indicating that the cattle molecule is not glycosylphosphotidylinositol (GPI)-anchored, but a conventional transmembrane protein. The presence of a GPI anchored form of cattle FcγRIII as described in human (Lanier *et al.*, 1988; Kurosaki and Ravetch, 1989) remains to be determined.

Additional *B. taurus* FCγRIII sequences have been described from cDNA prepared from five different B. *taurus* animals and the cDNA library used in the initial sequence in the database (Collins *et al.*, 1997). The results indicated that they all encoded a polypeptide of 250 amino acids but that changes in the nucleotide sequence resulted in differences in the predicted amino acid sequence. These amino acids changes were seen in the extracellular and transmembrane domains but not the cytoplasmic region. At each of the polymorphic sites only one nucleotide substitution was seen, indicating at least two genes which may or may not be different alleles. The polymorphism showed that the nucleotide changes at positions 17, 16, and 15 (CTG to TGC) were responsible for the proline and valine substitutions to leucine. Similarly, single nucleotide changes at positions 89 (A to G), 272 (G to A), 293 (C to T), and 637 (G to A) were responsible for the lysine to arginine, glycine to aspartate, alanine to valine, and valine to isoleucine substitutions. The single nucleotide substitution at position 633 (T to C) did not result in an amino acid change. (Collins *et al.*, 1997). However, the changes seen in the sequence derived from the *B. indicus* Kenyan Boran (F100) described here were not reported in that study. This may be an indication that the allele responsible for the substitution is unique for the African cattle subpopulation.

4.2 Polymorphism within bovine FcγRII (CD32) gene

A similar analysis to that described for CD16 identified an insertion of one codon within the extracellular domain of bovine FcγRII (CD32) based on comparisons with the published *B. taurus* sequence. The 3 bp insertion was observed in two out of three clones from BR37 and all three clones from F100. The remaining clone from BR37 did not have the insertion and showed 100% identity to the published sequence. The insertion of CAG at codon number 18 occurs after nucleotide 132 from the first nucleotide base of the forward primer and results in an additional Alanine. Allelic polymorphism within human FcγRIIa is associated with distinct differences in the capacity of monocytes to bind mouse IgG1 (Atsumi *et al.*, 1998). It was demonstrated that FcγRIIa of high responders (HR) and low responders (LR) had a nucleotide substitution of G to A resulting in a change of arginine (R) to histidine (H) at position 131 within the second extracellular domain.

The translation of the bovine sequence indicates that position 131 is occupied by tryptophan in the *B. taurus* sequence. This substitution in human FcγRIIa has been described as an example of functionally relevant polymorphism. H/R131, is associated with the binding affinity of human IgG2 (i.e. FcγRIIa-H131 isoform has a higher affinity than FcγRIIa-R131 for human IgG2 (Atsumi *et al.*, 1998). The occurrence of an extra alanine in the bovine CD32 may have some influence on the final configuration of the protein structure. However, the insertion occurs outside the Ig-superfamily domains demonstrated to have the binding regions (Hulett *et al.*, 1995). This suggests that the insertion may not have a direct influence in the affinity of the molecule to bovine IgG.

However, there is a need to express the two different bovine isotypes to demonstrate whether the insertion alters the affinity of the bovine CD32 for its ligand.

The data obtained from such a study is important because human CD32 has been used to demonstrate the link between polymorphism and variations in immune complex handling and therefore related disease pathogenesis. In a study on heparin-induced thrombocytopenia (HIT) it was speculated that reduced clearance of immune complexes in patients with the FcγRIIa-R/R131 allotype causes prolonged activation of endothelial cells and platelets, thus increasing the risk for thrombotic complications (Carslsson *et al.*, 1998). In a study on systemic lupus erythematosus (SLE) it was concluded that FcγRIIa polymorphism constitutes an additional factor that might influence the manifestation of SLE (Manger *et al.*, 1998). This shows the significance of the molecule in immune responses and hence a need to study it further and demonstrate the influence (if any) of the observed insertion and identify other possible polymorphic regions in bovine.

4.3 Polymorphism within bovine FcγRI (CD64) gene

In this study the first cDNA sequence for bovine FcγRI which previously had been partially sequenced only at the genomic DNA level (Symons and Clarkson, 1992) is described. Alignment of the cDNA sequence of the extracellular domain of FcγRI with the genomic DNA sequence corresponding to exons 1, 2 and 3 from European *B. taurus* identified three bp substitutions. These occur at positions 480 (T to C), 548 (G to A) and 564 (C to T) respectively. In addition, an undefined nucleotide base in the published genomic DNA sequence at position 417 was shown to be Adenine (A) in this study.

Following translation of the nucleotide sequence derived from the *B. indicus* animal and

comparison with the *B. taurus* genomic DNA sequence, the change at position 480, T to C was found to be non-synonymous (serine to proline) while the remainder at positions 612 and 628 represent silent or synonymous substitutions. Further analysis of allelic polymorphism at this locus in cattle is required as it encodes for the high affinity FcγR. Changes in the amino acid sequence such as that described here may results in functionally significant changes in binding patterns for bovine IgG.

4.4 Dectetion of polymorphism in the FcγR genes by PCR-SSCP

Polymorphism in cattle FcγRs genes has been described in this study and others (Collins *et al.*, 1997). However, cDNA analysis is not practical for studies involving large numbers of cattle due to the time and expense required in the preparation of RNA and cDNA. Therefore, PCR based genomic DNA methods that utilise the information derived from cDNA based studies such as that described here are required. These methodologies can be applied to large numbers of genomic DNA samples such as those prepared from cattle vaccinated with a neutralising antibody vaccine against *T. parva* as well genomic DNA (gDNA) samples available at ILRI representing all major cattle breeds from Asia and Africa. Results obtained in such studies would be of significance in determining links between the outcome of vaccination and FcγR allele polymorphism, the evolutionary history and origin of the FcγR alleles. By screening large numbers of vaccinated cattle a particular allelic form of FcγR may be associated with variable immune-response following vaccination and subsequent challenge. The result may also be used in genetic diversity studies where a given FcγR gene could be associated with the origin of a

particular cattle breed.

There are various alternatives in design of PCR based gDNA methods for FcγRs genes.

i. Cloning and sequencing of genomic DNA fragments.

ii. Polymerase chain reaction-restriction fragment length polymorphism.

iii. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE)

iv. Polymerase chain reaction-single strand conformational polymorphism.

