MAEDI-VISNA VIRUS: THE DEVELOPMENT OF SERUM AND WHOLE BLOOD IMMUNODIAGNOSTIC ASSAYS

Christoffel Hendrik Boshoff

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ABSTRACT

This thesis describes the development of serum and whole blood immunodiagnostic assays for Maedi-Visna virus (MVV). All previously described recombinant MVV ELISA assays utilised either the core p25 or transmembrane (TM) proteins alone, or combined, but as individual proteins. The p25 and TM genes of MVV were cloned individually into the pGEX-2T expression vector. Both proteins were expressed as a combined fusion protein in frame with glutathione S-transferase (GST). The purified recombinant antigens (GST-TM and GST-TM-p25) were used to develop a MVV ELISA. Sera from 46 positive and 46 negative sheep were tested using the GST-TM and GST-TM-p25 ELISAs and a commercial p25 EIA kit. A two-graph receiver operating characteristic (TG-ROC) analysis program was used to interpret the data. The GST-TM-p25 ELISA was more sensitive than the commercial assay which is based on the p25 antigen alone and more specific than the GST-TM ELISA. The GST-TM-p25 ELISA showed a sensitivity and specificity of 100%.

The human AIDS lentivirus transmembrane (TM) glycoprotein portion of the envelope viral protein has been identified as the antigen most consistently recognised by antibodies. There is suggestive evidence that the same applies to MVV as the GST-TM fusion protein, expressed in *E. coli*, has comparable sensitivity to the GST-TM-p25 fusion protein, but lacks specificity. However, due to the hydrophobic nature of the MVV TM protein, purification of the expressed fusion protein required lengthy purification protocols. This was despite the fact that only a truncated version of the TM protein was expressed. This prompted investigating an alternative expression system that could possibly circumvent the above mentioned problems. The yeast

Pichia pastoris is known to be suitable for the high-level expression of heterologous proteins which are secreted into the culture supernatant. These features made *P. pastoris* an attractive host for the expression of the hydrophobic TM protein of MVV. However, limited success was achieved as only low expression levels were obtained and detection and quantification was only accomplished by means of ELISA. Evaluation of the diagnostic performance of the *P. pastoris* expressed MVV TM-polypeptide was performed using a panel of 36 confirmed negative and positive sera, and evaluated using a TG-ROC analysis programme, which yielded an equal Se and Sp of 83%.

The use of a novel rapid immunoassay system, which allows the detection of circulating antibodies in whole blood, has been investigated for use as a MVV diagnostic assay. The central feature of this immunoassay lies in a monoclonal antibody against a glycophorin epitope present on all sheep erythrocytes. A Fab'-peptide conjugate was constructed by coupling a synthetic peptide, corresponding to a sequence from MVV TM protein, to the hinge region of the Fab' fragment of the anti-sheep erythrocyte antibody. Within the limited number of 10 seronegative and 10 seropositive samples the autologous red blood cell agglutination assay had a sensitivity of 90% and a specificity of 80%. Despite the limitations and difficulties encountered, the use of such rapid whole blood immunodiagnostic assays for MVV holds promise.

PREFACE

The experimental work described in this thesis was carried out in the department of Virology, University of Natal, Medical School, Durban and the Natal Institute of Immunology, Pinetown, from January 1993 to December 1995 under the supervision of Dr. D.F. York (University of Natal) and Dr. J.D. Conradie (Natal Institute of Immunology).

These studies represent original work by the author and have not been submitted in any other form to another university. Work of other authors has been duly acknowledged in the text.

(Christoffel Hendrik Boshoff)

Date:

The ultimate aim of the [hero's] quest [is] neither release nor ecstasy for oneself, but the wisdom and the power to serve others.

Joseph Campbell

This thesis is dedicated

to my wife

Helena

PUBLICATIONS

Some of the research in this thesis has been submitted to the following journals:

Boshoff, C.H., Dungu, B., Williams, R., Vorster, J., Conradie, J.D., Verwoerd, D.W. and York, D.F. (1997) Detection of Maedi-Visna virus antibodies using a single fusion transmembrane-core p25 recombinant protein ELISA and a modified receiver-operating characteristic analysis to determine cut-off values. *J. Virol. Methods* 63, 47-56.

Boshoff, C.H., Conradie, M., Green, F.A., York, D.F. and Conradie, J.D. (1997) Preparation and characterization of a monoclonal antibody against a species-specific antigen on sheep erythrocytes. Submitted to *Hybridoma.*

Boshoff, C.H., York, D.F. and Conradie, J.D. (1997) Development of a rapid whole blood agglutination test for Maedi-Visna virus. Submitted to *Onderstepoort J. Vet. Res.*

ABBREVIATIONS

Ab	antibody
AGID	agar gel immunodiffusion
AIDS	acquired autoimmune deficiency syndrome
AOX	alcohol oxidase
AP	alkaline phosphatase
BDB	bis-diazotized-benzidine
BIV	bovine immune deficiency virus
BMGY	buffered minimal glycerol-complex medium
BMMY	buffered minimal methanol-complex medium
BSA	bovine serum albumin
CAEV	caprine arthritis-encephalitis virus
COM2	pGEX-TM-p25 expression vector construct
CPE	cytopathic effect
DMEM	Dulbecco's modified Eagle's medium
DMEM-HAT	DMEM with hypoxanthine, aminopterin and thymidine
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassays
EIAV	equine infectious anaemia virus
ELISA	enzyme-linked immnosorbent assay
Fab'	antigen binding fragment of an intact antibody

$F(ab)_2$	intact antibody with Fc region removed
Fab'-Ac	Fab' with hinge thiols alkylated using iodoacetamide
Fab'-TNB	Fab' with hinge thiols attached to thiol on TNB molecule
Fab'-TNB-Ac	Fab'-TNB with remaining hinge thiols alkylated with iodoacetamide
Fc	portion of an intact antibody below the hinge region
FIV	feline immunodeficiency virus
gp	glycoprotein
GST	glutathione S-transferase
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
IMAC	immobilized metal affinity chromatography
i-ELISA	indirect ELISA
IgG	immunoglobulin class G
IPTG	isopropyl-β-D-thiogalactoside
IR	intermediate range
JSRV	jaagsiekte retrovirus
KDa	kilodalton
LTR	long terminal repeats
Mab	monoclonal antibody
MR	measurement range
MVV	Maedi-Visna virus
MW	molecular weight
Ni-NTA	nickel-nitrilotriacetic acid