Analysis of polymorphism by cloning and sequencing DNA fragments will effectively identify all the nucleotide differences present in these genes. However, this method is not practical (although with recent advances in sequencing technologies this is becoming less true) because of the time, effort and expense involved. The use of PCR-RFLP requires that the sequences are known and that the polymorphism affects a DNA restriction enzyme site. This method is generally not applicable in analysis of polymorphism in DNA fragments, which have not been sequenced before i.e. for identification of novel allelic polymorphism.

Analysis of polymorphism in a given gene by PCR-DGGE requires first of all the amplification of each exon in the gene by use PCR exon specific primers. The PCR fragments obtained are resolved in 9% polyarylamide gel with a denaturing gradient of 30-50% between the lowest and the highest concentration of denaturant (Hayes *et al.*, 1999). This method has been reported to accurately detect base changes in the TNF-α gene promoter by altered band patterns induced by the presence of polymorphisms at positions -376, -308, -238, -163 (Patino-Garcia *et al.*, 1999)

Single strand conformational polymorphism is a widely used method for detecting mutations in DNA fragments (Orita *at el.*, 1989; Sarkar *et al.*, 1992). This method may be

used in the detection of single base changes in amplified DNA fragments resolved on non-denaturing polyacrylamide gels. In determining the extent of the bovine FcγR genes polymorphism, fragments of approximately 250 bp were obtained by design of primers to amplify the exons encoding the extracellular domains in CD32 and CD64. PCR-SSCP has been shown to work optimally in the detection of polymorphism with fragments of 200-300 bp (Orita *et al.*, 1989) but may be successfully used to detecting polymorphism in the fragments up to 400 bp (Sarkar *et al.*, 1992). Here we describe an initial SSCP analysis on 10 cattle genomic DNA samples obtained from severe reactors and non-reactors in p67 vaccination trial. There were no observable differences between the cattle PCR amplified fragments when analysed as single strands by SSCP for the two exons encoding the extracellular region of CD32. However, there was clear difference between the cattle samples and the positive controls (buffalo and sheep samples) indicating that the method is efficient in detecting nucleotide base substitutions. Due to time limitation it was not possible to do further analysis on these genes. The methods described here may be used to screen for polymorphism in large numbers of DNA samples in an attempt to determine if there is a link between FcγRs polymorphism and the outcome of vaccination of cattle against *T. parva* using a novel subunit vaccine.

4.5 Conclusions and recommendations

The sequence analysis of cDNA clones encoding CD16, CD32 and CD64 from two *B. indicus* Kenyan Boran cattle indicate that each is highly conserved when compared with published *B. taurus* sequences. However, the limited amino acid diversity described here indicates subtle differences possibly between these two subspecies of cattle. To investigate this further, the study aimed to develop rapid PCR based methods to analyse

allelic FcγR polymorphism in large numbers of samples from a diverse range of cattle populations. This initially focused on CD32 because of the functionally important human polymorphism and aimed to use the SSCP method for analysis of single base pair substitutions in genomic DNA samples. To accurately test this method we required a positive control with known but very limited number of nucleotide differences. As such the sequence of the African buffalo (*Syncerus caffer*) and sheep (*Ovis aeris*) CD32 was determined. The sheep sequence showed 11 nucleotide base substitution while the buffalo sequence showed only 2 nucleotide base substitution. The first change, T to C result in the substitution of isoleucine by threonine while the second change, A to T result in the substitution of glutamine by leucine. These results show the significance of the single nucleotide base substitutions within cattle populations. This is because cattle and the African buffalo (*Syncerus caffer*) are estimated to be separated from a common ancestor by 10 million years (Modi *et al.*, 1996). There are two subspecies of cattle *B. indicus* and *B. taurus* which readily interbreed to produce fertile offspring (Epstein and Mason, 1984). Cattle may have been domesticated in a single process between 8000 and 10000 years ago near the Indus valley from the wild ox (*Bos primegenius nomadicus*) with *B. taurus* being first to be domesticated and *B. indicus* cattle later evolved by developing an arid-adapted physiology (Epstein and Mason, 1984). A more recent theory suggests that the domestication of cattle may have been a result of two independent events (Loftus *et al.*, 1994a). This view is supported by the observation that African and European cattle seem to have one mitochondrion DNA lineage whereas Asian cattle have a different lineage. The lineages diverged 200000-1 million years ago, long before the domestication process. These and the view that African cattle may have been a result of

an independent domestication process in Africa (Grison, 1991) indicate that differences occur between cattle from the three continents. The polymorphism observed in the three FcγR genes in African cattle may have *Taurine*, *Indicus* and Asian origins as all three populations are mixed in African Zebu cattle. It would be interesting to investigate if the insertion of alanine in CD32 also occurs in the African buffalo sequence. Such a study in addition to the two substitutions observed in this study would add more data to the study of the genetic divergence of two species.

In conclusion, the bovine FcγR genes are highly conserved between *taurine* and African *indicus* cattle with only limited allelic polymorphism described here. Analysis of the buffalo CD32 sequence revealed only two nucleotide substitutions despite 10 million years since a common ancestor. Such conservative evolution suggests intense functional constraints on the FCγR, which is likely to be a reflection of their biological function. The limited allelic polymorphism observed in this study is therefore significant and may change the three dimensional structure of the encoded molecules with possible functional differences. The two changes observed in CD16 involved substitution of proline from the protein structures. In addition, the change in CD64 involves substitution of serine by proline. Proline is a significant amino acid in determining the secondary structure of the protein molecule by formation of kink bonds. This structure will in turn determine the folding of the protein to acquire the three dimensional structure and ultimately the biological function. This is deduced from similar polymorphic studies in human where a single nucleotide substitution in FcγRIIa result into two alleles encoding for proteins with different binding affinities for hulgG2.

Additional work is needed to complete the SSCP analysis of all the exons encoding the

extracellular domains of CD16, CD32, CD64 and the novel cattle FcγR. The method may then be applied to gDNA samples from additional breeds in Africa, Asia and Europe. The results obtained may identify the relationship between the polymorphism within these genes and the origin of diversity in cattle. There is also a need to screen additional p67 vaccinated animals representing the extreme ends (severe reactors to none reactors) to determine if polymorphism in bovine FcγRs has an effect on the outcome of p67 vaccination and challenge. The gDNA based methods described in this study may form an important part of such screening.