O.D.	optical density
ORF	open reading frame
p	protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS/Azide	PBS with sodium azide
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethyleneimine
PMSF	phenylmethylsulfonyl fluoride
POD	peroxidase
RBC	red blood cell
RD	regeneration dextrose- top agar
RDB	regeneration dextrose base
RT	reverse transcriptase
SA-OMVV	South African ovine maedi-visna virus
SDS	sodium dodecyl sulphate
Se	sensitivity
SIV	simian immunodeficiency virus
Sp	specificity
SPA	sheep pulmonary adenomatosis
TG-ROC	two-graph receiver operating characteristic
TM	transmembrane protein
TNB	2-nitro-5-thiobenzoate

Tris	tris(hydroxymethyl)methylamine
TST	tris-buffered saline with Tween 20
U	unit
VRP	valid range proportion
YPD	yeast extract-peptone-dextrose medium

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CHAPTER 1

GENERAL INTRODUCTION

1.1 A BRIEF HISTORY

The virus family *Retroviridae* is divided into three subfamilies: *Oncovirinae*, *Lentivirinae* and *Spumavirinae*. The name lentivirus is derived from a group of infectious diseases associated with slow infections (Sigurdsson, 1954), distinguishing them from chronic diseases such as tuberculosis. Lentiviral disease in sheep was first described as a progressive pneumonia in South Africa by Mitchell in 1915 (Palsson, 1976). This was followed in 1923 by a report of severe chronic interstitial pneumonia of sheep in Montana that resulted in wasting and eventual death (Marsh, 1923).

Maedi-Visna virus (MVV) was recognized as a pathogen of sheep as a result of an epidemic of a progressive pneumonia ("maedi"), accompanied in some cases by progressive paralysis ("visna") which arose in Icelandic sheep flocks between 1939 and 1952 (Sigurdardottir and Thormar, 1964). One hundred and fifty thousand animals were lost due to the disease and a further 650 000 destroyed in a successful attempt to control the disease (Sigurdsson, 1954). Transmission of the disease to healthy animals using tissue preparations from affected sheep was subsequently demonstrated (Sigurdsson *et al*, 1957) and in 1967, a single virus was shown to be the causative agent of both maedi and visna, hence the term maedi-visna virus (Gudnadottir and Palsson, 1967). This virus was subsequently characterized as a nononcogenic, exogenous retrovirus of the *Lentiviridae* subfamily (Cutlip and Laird, 1976). MVV infection has now been recognized in numerous countries, including the Netherlands where it is known as *zwoegerziekte* (De Boer, 1970), Belgium (Bironf and Deluyker,

1985), South Africa (Querat *et al.*, 1990), the U.S.A., where it is sometimes known as ovine progressive pneumonia (Cutlip and Laird, 1976), Greece (Seimenis *et al.*, 1985) and the U.K. (Dawson *et al.*, 1990; Watt *et al.*, 1990). In none of these countries has it yet had the same economic impact as in the Icelandic outbreak, although it is of importance in flocks with a high incidence of infection or in pedigree flocks.

Lentiviruses have also been isolated from other animal species including the caprine arthritis-encephalitis virus (CAEV) of goats (Crawford *et al*, 1980) and the equine infectious anaemia virus (EIAV) of horses (Vallee and Carre, 1904). Interest in lentiviruses was greatly increased when it was realized that the causative agents of human acquired immunodeficiency syndrome (AIDS) (HIV-1 and HIV-2) were also members of the lentivirus subfamily (Gonda *et al.*, 1985; Sonigo *et al.*, 1985). The search for AIDS animal models rapidly led to the isolation of simian immunodeficiency virus (SIV) of monkeys (Daniel *et al.*, 1985), bovine immunodeficiency virus (BIV) of cattle (Gonda *et al.*, 1988; Beatty, *et al.*, 1992), and feline immunodeficiency virus (FIV) of cats (Pederson *et al.*, 1987). This taxonomic group of viruses is based upon similarities in virion morphology, protein serological properties, genomic organization, nucleotide sequences, host cell specificities and patterns of pathogenesis. It is probable that more animal lentiviruses will be identified in future.

1.2 THE MAEDI-VISNA VIRUS GENOME

MVV like other retroviruses, possesses a positive sense single-stranded RNA genome (Brahic *et al.*, 1977), and replicates via a DNA intermediate (Haase and Varmus, 1973). Each virion contains two RNA molecules and is therefore dimeric. The size of the MVV RNA genome varies from 9202 nucleotides for Icelandic strain 1514 (Sonigo

et al., 1985), 9203 nucleotides for a British isolate EV1 (Sargan *et al.*, 1991) to 9256 nucleotides for SA-OMVV (Querat *et al.*, 1990). Figure 1.1 shows the general organisation of the genome which is similar for most retroviruses.

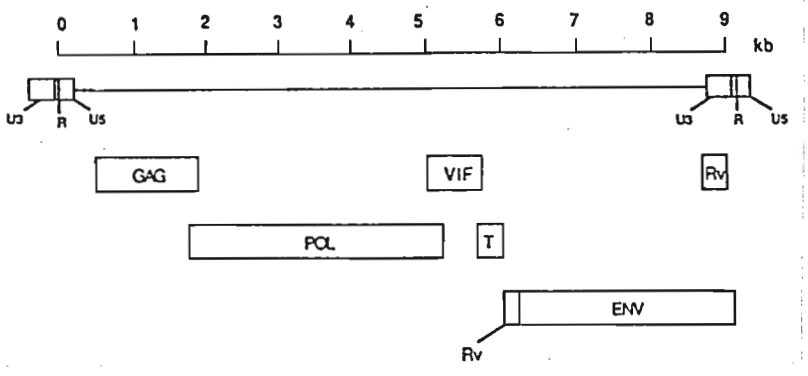

Figure 1.1 The MVV genome. The position of the U3, R and U5 regions of the LTR and of the major open reading frames of MVV are shown. Approximate nucleotide positions are indicated by the scale bar. T, *tat*; Rv, *rev*.

The genome contains three major structural genes. From the 5' end the *gag* gene codes for the core proteins of the virus, *pol* codes for the RNAse H, protease, integrase and reverse transcriptase enzymes, while the *env* gene at the 3' end codes for the viral envelope glycoprotein. At both ends are the long terminal repeats (LTRs). These LTR sequences are divided into the U3, R and U5 regions. They also contain the tRNA primer binding site, and a polypurine tract which acts as the initiation site for positive strand DNA synthesis. A well defined TATA box starts at nucleotide 438 and a CCAAT consensus sequence begins at bases 352 and 364 (Hess *et al.*, 1985). Studies on the role of retroviral LTRs revealed that they contain target sequences that regulate the level of transcription and thereby viral replication in cells. Another feature of lentiviral genomic organization, is the presence of several additional open reading frames (ORFs) located between the *pol* and *env* genes and in the *env* region (Davis and Clements, 1988). These small ORFs encode proteins that regulate gene expression,

control production of infectious viral particles and are involved in the disease manifestation *in vivo*. These genes also show little nucleotide or amino acid homology among the different lentiviruses and may be one of the factors determining the species-specificity of each lentivirus. An additional ORF, termed W, in SA-OMVV (Querat *et al.*, 1990) has been shown to exist without any significant sequence homology to other lentiviral ORFs. However an open reading frame with sequence homology to HIV *vif* (Strebel *et al.*, 1987) has been shown to be present in the MVV genome.

1.3 MVV PROTEINS

A general model of a lentivirus virion is presented in Figure 1.2. The virus has a diameter of approximately 100 nm with a lipid envelope enclosing an oblong core containing the diploid RNA genome.

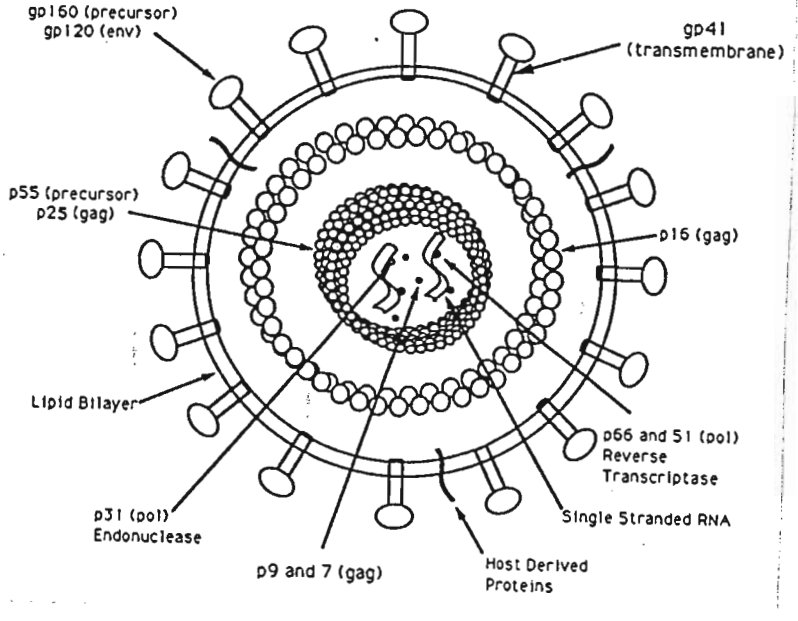

Figure 1.2 The major structural components of MVV.

The major precursor of the *gag*-encoded proteins is a non-glycosylated molecule of 55 KDa (Pr55gag) (Vigne *et al.*, 1982) which is cleaved intracellularly to form the mature

proteins. The precursor polyprotein is transported to the site of virus assembly at the plasma membrane where it is processed by the viral protease during budding into the native structural proteins found in virus particles. The core proteins of lentiviruses constitute the predominant protein components of the virus particle accounting for up to 90% of the virus protein. The p16 matrix protein, located immediately beneath the envelope lipid bilayer is myristylated at its amino-terminus. The virus particle contains enough matrix protein to form a continuous monolayer, which is believed to be noncovalently associated with the envelope transmembrane (TM) protein. The major capsid shell protein is the most abundant protein in lentiviruses and is the major component of the core shell. The MVV major capsid protein has an approximate mass of 25 KDa and displays significant sequence homology within this protein group of viruses which is reflected by serological cross-reactivities. The third core protein of MVV is the highly basic ribonucleoprotein that is bound to the viral RNA, presumably to form a helical ribonucleoprotein complex. The ribonucleoprotein has a molecular mass of 14 KDa and is rich in lysine and arginine residues and contains a cys-his box, which is characteristic of various nucleic acid binding proteins and perhaps essential for reverse transcription and assembly (Meric and Goff, 1989). In summary, the order of *gag* proteins within the precursor is NH_2-p16-p25-p14-COOH.

The *pol* gene encodes the reverse transcriptase (RT) enzyme of which 5-10 copies are associated with the viral RNA in each virion. The MVV reverse transcriptase is Mg^{2+} dependent but has similar pH, substrate and temperature requirements to the Mn^{2+} reverse transcriptases of the type-C retroviruses (Lin and Thormar, 1970). By alignment with other retroviral *pol* sequences the conserved domains of visna virus *pol*,

corresponding to reverse transcriptase and endonuclease/integrase, are readily identified (McClure *et al.*, 1987). However, production of the predicted protease and integrase proteins have not been directly demonstrated, although cleavage of the gag-Pol precursor molecules (Vigne *et al.*, 1982) and integration of the MVV genome (Vigne *et al* 1985) suggest that both these functions may be present.

The third major structural protein of MVV has been shown to be the only glycosylated protein and is encoded by the *env* gene. These proteins are the primary targets for host immune responses and they mediate a number of important biological functions including binding to receptors and membrane fusion. The outer membrane glycoprotein is referred to as gp135 and the transmembrane glycoprotein (TM) as gp46. The outer membrane protein and the TM proteins are synthesized initially by translation of a spliced subgenomic mRNA to produce a polyprotein with the outer membrane protein as the amino-terminal component and the transmembrane as the carboxy-terminal component.

Recently a general model has been proposed for the amino-terminal segment of TM protein of retroviruses (Gallaher *et al.*, 1989). This scheme, presented in Figure 1.3, demonstrates the conserved structural features proposed for the amino-terminal end of lentiviral TM proteins.