REFERENCE

1. Ainsworth, P.J., Surh, L.C. and Coulter-Mackie, M.B. (1991). Diagnostic single strand conformational polymorphism, (SSCP): A simplified non-radioisotopic method as applied to a Tay-sachs B1 variant. *Nucleic Acids Research,* **25;19(2)**:405-406.

2. Allen, J.M. and Seed, B. (1998). Nucleotide sequence of the three cDNAs for human high affinity Fc receptor (FcRI). *Nucleic Acids Research,* **16**:11824.

3. Allen, J.M. and Seed, B. (1989). Isolation and expression of functional high-affinity Fc receptor cDNAs. *Science,* **243**:378-380.

4. Amigorena, S., Salamero, J., Davoust, J., Fridman, W.H. and Bonnerot, C. (1992). Tyrosine-containing motif that transduces cell activation signals also determine internalisation and antigen presentation via type III receptors for IgG. *Nature,* **358**: 337-341.

5. Amigorena, S., Bonnerot, C., Drake, J., Choquet, D., Hunziker, W., Guillet, G.G., Webster, P., Sautes, C., Mellman, I. and Friman, W.H. (1992). Cytoplasmic domain heterogeneity and function of IgG Fc receptors in B-lymphocytes. *Science,* **256**:1808-1812.

6. Anderson, C.L. and Abraham, N.G. (1980). Characterisation of the Fc receptor for IgG on human macrophages cell line, U937. *The Journal of Immunology,* **125**:2735-2741.

7. Anderson, C.L. and Looney, R.J. (1986). Review: Human leukocyte IgG Fc receptor. *Immunology Today,* **7**:246-266.

8. Anderson, C.L., Shen, L., Eicher, D.M., Wewres, M.D. and Gill, J.K. (1990). Phagocytosis mediated by three distinct Fcγ receptor classes on human leukocytes. *Journal of Experimental Immunology,* **171**:1333-1345.

9. Altschul, S., Gish, W., Miller, W., Myers, E. and Lipman, J. (1990). Basic local alignment search tool. *Journal of Molecular Biology,* **215**:403-410.

10. Atsumi, T., Caliz, R., Amengaul, O., Khamashta, M.A. and Hughes, G.R. (1998). Fc gamma receptor IIA H/R131 polymorphism in patients with antiphospholipid antibodies. *Thrombosis and Haemostasis,* **79(5)**:924-927.

11. Azzoni, L., Anegon, I., Calabretta, B. and Perrusia, B. (1995). Ligand binding to Fc gamma R induces c-myc-dependent apoptosis in IL-2-stimulated NK cells. *The Journal of Immunology,* **15;154(2)**:491-499.

12. Bailer, G., Telford, D., Guibins, E., Toshika, K. and Altman, A. (1993). Improved specificity of RT-PCR amplification using nested cDNA primers. *Nucleic Acids Research,* **21**:1329-1330.

13. Ballingall, K.T., Luyai, A. and McKeever, D.J. (1997). Analysis of genetic diversity at the DQA loci in the African cattle: evidence for a BoLA-DQA3 locus. *Immunogenetics,* **46**:237-244.

14. Brooks, D.G., Qui, W.Q., Luster, A.D. and Ravetch, J.V. (1989). Structure and expression of human IgG FcRII (CD32). Functional heterogeneity is encoded by the alternatively spliced products of multiple genes. *The Journal of Experimental Medicine,* **170**:1369-1386.

15. Carlsson, I.E., Santoso, S., Baurichter, G., Kroll, H., Papenberg, S., Eichler, P., Westerdaal, N.A., Kiefel, V., van de Winkel, J.G. and Greinacher, A. (1998). Heparin-induced thrombocytopenia: New insights into the impact of the Fcgammalla –R-H131 polymorphism. *Blood,* **92(5)**:1526-1531.

16. Chein, A., Edgar, D. and Trela, J. (1976). Dedioxynucleotide polymerase from the extreme thermophile, Thermus aquaticus. *Journal of Bacteriology,* **127**:1550-1557.

17. Chirgwin, J.M., Prybyla, A.E., Raymond, J., MacDonald, J. and Ruther, W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched with ribonuclease. *Biochemistry,* **18(24)**:5294-5299.

18. Chizyuka, H.G. and Mulilo, J.B. (1990). Methods currently used for the control of multi-host ticks: their validity and proposals for future control strategies. *Parassitologia,* **32**:127-132.

19. Clark, JM (1988). Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. *Nucleic Acid Research* **16**:9677-9678

20. Cohen, L., Sharp, S. and Kulczycki, A. (1983). Human monocytes, B lymphocytes and non-B lymphocytes each have structurally unique Fc receptor. *The Journal of Immunology,* **131**:378-383.

21. Collins, R.A., Gelder, K. and Howard, C.J. (1997). Nucleotide sequence of cattle FcGRIII: Its Identification in $\gamma\delta$ T cells. *Immunogenetics,* **45(6)**:440-443.

22. Cunningham, M.P., Brown, C.G., Burridge, M.J., Musoke, A.J., Purnell, R.E., Radley, D.E. and Sempebwa, C. (1974a). East Coast fever: Titration in cattle of suspensions of *Theileria parva* derived from ticks. *British Veterinary Journal,* **130**:336-345.

23. Davis, W., Harrison, P.T., Hutchinson, M.J. and Allen, J.M. (1995). Two distinct regions of FcγRI initiate separate signalling pathway involved in endocytosis and phagocytosis. *The EMBO Journal,* **14**:432-441.

24. Dhar, S., Malhotra, D.V., Bushan, C. and Gautam, O.P. (1986). Chemotherapy of *Theileria annulata* infection with Buparvaquone. *Veterinary record,* **119**:635-636.

25. Dobbelaere, D.A., Spooner, P., Barry, W. and Irvin, A. (1984). Monoclonal antibody neutralizes the sporozoites stage of different *Theileria parva* stocks. *Parasite Immunology,* **6**:635-636.

26. Dolan, T., Radley, D., Brown, C., Cunningham, M., Morzaria, S.P. and young, A. (1980). East Coast fever: Further studies on the protection of cattle immunised with a combination of *theilerial* strains. *Veterinary parasitorogy,* **6**:325-332.

27. Dolan, T.T. (1981). Progress in the chemotherapy of *Theileriosis*. In: Advances in the control of *Theileriosis*. (Irvin, A.D., Cunninghan, M.P. and Young, A.S. eds) pp 186-208, Martinus Nijhoff, the Hague.

28. Dolan, T.T., Linyonyi, A., Mbogo, S.K. and Young, A.S. (1984). Comparison of long acting oxytetracycline and parvaquone in immunisation against East Coast fever by infection and treatment. *Research in Veterinary Science,* **37**:175-178.

29. Dolan, T.T. (1986). Chemotherapy of East Coast fever, the long term growth rate, carrier state and disease manifestation of Parvaquone-treated cattle. *Journal of Comparative Medicine*, **96**:137-145.

30. Elberg, J.C., Lin, C.T., Lau, D., Unkeless, J.C. and Kimberly, R.P. (1995). The calcium dependence of human FcΥ receptor-initiated phagocytosis. *The Journal of Biological Chemistry,* **270**:2230-2237.

31. Ellis, S.A., Braem, K. and Morrison, W.I. (1992). Transmembrane and cytoplasmic domain sequences demonstrate at least two expressed bovine MHC class 1 loci. *Immunogenetics,* **37**:49-56.

32. Epstein, H. and Mason, L. (1984). Origin and domestication of cattle: In evolution of domesticated animals (Ed:Mason L). 1st edition. Longman, New York, pp 65-27.

33. Gelhaus, A., Schnitter, L., Melhitz, D., Horstmann, R. and Meyer, C. (1995). Sequence and PCR-RFLP analysis of 14 novel BoLA-DRB3 alleles. *Animal Genetics,* **26**:147-153.

34. Ernst, L.K., van de Winkel, G.J., Chui, I.M. and Anderson, C.L. (1992). Three genes for human Fc receptor for IgG (FcΥRI) encode four distinct translation products. *The Journal of Biological Chemistry,* **267**:1592-1600.

35. Fawcett, D.W., Buscher, G. and Doxsey, S. (1982b). Salivary gland of the tick vector of East Coast fever. 4. The ultrastructure of sporogony in *Theileria parva*. *Tissue and cell,* **14**:183-206.

36. Fawcett, D.W., Doxsey, S., Stagg, D.A. and Young, A.S. (1982a). The entry of sporozoites of *Theileria parva* into bovine lymphocytes *in vitro*: Electron microscopic

observations. *European Journal of Cell Biology,* **27**:10-21.

37. Fawcett, D.W., Musoke, A. and Voigt, W. (1984). Interaction of sporozoites of *Theileria parva* with bovine lymphocytes *in vitro*. Early events after invasion. *Tissue and Cell,* **16**:873-883.

38. Goddeeris, B.M., Balwin, C.L., Ole-MoiYoi, O. and Morrison, W.I. (1989). Improved method for purification and depletion of monocytes from bovine peripheral blood mononuclear cells. *Journal of Immunological Methods,* **89**:165-173.

39. Gosselin, E.J., Wardwell, K., Gosselin, D.R., Alter, N., Fisher, J.L. and Guyre, P.M. (1992). Enhanced antigen presentation using human FcγI receptor (monocyte/macrophage)-specific immunogens. *The Journal of Immunology,* **149**:3477-3481.

40. Good, M.F., Miller, L.H., Kumar, S., Quakyi, I.A., Keister, D., Adams, J.H., Moss, B., Berzofsky, J.A. and Carter, R. (1988). Limited immunological recognition of critical malaria vaccine candidate antigens. *Science*, **28;242(4878)**:574-577.

41. Gray, A.R. (1991). International Laboratory for Research on Animal Diseases: Research achievements, problems and future planning. *Kenya Veterinarian* **11**:16-18.

42. Grison, C. (1991). *African Archaeological Review,* **9**:119-144.

43. Guyre, P.M., Morganelli, M.P. and Miller, R. (1983). Recombinant immune interferon increases immunoglobulin G Fc receptors on cultured human mononuclear phagocytes. *Journal of Clinical Investigation,* **72**:393-397.

44. Hananhan, D. (1983). Studies on Transformation of *E. coli* with plasmids. *Journal of Molecular Biology,* **16**:557-580.

45. Hayashi, K. (1992). PCR-SSCP: A method for detection of mutations. *Genes Analysis Techniques Applications,* **9**:73-79.

46. Hayes, V.M., Wu, Y., Osinga, J., Mulder, I.M., van der Vlies, P., Buys, C.H. and Hofstra, R. (1999). Improvement in gel composition and electrophoretic conditions for broad-range mutations analysis by denaturing gradient gel electrophoresis. *Nucleic acids Research,* **27**:20-29.

47. Hogarth, P.M., Hibbs, L., Bonadonna, L., Scott, BM., Witort, G.A., Pietersz, G.A. and McKenzie, I.F. (1987). The mouse Fc receptor for IgG (Ly-17): Molecular cloning and specificity. *Immunogenetics,* **26**:161-168.

48. Howard, C., Taylor, G. and Browlie, J. (1980). Surface receptors for immunoglobulins on bovine polymorphonuclear neutrophils and macrophages. *Research in Veterinary Science,* **29**:128-130.

49. Hulett, M. and Hogarth, P. (1994). Molecular basis of Fc receptor function. *Advances in Immunology,* **57**:1-127.

50. Hulett, M.D., Witort, E., Brinkworth, R.I., McKenzie, I.F.C. and Hogarth, P.M. (1995). Multiple regions of human FcγRII (CD32) contribute to binding of IgG. *The Journal of Biological Chemistry,* **36**:21188-21194.

51. Irvin, A., Ocama, J. and Spooner, P. (1982). Cycle of bovine lymphoblastoid cells parasitised by *Theileria parva. Research in Veterinary Science,* **33**:298-304.

52. Irvin, A. and Mwamachi, A. (1983). Clinical and diagnostic features of East Coast fever (*Theileria Parva*) infection of cattle. *Veterinary Record,* **113**:192-304.

53. Irvin, A. and Morrison, I. (1987). Immunopathology, immunology and immunoprophylaxis of *Theileria* infection. In: Immune response in parasitic infections: Immunology, Immunopathology and immunoprophylaxis (Soulsby, E.J.L. ed) pp 223-274 CRC Press, Boca Raton, FL.