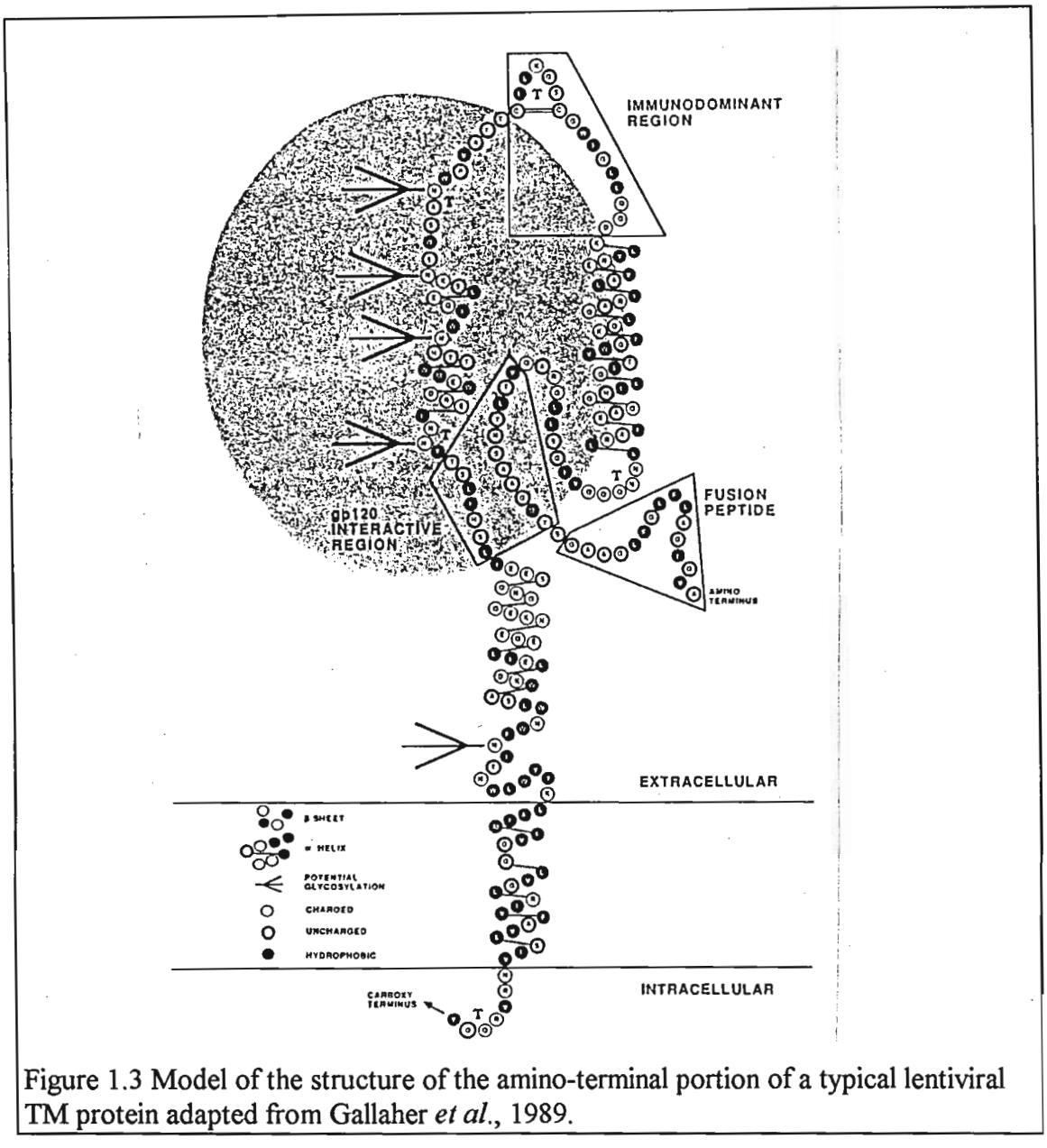

Figure 1.3 Model of the structure of the amino-terminal portion of a typical lentiviral TM protein adapted from Gallaher *et al.*, 1989.

Overall the TM protein is predicted to be a fibrous structure with a significant degree of sidedness; all of the immunodominant sites are located at the apex and on one side of the molecule and the potential N-linked glycosylation sites on the other side. Several other common features are also evident in this model. Firstly, the laterally extended

amino terminus contains the fusion peptide, which presumably acts as a membrane insertional hairpin similar to the hydrophobic core of signal sequences. This is followed by an internally looping structure rich in serine and threonine that is postulated to interact with gp135, and by an ascending amphipathic helix that is a predicted T-cell epitope. This extended helix leads to the apex of the molecule containing an immunodominant epitope that is possibly stabilized by the characteristic conserved vicinal cysteines. The apex is followed by an extended α-helix containing the potential glycosylation sites. The descending helix terminates in a bend also rich in serine and threonine and is believed to interact with gp135. This region is a highly charged region which can form an α-helix stabilized by multiple ion pairs. After a helix-bending tryptophan-rich region external to the lipid bilayer, there appears to be a hydrophobic membrane spanning α-helix that is terminated by a sharp turn just as the protein emerges from the lipid bilayer into the cell cytosol.

In infected cells, the envelope polyprotein remains membrane associated, is rapidly glycosylated, and then assembled into the plasma membrane. The outer membrane and transmembrane proteins remain associated by noncovalent interactions, eventually forming the trimer complex found in mature virus particles. The outer membrane protein is loosely bound to the cell membrane or the viral envelope and is readily shed from the surface of the cell or virus. In contrast, the transmembrane protein is bound tightly, presumably traversing the lipid bilayer one or more times. The outer membrane protein of all three visna viruses shows areas of highly conserved sequence as well as areas of high variability (Sargan *et al.*, 1991). Generally it is thought that variable residues are grouped at sites which are largely in hydrophilic and possibly external

positions in the Env protein (Hopp and Woods, 1981; Emini *et al.*, 1985) consistent with variations being fixed by immune selection. Selection acting upon antigenic variants of visna virus Env is well documented (Narayan *et al.*, 1978, 1981) and it is also indicated that the outer membrane protein of MVV is probably the major target for neutralization of virus infectivity by specific antisera (Scott *et al.*, 1979). The external part of the Env protein also contains 23 potential N-linked glycosylation sites (Querat *et al.*, 1990). Sialic acid residues have been demonstrated on the surface of MVV particles (August *et al.*, 1977), however the levels do not appear to be as high as in CAEV, and no clear functional role has been assigned to them. This is in contrast to CAEV where the residues are believed to have an important role in protection of the virus from neutralizing antibodies, probably through steric hindrance (Huso *et al.*, 1988).

Of the proteins encoded by the small ORFs, the *tat* gene encodes a protein of 10 KDa which acts as a positive transactivator of viral transcription (Davis and Clements, 1989). The *rev* gene encodes a 19 KDa polypeptide (Mazarin *et al.*, 1990) necessary for the cytoplasmic expression of the incompletely spliced *env* mRNA (Tiley *et al.*, 1990) and the *vif* gene encodes a 29 KDa protein detectable late in the lytic cycle *in vitro* (Audoly *et al.*, 1992) but it's function is still unclear.

1.4 THE MVV LIFE CYCLE

As with the other lentiviruses MVV characteristically displays very different patterns of transcription and replication *in vitro* and *in vivo.* In permissive cells *in vitro,* MVV causes extensive cytopathic effects (CPE) with formation of multinucleated giant cells

and the release of free infectious virus into the culture medium. Binding of MVV to a cellular receptor is followed by viral fusion, entry and uncoating (Figure 1.4).