54. Kupiec, J., Giron, M., Vilette, D., Jeltsch, J. and Emanoil-Ravier, R. (1987). Isolation of high affinity-molecular-weight DNA from eukaryotic cells by formamide treatment and dialysis. *Analytical Biochemistry,* **164(1)**:53-59.

55. Kurosaki, T. and Ravetch, J. (1989). A single amino acid in the GPI attachment domain determines the membrane topology of FcγRIII. *Nature (London),* **342**:805-807.

56. Kurosaki, T., Gander, I., Wirthermueller, U. and Ravetch, J. (1992). The beta subunit of the FcεRI is associated with the FcγRIII on mast cells. *The Journal of Experimental Medicine,* **175**:447-457.

57. Lanier, L., Ruitenburg, J. and Phillips, H. (1988). Functional and biochemical analysis of CD16 antigen on natural Killer cells and granulocytes. *The Journal of Immunology,* **141**:3478-3485.

58. Lipman, D.L. and Pearson, W.R. (1985). Rapid and selective protein similarity searches. *Science*, **227**:1435-1441.

59. Loftus, R., MacHugh, D., Bradley, D., Sharp, P. and Cunnigham, P. (1994). Evidence for two independent domestication of cattle. *Proceedings of the National academy of Sciences, USA.*, **91**:2757-2761.

60. Looney, R., Abraham, N. and Anderson, C. (1986). Human monocytes and U937 cells bear two distinct Fc receptors for IgG. *The Journal of Immunology*, **136**:1641-1646.

61. Manger, K., Repp, R., Spriewald, B., Rascu, A., Geiger, A., Wassmuth, R., Westerdaal, N., Wentz, B., Manger, B., Kalden, J. and van de Winkel, J. (1998). Fc

gamma receptor IIa polymorphism in Caucasian patients with systemic lupus erythematosus: Association with clinical symptoms. *Arthritis and Rheumatism*, **41(7)**:1181-1189.

62. McHardy, N., Haigh, A. and Dolan, T. (1976). Chemotherapy of *Theileria parva* infection. *Nature*, **261**:698-699.

63. McHardy, N. (1978). *In vitro* studies on the action of menoctone and other compounds on *Theileria parva* and *T. annulata*. *Annals of Tropical Medicine and Parasitology*, **72**:501-511.

64. McHardy, N., Hudson, A.T., Morgan, D.T., Rae, D.G. and Dolan, T. (1983). Activity of 10 napthoquinones, including parvaquone (993c) and menoctone, in cattle infected with *Theileria parva*. *Research in Veterinary Science*, **35**:347-352.

65. Mcquire, T., Musoke, A. and Kurtti, T. (1979). Functional properties of bovine IgG1 and IgG2: Interaction with complement, macrophages, neutrophils and skin. *Immunology*, **38**:249-256.

66. Mehlhorn, H. and Schien, E. (1984). The piroplasm: Life cycle and sexual stages. *Advances in Parasitology*, **23**:37-103.

67. Modi, W.S., Gallangher, D.S. and Wamack, J.E. (1996). Evolutionary histories of highly repeated DNA families among the Artiodactyla (mammalia) (1996). *The Journal of Molecular Evolution*, **42(3)**:337-349.

68. Moll, G., Lohding, A., Young, A. and Leicth, B. (1986). Epidiemology of *Theileriosis* in calves in an endemic area of Kenya. *Veterinary Parasitology*, **19**:255-273.

69. Musisi, F. (1990). Methods currently used for control of East Coast fever: Their validity and proposal for future control strategies. *Parrasitologgia*, **32**:15-22.

70. Musoke, A., Nantulya, V. Rurungirwa, F and Buscher, G. (1984). Evidence for a common protective antigenic determinant on sporozoites of several *Theileria parva* sporozoites. *Molecular and Biochemical Parasitology*, **51**:17-28.

71. Musoke, A. Morzaria, S., Nkonge, C., Jones, E. and Nene, V. (1992). A recombinant sporozoite surface antigen of *Theileria parva* induces protection in cattle. *Immunology*, **89**:514-518.

72. Mullis, K. and Faloona, F. (1987). Specific synthesis of DNA *in vitro* via a polymerase-catalysed chain reaction. *Methods in Enzymology*, **155**:335-350.

73. Nishino, Y., Tajima, S. and Aida, Y. (1995). Cattle cDNA encoding a new allele of the MHC class II DQA1 gene. *Immunogenetics*, **42**:306-307.

74. Osman, N., Kozak, C., McKenzie, I. and Hogarth, P. (1992). Structure and mapping of the gene encoding mouse high affinity Fc gamma receptor I and chromosomal location of the human Fc gamma RI gene. *The Journal of Immunology*, **148(5)**:1570-1575.

75. Orita, M., Suzuki, Y., Seikiya, T and Hayashi, K. (1989). A rapid and sensitive detection of point mutations and genetic polymorphism using chain reaction. *Genomics*, **5**:874-879.

76. Park, J-G. and Schreiber, A. (1995). Determinants of the phagocytic signal mediated by the type IIIA Fc gamma receptor, FcγRIIIA: Sequence requirements and interaction with protein-tyrosine kinases. *Proceedings of the National academy of Sciences, USA*..**92**:7381-7385.

77. Patino-Garcia, A., Sotillo-Pineiro, E., Modesto, C. and Sierrasesumaga, L. (1999). Screening of human tumor nectrosis factor-alpha (TNF-alpha) gene promoter polymorphism by PCR-DGGE analysis. *Mutation Research*, **406(2-4)**:121-125.

78. Peper, R., Tina, W. and Mickelson, M. (1968). Purification of lymphocytes and platelets by gradient centrifugation. *Journal of Laboratory and Clinical methods*, **72**:842-848.

79. Perrusia, B., Dayton, T., Lazarus, R., Fanning, V. and Trinchieri, G. (1983). Immune interferon induces the receptor for monomeric IgG1 on human monocytic and myeloid cells. *The Journal of Experimental Medicine*, **158**:1092-1113.

80. Pruett, J.H. (1999). Immunological control of arthropod ectoparasites-review. *International Journal for Parasitology*, **29**:25-32.

81. Qui, W., de Bruin, D., Brownstein, B., Pearse, R. and Ravetch, J. (1990). Organisation of human and mouse low affinity FcγR genes: Duplication and recombination. *Science*, **248**:732-735.