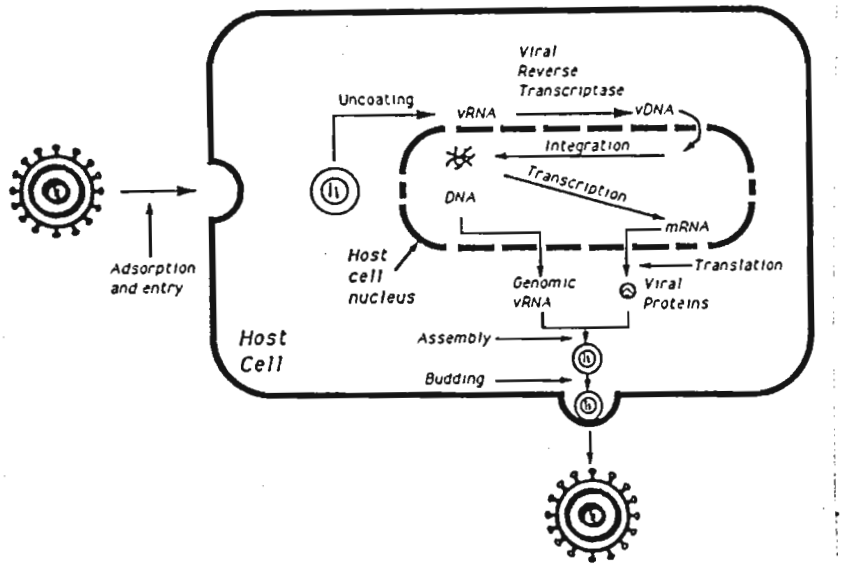

Figure 1.4 Schematic presentation of a lentivirus replication cycle (Constantine, *et al.*, 1992.

The first step in viral transcription and translation is reverse transcription of the viral single-stranded RNA (ssRNA) to form double-stranded DNA (dsDNA) (Sonigo *et al.*, 1985). The integration state of visna DNA is not clear. It has been claimed (Harris *et al.*, 1984) that there was no detectable integration of MVV DNA into host chromosomal DNA during a lytic infection. However, integration has been reported (Vigne *et al.*, 1985) when infecting ovine foetal cornea cells but not choroid plexus fibroblasts. dsDNA serves as the template for transcription of viral genes, and for the

full-length genomic RNA which is eventually packaged into virions. The small, fully spliced transcripts are believed to encode the *tat* (Gourdou *et al.*, 1989) and *rev* messages (Tiley *et al.*, 1990). In MVV infection, Rev appears to be at least dual-functional; it can increase expression of mRNAs from the LTR (Mazarin *et al.*, 1988) and acts to allow the expression of singly spliced *env* mRNAs in the cytoplasm (Tiley *et al.*, 1990), the appearance of which is a critical event in progression of a lytic infection *in vitro*. Lytic infection leads to massive amplification of viral genomes with reports of cells containing in excess of 4000 copies of viral RNA (Brahic *et al.*, 1977).

The viral life cycle *in vivo,* however, is interrupted and this has important consequences for viral persistence. Virus cannot usually be recovered from tissue homogenates from infected animals, but if these are explanted, or if the tissues are maintained in culture with a permissive cell type, then infectious virus can be detected. This is consistent with a block on viral replication *in vivo* which is relieved under tissue culture conditions. Restricted viral RNA expression has been reported in monocytes obtained from the lateral ventricles of experimentally infected sheep (Peluso *et al.*, 1985) and restricted viral RNA levels have been demonstrated in monocyte/macrophage precursor cells in the bone marrow (Gendelman *et al.*, 1985). Although much remains to be discovered about the restricted life cycle of MVV *in vivo*, it is probable that interaction of cellular factors with the viral genome, and perhaps particularly the LTR, may play a critical role in limiting viral replication under the majority of *in vivo* conditions, and in relieving this block on the viral life cycle in tissue culture or when a latently infected cell is activated.

1.5 TROPISM OF MVV

In vivo, MVV has been shown only to infect sheep and goats (Banks *et al.*, 1983). It has been suggested (Houwers *et al.*, 1989) that different sheep breeds may differ in their susceptibility to MVV but there are difficulties in distinguishing breed variations from inbred familial effects. Cells of the monocyte/macrophage lineage are the major target cell of MVV *in vivo*. Macrophages were shown to be infectable *in vitro* and macrophages from infected animals produced virus detectable by a co-cultivation assay with fibroblasts (Narayan *et al.*, 1982). Clusters of infected macrophage precursors have been found in the bone marrow, which may act as a viral reservoir (Gendelman *et al.*, 1985). Such a reservoir is essential for the continued survival of the virus within the host as blood monocytes and tissue macrophages are relatively short-lived. There are no convincing reports of MVV infection of lymphocytes.

1.6 GENETIC VARIATION

Because the reverse transcriptase is prone to make errors when viral RNA is transcribed into DNA the mutation frequency of lentiviruses is quite high. Two different isolates of the same virus are rarely if ever completely identical in genetic sequence (Woodward *et al.*, 1994). This gives rise to alterations of the structure and antigenic properties of the virus and allows it to circumvent established immune responses (Clements *et al.*, 1988). The result is antigenic variants that can elude immune surveillance and propagate even in the presence of fully competent host immune systems. Certain regions of the MVV genome are thought to be hypervariable, especially parts of the *env* gene (Narayan *et al.*, 1978). Lentivirus isolates recovered from different infected hosts may differ in overall nucleotide and deduced amino acid

sequences by as much as 15-20%. When strains of ovine lentiviruses from different parts of the world are compared, certain differences in biological characteristics are revealed. They show different growth in cell culture, some are more lytic than others (Querat *et al.*, 1984) and it has also been shown that the original infecting virus type persists in the animal in coexistence with the new variants (Lutley *et al.*, 1983). Studies showing virus mutation during the course of MVV infection have also been reported (Woodward *et al.*, 1995). No link could, however, be established between the progression of the disease and the appearance of new variants. The disease spectrum also differs. Although interstitial pneumonia appears to be a common manifestation of ovine lentivirus infection in all countries, arthritis has only been reported in the U.S.A. (Carey and Dalziel, 1993). Clinical symptoms of the central nervous system (visna) have very rarely been seen outside Iceland (Zink and Johnson, 1994). Host genetic factors have also been shown to be important in determining the outcome of MVV infection although the influence of the virus strain phenotype cannot be ruled out. Consequently, it is likely that the outcome of a lentivirus infection depends on the interaction of both host and viral genes (de la Concha-Bermejillo *et al.*, 1995). Genetic variation is a factor that has to be taken into account when selecting proteins to be used in diagnostic assays for MVV (see Chapter 2).