82. Radley, D., Brown, C., Burridge, M., Cunnigham, M., Kirimi, I., Purnell, R. and Young, A. (1975a). East Coast fever: 1. Chemoprophylatic immunisation of cattle against *Theileria parva* (Muguga) and five *Theilerial* strains. *Veterinary Parasitology*, **1**:35-41.

83. Radley, D., Brown, C., Cunnigham, M., Kimber, C., Musisi, F., Puyne, R., Purnell, R., Stagg, S. and Young, A. (1975b). East Coast fever: 3. Chemoprophylatic immunosation of cattle using oxytetracycline and a combination of *Theilerial* strains. *Veterinary Parasitology*, **1**:51-60.

84. Radley, D., Young, A., Brown, C., Burridge, M., Cunnigham, M., Musisi, F. and Purnell, R. (1975c). East Coast fever: 2. Cross-immunity trials with a Kenyan strain of *Theileria lawrencei*. *Veterinary Parasitology*, **1**:43-50.

85. Radley, D., Young, A., Grootenhuis, J., Cunnigham, M., Dolan, T. and Morzaria, S.

(1979). Further studies on immunisation of cattle against *Theileria lawrencei* by infection and treatment. *Veterinary Parasitology,* **1**:43-50.

86. Raknes, G., Skeie, G., Gilhus, N., Aadland, S. and Vedeler, C. (1998). FcγRIIA and FcγIIIB polymorphism in myasthenia gravis. *Journal of Neuroimmunology,* **81(1-20)**:173-176.

87. Ravetch, J. and Perrusia, B. (1989). Alternative membrane forms of FcγRIII (CD16) on human NK cells and neutrophils: Cell-type specific expression of two genes which differ in single nucleotide substitutions. *The Journal of Experimental Medicine,* **170**:481-497.

88. Ravetch, J. and Kinet, J. (1991). Fc receptors. *Annual Review of Immunology,* **9**:457-492.

89. Rosenfeld, S. and Anderson, C. (1989). Fc receptors of human platelets, In kunicki T.J. and J.N. George (ed). *Platelets Immunology*. J.B. Lippincott, Philadelphia.

90. Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G., Erlich, H. and Arnheim, N. (1985). Enzymatic amplification of beta-globulin genomic sequences and restriction site analysis for diagnosis of single cell anaemia. *Science,* **20;230(47320)**:1350-1354.

91. Saiki, R., Geifand, D., Staffel, S., Scharf, S., Higushi, R., Horn, G., Mullis, K. and Erlich, H. (1988). Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. *Science,* **239**:487-491.

92. Sambrook, J., Fritich, E. and Maniatis, E. (1989). Molecular Cloning, a Laboratory Manual. (2nd edition). *Cold Spring Habour Laboratory Press.*

93. Sammatino, L., Webber, L.M., Hogarth, P.M., McKenzie, I. and Garson, O.M. (1988). Assignment of the gene coding for human FcRII (CD32) to bands q23q24 on chromosome 1. *Immunogenetics,* **28**:380-381.

94. Sanger, F., Nickler, S. and Coulson, R. (1977). DNA sequencing with chain terminating inhibitors. *Proceedings of the National academy of Sciences, USA.,* **74**:5463-5467.

95. Sarkar, G., Youn, H. and Sommer, S. (1992). Screening for mutations by RNA single-stranded conformation polymorphism (rSSCP): Comparison with DNA-SSCP. *Nucleic Acids research,* **20(4)**:871-878.

96. Scharenberg, A.M. and kinet, J.P. (1996). The emerging field of receptor-mediated inhibitory signalling: SHP or SHIP? *Cell,* **13;87(6)**:961-964.

97. Schien, E., Warnecke, M and Kirmse, P. (1977). Development of *Theileria parva* in the guts of *Rhiphicephalus appendiculatus* (Neumann. *Parasitology,* **75**:309-316.

98. Sears, D., Osman, N., Tate, B., McKenzie, I. and Hogarth, P.M. (1990). Molecular cloning and expression of mouse high affinity Fc receptor for IgG. *The Journal of Immunology*, **144**:371-378.

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

100. Stephens, A., Brown, A., Chandanayingyong, D., Webster, H.K., Sirikong, M., Longta, P., Vangseratthana., Gordon, D.M., Lekmak, S. and Rungruang, E. (1995). The presence of the HLA class II allele DPB1*0501 in the ethnic Thais correlate with an enhanced vaccine-induced antibody response to a malaria sporozoite antigen. *European Journal of Immunology*, **25**:3142-3147.

101. Strohman, R.C., Moss, P.S., Micou, E., Spector, D., pryzybyla, A. and Peterson, B. (1977). Messenger RNA for myosin polypeptides: Isolation from a single myogenic cell culture. *Cell*, **10**:265-273.

102. Stuart, S.G., Simister, N.E., Clarkson, S.B., Kacinski, B.M., Sahpiro, M. and Mellman, I. (1989). Human IgG Fc receptor (hFcRII; CD32) exists as multiple isoform in macrophages, lymphocytes and IgG-transporting placental epithelium. *The EMBO Journal*, **8**:3657-3666.

103. Strul, K. (1985). Simplified technique of purifying DNA from agarose gels. *Biotechniques*, **3**:452.

104. Symons, D.B. and Clarkson, C.A. (1992). Genomic organisation and sequence of the extracellular domain exons of the bovine FcγRI, and evidence for restricted binding of ruminant IgG to U937 cells. *Molecular Immunology*, **29**:1407-1413.

105. Tuijnman, W.B., Capel, P.J. and van de Winkel, J.G. (1992). Human low-affinity IgG receptor FcγRIIa (CD32) introduced into mouse fibroblasts mediates phagocytosis of sensitised erythrocytes. *Blood*, **79**:1651-1656.

106. Van de Herik-oudijk, I.E., Terbekke, M.W., Tempelman, N.V. and Capel, P.J. (1994). Functional difference between two Fc receptor ITAM signalling motifs. *Blood*, **86**:3302-3307.

107. Vivier, E. and Rochet, N., Ackerly, M., Petrini, J., Levine, H., Daley, J. and Anderson, P. (1992). Signalling function of reconstituted CD16: Zeta: Gamma receptor complex isoforms. *International Immunology*, **4(11)**:1313-1323.

108. Wang, A.V., Scholl, P.R. and Geha, R.S. (1994). Physical and functional association of the high affinity immunoglobulin G receptor (Fc gamma RI) with the kinases Hck and Lyn. *The Journal of Experimental Medicine*, **180(3)**:1165-1170.

Hunkank, R.L., Luster, A.D. and Ravetch, J.V. (1988). Functional regulation of a ...nocyte-specific receptor, FcγRα. *The Journal of Experimental Medicine*, ...1925.

...Wilsden, P. (1997). Novel vaccines for ectoparasites. *Veterinary parasitology*, ...211.

...Williams, A.F. and Barclay, A.N. (1988). The immunoglobulin superfamily domains ...surface recognition. *Annual Review of Immunology*, **6**:381-405.

...Young, A.S., Leitch, B.L. and Omwonyo, P.L. (1979). Induction of infective stages ...*parva* by exposure of host ticks to high temperature. *Veterinary Record*, ...533.

...Young, A.S., Leitch, B.L. and Mutugi, J.J. (1984). Some factors controlling the ...of sporogony of *Theileria parva* in its ticks vector *Rhipicephalus ...International Journal for Parasitology*, **14**:97-102.

...Zhang, G., Young, J.R., Tregaskes, C.R. and Howard, C.J. (1994). Cattle FcγRII: ...cloning and ligand specificity. *Immunogenetics*, **39**:423-427.

...Zhang, G., Young, J.R., Tregeskes, C.A., Sopp, P. and Howard, C.J. (1995). ...of a novel class of mammalian Fcγ Receptor. *The Journal of Immunology*, ...1534-1541.

...Zhu, M., Clark, S.E. and Gomezsachez, C.E. (1995). Universal cloning method by ...energy. *Biotechniques*, **19**:34-35.

APPENDICES

APPENDIX A: BUFFERS AND SOLUTIONS

A.1 SOLUTIONS AND BUFFERS FOR CELL CULTURE

A.1.1. Alsevers solution (1 litre)

0.55 g citric acid
20.5 g dextrose
NaCl
8.0 g sodium citrate

A.1.2 Tris Ammonium Chloride

Solution A: 20.6 g Tris [Tris (Hydroxymethyl) Aminomethane]
Solution B: 8.3 g Ammonium Chloride
Solution A and B are mixed in a ratio of 1:9 and adjusted to pH 7.2

A.1.3 Phosphate buffered saline pH 7.4 (1 litre)

1.15 g Na_2HPO_4 anhydrous
0.2 g KH_2PO_4
8.0 g NaCl
$MgCl_2 (H_2O)_6$
0.13 g $CaCl_2$

A.1.4 RPMI 1640

A.1.5 Cell culture medium

500 ml RPMI 1640
50 ml fetalclone II (heat inactivated)
5 ml gentamycin (50 µl/ml)
5 ml 2 mM L-Glutamine
500 µl βmercaptoethanol 350 µl into (100 ml RPMI)

A.2 BUFFERS USED IN DNA MANIPULATION

A.2.1 RBC lysis solution

109.536 g sucrose
10 ml 1M Tris-HCl pH 7.5
5 ml 1 M $MgCl_2$
10 ml Triton 100

water added to make 1 litre

A.2.2 Solution B

15 ml 0.5M NaCl
5 ml 0.5M EDTA (pH 8.0)
80 ml water

A.2.3 Proteinase K/SDS

0.5 ml 10% SDS
0.5 ml proteinase K stock (8 mg/ml)

A.2.4 Saturated salt

379.6 g NaCl
Water added to make 1 litre

A.2.5 10 x AMV-RT buffer

100 mM Tris-HCl pH 8.8
500 mM KCl
1.0% Triton X-100

A.2.6 10 x *Taq* DNA polymerase buffer (for PCR)

100 mM Tris-HCl pH 9.0
500 mM KCl
1.0% Triton X-100

A.2.7 10 x DNA ligase buffer

300 mm Tris-HCl pH 7.8
100 ml $MgCl_2$
100 mM dithiothreitol (DTT)
10 ml ATP

A.3 BUFFERS FOR ELECTROPHORESIS

A.3.1 50 x TAE buffer (1 litre)

242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA pH 8.0

water added to make 1 litre

A.2.2 Solution B

15 ml 0.5M NaCl
5 ml 0.5M EDTA (pH 8.0)
80 ml water

A.2.3 Proteinase K/SDS

0.5 ml 10% SDS
0.5 ml proteinase K stock (8 mg/ml)

A.2.4 Saturated salt

379.6 g NaCl
Water added to make 1 litre

A.2.5 10 x AMV-RT buffer

100 mM Tris-HCl pH 8.8
500 mM KCl
1.0% Triton X-100

A.2.6 10 x *Taq* DNA polymerase buffer (for PCR)

100 mM Tris-HCl pH 9.0
500 mM KCl
1.0% Triton X-100

A.2.7 10 x DNA ligase buffer

300 mm Tris-HCl pH 7.8
100 ml $MgCl_2$
100 mM dithiothreitol (DTT)
10 ml ATP

A.3 BUFFERS FOR ELECTROPHORESIS

A.3.1 50 x TAE buffer (1 litre)

242 g Tris base
57.1 ml glacial acetic acid
100 ml 0.5 M EDTA pH 8.0

A.3.2 6 x sample loading buffer

0.25% bromophenol blue
0.25% Xylene cyanol FF
15% Ficoll in water

A.3.4 10 x TBE buffer (1 litre)

108 g Tris base
55 g Boric acid
40 ml 0.5 M EDTA pH 8.0

A.4 BUFFERS USED IN PURIFICATION OF DNA BY GENECLEAN

A.4.1 Gene clean new wash buffer (in 50% ethanol)

29 mM Tris-Cl pH 7.5
1M NaCl
1 mM EDTA

A.4.2 Cell resuspension buffer (for gene clean)

50 mM Tris-Cl pH 7.5
10 mm EDTA
100 µg Ribonuclease/ml

A.4.3 Column wash buffer (in 50% ethanol)

0.2 M NaCl
20 mM Tris-Cl pH 7.5
5 mM EDTA

A.5 ALKALINE LYSIS BUFFERS FOR MINIPREPARATION OF PLASMID DNA

A.5.1 Cell resuspension buffer (solution 1)

50 mM glucose
25 mM Tris-Cl pH 8.0
10 mM EDTA pH 8.0

A.5.2 Cell lysis buffer (solution 2)