1.7 CHARACTERISTICS OF DISEASE

In sheep, clinical disease caused by lentiviruses generally occurs only in adult animals, although in flocks with a high incidence of infection, yearlings may occasionally be affected. The extraordinarily long incubation period for MVV, noted during initial outbreak as one to three years or more (Sigurdsson, 1954) is followed by the onset of

disease symptoms with little subsequent regression and a generally progressive clinical course. The most common manifestation of lentiviral infection in sheep, regardless of geographical location, is chronic interstitial pneumonia (Marsh, 1923), characterized by thickened, often fibrotic, interalveolar septa and dense interstitial, perivascular and peribronchial infiltrates of lymphocytes and macrophages (Cutlip *et al.*, 1985; Narayan and Clements, 1989; Bulgin, 1990). This explains the most commonly reported symptom of dyspnoea, which becomes particularly obvious if the affected animal is exercised. Post-mortem examination usually reveals lungs of two to four times the mass of normal lungs with decreased elasticity and a degree of fibrosis (Sigurdsson, 1954). A large proportion of infected sheep also develop an indurative mastitis with intense periductal and interstitial lymphocytic infiltrates (Van der Molen *et al.*, 1985). A small percentage of infected animals have arthritis, most often of the carpal joints, but occasionally involving other joints (Oliver *et al.*, 1981). Neurological disease occurs only rarely in infected sheep, although during the Icelandic epizootic it was a relatively common manifestation in some flocks (Sigurdsson and Palsson, 1958). Wasting commonly accompanies the above disease syndromes and is frequently the initial indicator of infection. Such sheep are also immunocompromised and susceptible to secondary infections (Myer *et al.*, 1988). In certain flocks, MVV is found as a co-infection with Jaagsiekte retrovirus (JSRV), the causative agent of sheep pulmonary adenomatosis (Payne *et al.*, 1986). There have been tentative suggestions of *in vivo* synergism between the two retroviruses (DeMartini *et al.*, 1987), based on accelerated development of lesions when both viruses are present.

1.8 TRANSMISSION OF DISEASE

The major route of transmission of MVV is horizontal spread via respiratory route, and is probably exacerbated by close housing practices. In flocks in which the length of exposure of lambs to infected ewes was varied, there was a rising incidence of transmission with increasing periods of contact, rising to 81% of lambs showing evidence of MVV infection when the flock was maintained intact for one year (De Boer *et al.*, 1979). Horizontal transmission between adult sheep has also been demonstrated for flocks maintained under standard management conditions (Houwers and Van der Molen, 1987). Concurrent MVV and sheep pulmonary adenomatosis (SPA) infection may lead to increased transmission of MVV infection (Dawson *et al.*, 1990), possibly as a consequence of the increased number of alveolar macrophages in SPA lungs contributing to an increase in MVV replication. There is little clear evidence for vertical transmission of MVV. Fifty lambs taken from their infected dams at birth (De Boer *et al.*, 1979) all remained uninfected and no direct evidence for transplacental transmission could be demonstrated in the experiments of Sihvonen (1980). Unlike CAEV, in which it has been clearly demonstrated that maternal colustrum is a major infectious route (Ellis *et al.*, 1983), such a mechanism has not been unequivocally demonstrated for MVV, despite the presence in ovine milk of cells from which virus can be intermittently isolated (Sihvonen, 1980). Sexual transmission of MVV has not been reported. There have also been no reports of an invertebrate vector, such as that implicated in transmission of EIAV (Narayan and Clements, 1989).

1.9 HOST IMMUNE RESPONSES TO MVV

Infection by lentiviruses results in both humoral (antibody-mediated) and cellular immune responses against viral antigens. Antibodies reacting with core, envelope, reverse transcriptase and other viral enzymatic and regulatory gene products are often found in infected hosts. Studies using experimentally infected sheep have demonstrated the generation of complement-fixing and neutralizing antibodies to MVV (Gudnadottir and Kristindottir, 1967). Although sheep develop antibodies which are described as neutralizing this is often a reflection of an *in vitro* assay technique involving prolonged prior incubation of MVV with serum and may not be an accurate representation of the *in vivo* activity of these immunoglobulins. Incubation of MVV with neutralizing antiserum for 15 minutes results in no detectable decrease in virus infectivity. A possible consequence of this *in vivo* is that the rate of virus binding to cells may be faster than the rate of neutralization (Kennedy-Stoskopf and Narayan, 1986). Neutralization *in vitro* has been shown to involve immunoglobulin of the IgG_1 subclass and to be complement-independent (Kennedy-Stoskopf and Narayan, 1986). The viral proteins against which the humoral response is mounted were determined in a longitudinal study of experimentally and naturally MVV-infected lambs. By immunoblotting it was shown that the first antibody to be generated was anti-p25 followed by anti-gp135, anti-p16 and anti-p14 (Kajikawa *et al.*, 1990), with some minor variation between sheep (Figure 1.5).

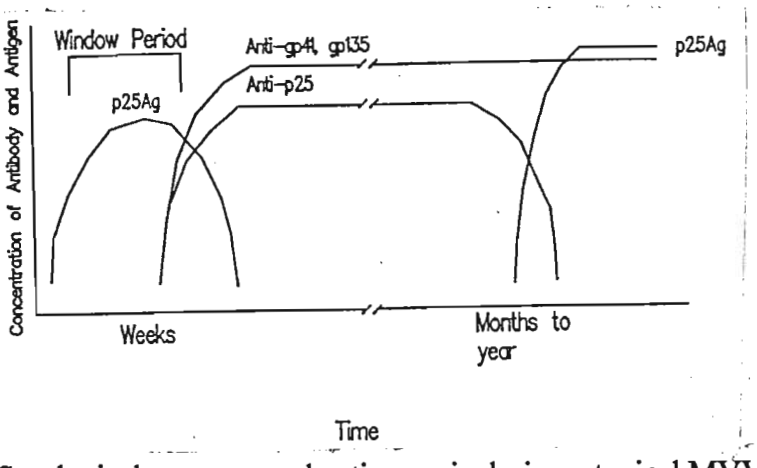

Figure 1.5 Serological response and antigenemia during a typical MVV infection (Houwers and Nauta, 1989; Kajikawa *et al.*, 1990).

Antibodies to the 11 KDa Rev protein have also been demonstrated in experimentally-infected sheep (Vigne *et al.*, 1990). However, the functional significance *in vivo* of the reactivity to each of these viral proteins is uncertain. It is unclear if antibodies to gp135 are protective, or if some may have a deleterious immunopathological effect. In CAEV, the titres of anti-gp135 in the synovia correlated directly with disease severity (Knowles *et al.*, 1990) and in studies of lambs experimentally infected with MVV strains of different *in vitro* replication characteristics, the mean antibody titre to gp135 was three times higher in lambs which developed lymphoid interstitial pneumonia (Kajikawa *et al.*, 1990). Although this could suggest a pathological role for anti-gp135 antibodies, it might also simply indicate that viruses which replicate to a higher titre *in vivo* generate more profound lesions, and coincidentally also stimulate a higher level of anti-gp135 antibodies by the presence of a greater antigenic dose.

Little data is available on the cell-mediated response to MVV infection. An early cell-mediated immune response to MVV has been demonstrated one week following experimental intracerebral inoculation (Griffin *et al.*, 1978) or four to six weeks

following respiratory tract infection (Sihvonen, 1981). This declines to control levels four to six weeks (Griffin *et al.*, 1978) or eight to twelve weeks (Sihvonen, 1981) post-inoculation and is typical of the cell-mediated response to an acute infection. The available evidence therefore suggests that MVV persists in an infected animal despite an active immune response to the virus. The immune response appears to be incapable of clearing the virus in the early stages of infection, thereby permitting establishment of infection in the host tissues, and virus continues to be produced subsequently despite the continuing specific immune response.

1.10 DIAGNOSTIC ASSAYS

Serological screening tests designed to detect lentiviral antibodies are many in number and variety, and are the most common approach for detecting infection. Since its first application by Engvall and Perlman (1971), the indirect enzyme-linked immunosorbent assay (i-ELISA) technique has been widely used for the serodiagnosis of various infectious diseases. For animal lentiviral infections, the i-ELISA has been applied to detect antibodies against caprine-arthritis encephalitis virus (CAEV) (Banks *et al.*, 1983), equine infectious anaemia virus (EIAV) (Suzuki *et al.*, 1982) and maedi-visna virus (MVV) (Houwers *et al.*, 1982; Vitu *et al.*, 1982).

As a first step in performing the technique, a suitable preparation of the viral antigen is required to prevent undesirable background which might affect the sensitivity and specificity of the test (Voller *et al.*, 1982). Houwers and Gielkens (1979) were the first to report the use of the conventional i-ELISA for MVV. Their antigen preparation required high speed centrifugation on a cushion of sucrose followed by ether

extraction. Ethyl ether is an organic solvent used to disrupt lipid viruses but is fairly ineffective in removing nonviral components (Philipson, 1967). In order to reduce the amount of cellular proteins interfering with the test, Vitu *et al.* (1982), stressed the importance of a more purified preparation of the viral antigen. They applied consecutive zonal centrifugation in density gradients such as sucrose to obtain good discrimination between positive and negative serum samples. McGrath *et al.* (1978), indicated that retroviral activity is sensitive to the osmotic shock of suspension in sucrose gradients and that the conventional pellet/banding technique had an adverse effect on the virus population. In order to reduce the problems associated with the production of MVV antigen a novel approach was taken by Simard and Briscoe (1990), by treating the virus particles with sodium dodecyl sulphate (SDS) instead of organic solvents. The SDS-treated antigen resulted in higher ELISA signals with positive serum and better discrimination between positive and negative serum samples from specific pathogen free sheep experimentally inoculated with the virus.

A variety of different ELISAs are available and are classified as either indirect, competitive, sandwich or capture assays. The most popular type of ELISA for the detection of MVV has been the i-ELISA (Figure1.6).

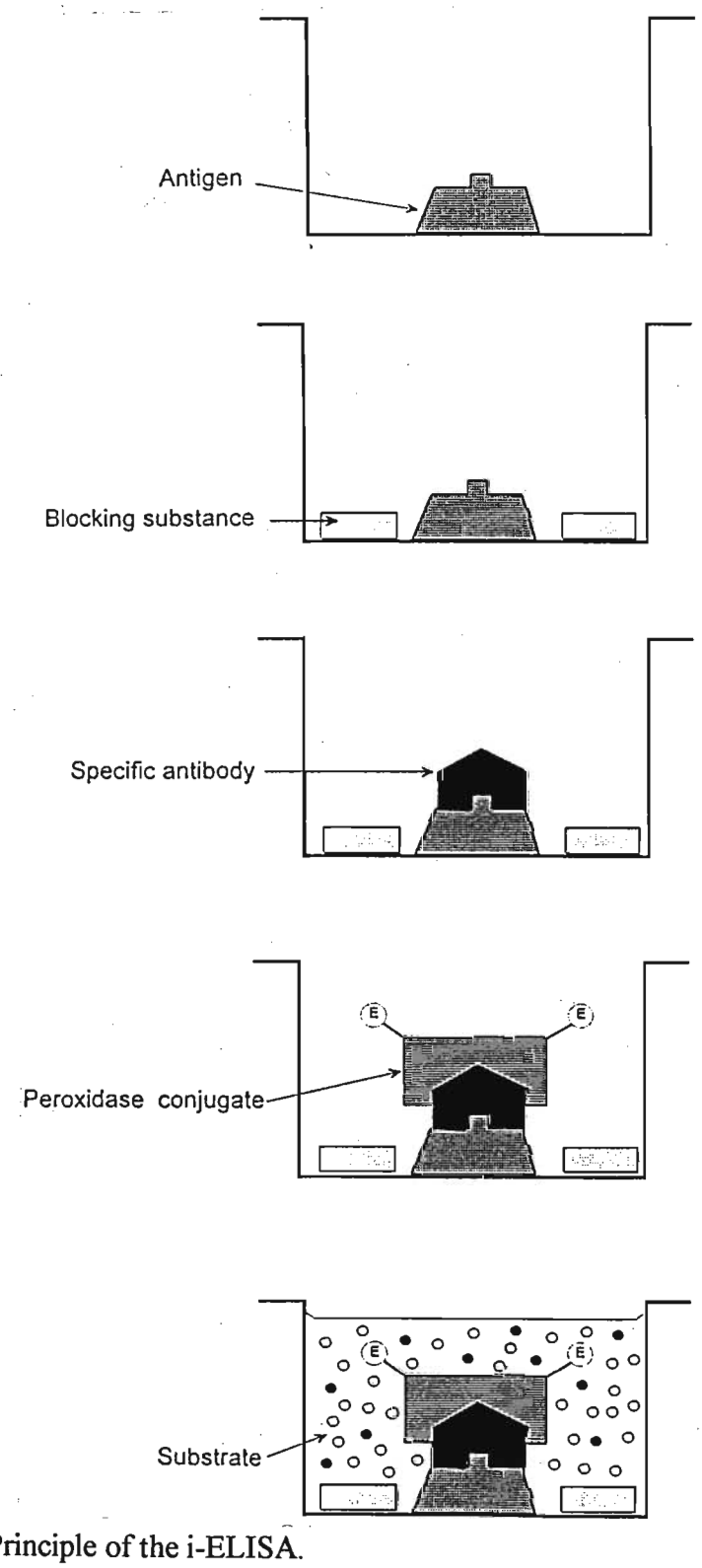

Figure 1.6 Principle of the i-ELISA.