0.2 N NaOH
10% SDS

A.5.3 Neutralising buffer (solution 3)

5 M potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Water	28.5 ml

Resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

A.6 BUFFERS USED IN DNA SEQUENCING

A.6.1 10 x polynucleotide kinase buffer

500 mM Tris-HCl pH 7.5
100 mM $MgCl_2$
500 mM Dithiothreitol
mM spermine

A.6.2 5 x sequencing buffer

250 mM Tris-HCl pH 9.0
10 mM $MgCl_2$

A.6.3 Sequencing stop solution

10 mM NaOH
95% Formamide
0.5% Bromophenol blue
0.05% xylene cyanole

A.7 BUFFERS AND SOLUTION FOR SSCP ANALYSIS

A.7.1 Silver reaction

12.5 µl$AgNO_3$ (1 % w/v)
Make upto 125 ml with deionised water
125 µl formaldehyde (37%)
Store $AgNo_3$ solution at 4^0C

A.7.2 Developing solution

3.125 g Na_2CO
125 µl Formaldehyde (37%)
125 µl Sodium thiosulphate (12% w/v)

A.7.3 Preservation solution

75 ml ethanol
11.5 ml glycerol (87% w/v)
Make up to 250 ml with deionised water.

APPENDIX B: BACTERIAL MEDIA AND PLATES

B.1 2 XYT medium (per litre)

16 g bactotyptone
10 g yeast extract
5 g NaCl

Ampicilin (Penbrithin, Dawa pharmaceuticals Ltd, Nairobi, Kenya)was dissolved in concentration of 100 mg/ml each and stored at –20 ^{0}C. Ampicilin was added to the media at a concentration of 50 μg/ml.

To prepare 2 XYTH plates, 15.0 g/l technical agar (DIFCO) was added, and the mixture was poured in 15 x 15 large plastic petri dishes with 60 ml of the agar per plate.

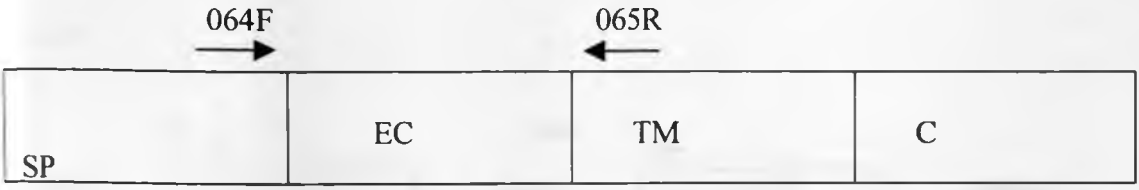
When required, ampicilin and tetracyclin were added to the autoclaved agar after it had cooled to 60^{0}C before pouring. All plates were dried by incubation at 37^{0}C, with lids slightly open, overnight.

B.2 NZCYM medium for plates

5.0 g Yeast extract
5.0 g NaCl
12.0 g technical agar
NZ Amine-A 10.0 g
Casamino acids 1.0 g
1.71 g $MgCl_2$

APPENDIX C: PRIMERS FOR PCR AMPLIFICATION OF BOVINE CD16, CD32 AND CD64.

a.

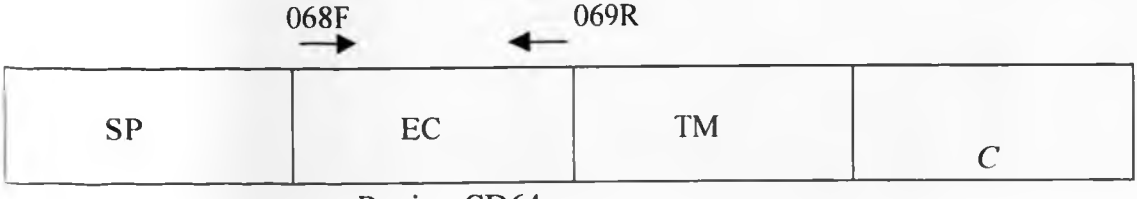


Bovine CD16

b.

Bovine CD32

c.

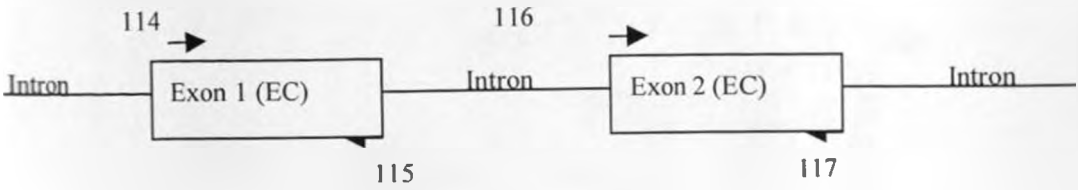


Bovine CD64

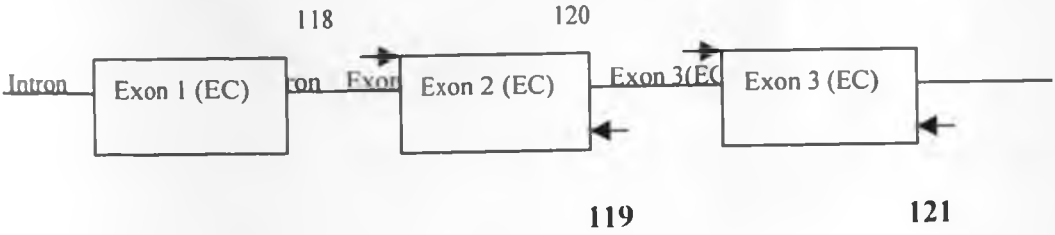
Representation of the primers used in the PCR amplification of Bovine FcγRs (a. CD16; b. CD32; c. CD64) from cDNA, the arrows indicte the direction of amplification. Abbreviation for regions: SP, *signal peptide*; EC, *extracellular*; TM, *transmembrane*; C, *cytoplasmic*. F, forward primer and R, Reverse primer.

APPENDIX D: Primers for amplification of exons encoding for Extarcellular regions of FcγRs from genomic DNA

a. CD32



b. CD64



Representation of primers used in the PCR amplification of exons encoding bovine FcγRs from genomic DNA. The arrows indicate the direction of amplification.

APPENDIX E: A plasmid map of pGEM-T Easy vector