In the i-ELISA, test serum is added to the solid phase containing the antigen and is incubated for a specified time and at a particular temperature. If anti-MVV antibodies

are present in the serum they will bind to the antigen on the solid phase. Following a wash step, conjugate (anti-sheep immunoglobulin labelled with an enzyme) is added and incubated. During this step, the anti-MVV antibody attached to the bound antigen will bind the conjugate. Another wash step removes excess conjugate, and substrate is added. During the subsequent incubation, the conjugate will modify the substrate to produce a colour. The i-ELISAs produce more colour as the unknown antibody concentration in the sample increases (Figure. 1.3). Conversely, with small amounts or an absence of antibody, smaller quantities of conjugate will bind, resulting in less substrate cleaved, less colour produced, and a lower O.D. value. Therefore, the O.D. value is directly proportional to antibody concentration in the sample.

First generation assays based on viral lysate derived antigens are good tests but generally lack specificity. This is due to the presence of contaminating components derived from the cells in which the virus was propagated. If an unknown sample contains antibodies that react with these contaminating components, a false-positive reaction may occur. In addition, viral lysate tests are usually not 100% sensitive, probably because the high sample dilution needed to attain acceptable specificity eliminates the detection of small amounts of antibody. Also, contaminating cellular substances can occupy sites on the solid phase, thereby decreasing the space that is available for optimal quantities of the MVV antigen to attach. With less antigen attached to the solid support, less antibody can be detected and the indicator signal (colour) will be low.

Second and third generation assays utilise selected antigens. These precisely targeted portions of MVV are immunodominant epitopes that have a better chance of binding significant antibodies to MVV. In most cases these antigens are so called recombinant antigens. Recombinant antigens are produced when a portion of the MVV genome, which codes for a specific protein of interest, is inserted into a plasmid vector e.g. bacterial plasmids. Expression of the viral protein is under control of the plasmid promoter sequence which, when induced, results in the production of the gene product (antigen) by the recombinant plasmid. Since these recombinant plasmid containing cells can be grown easily in culture, they produce large quantities of the antigen. In addition, these antigens have the advantage of being easier to produce and purify. This results in the production of specific antigens that can be used to develop serologic tests with a high specificity (Reyburn *et al.*, 1992; Kwang *et al.*, 1993; Keen *et al.*, 1995).

All ELISA tests are easy to perform, but require careful adherence to procedures. Any deviation in incubation times, temperatures or volumes can result in dramatic changes in test results. Wash procedures must be thorough, and reagents must be prepared exactly in the same way each time. These prerequisites, therefore, confine ELISA type assays to a laboratory setting.

1.11 RED CELL AGGLUTINATION AS AN IMMUNOASSAY SYSTEM

All microparticulate antigens (e.g. bacteria, yeast, leukocytes and erythrocytes) may be agglutinated by antibodies to antigenic determinants on their surface and this phenomenon has been exploited widely in the study of cell and micro-organism surface antigens and in the detection of antibodies. Tests employing agglutination as the

indicator system have been used for diagnosing infectious diseases for many years because they are generally very sensitive. However, specificity is sometimes compromised. The ability of red blood cells (RBCs) to be agglutinated, or cross-linked, has been used to monitor antigen-antibody interactions (reviewed by Coombs 1981, 1987, 1989). When antigens are bound without specific attachment (passively adsorbed onto carriers) the technique is referred to as passive agglutination. If RBCs are used as the carrier system, the assay is called a passive haemagglutination assay. During the agglutination reaction, a lattice network is formed between the antigen-coated particles and the antibody, as antibody in the sample reacts in the system.

This reaction brings about the clumping (agglutination) of particles (Figure 1.7). The agglutination is visible with the naked eye.

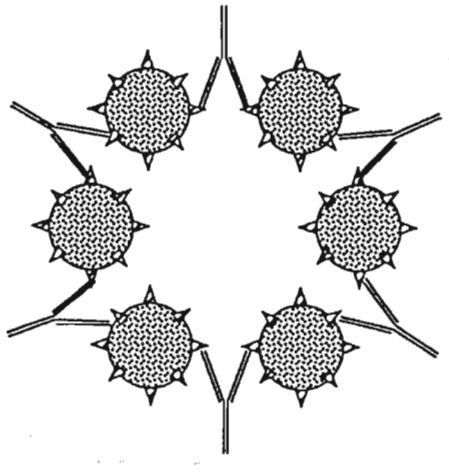

Figure 1.7 Formation of the lattice network seen as visible agglutination.

Protein antigens can be adsorbed onto red cells by prior treatment of the cells with tannic acid (Boyden, 1951). One recent example was a test for HIV-1 antibodies which

used sheep red cells coated with a recombinant antigen of the virus (Vasudevachari *et al.*, 1989). Cross-linking reagents such as bis-diazotized-benzidine (BDB) (Stavitsky and Arquilla, 1958) have also been used with success.

One possible problem with agglutination assays is the phenomenon of prozone reactions. Prozone refers to the inhibition of agglutination when excess antibody is present, thereby preventing the optimal combination of antibody and antigen. In this case, the lattice network may not be sufficient to produce a visible agglutination; the high concentration of antibody binds to antigenic sites in such a manner that cross-linking of the complexes cannot occur (Figure 1.8).

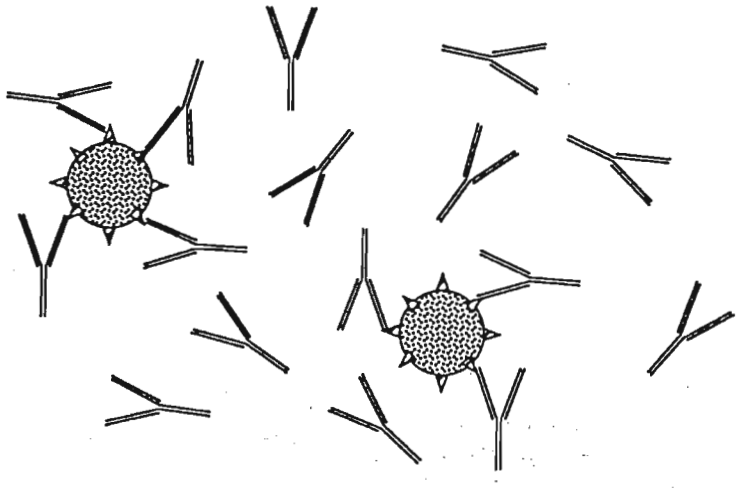

Figure 1.8 Absence of agglutination due to prozone (antibody excess).

This may result in a false-negative reaction (no agglutination) when higher concentrations of reacting antibody are present in the serum. In the case of antibody excess and the occurrence of the prozone phenomenon, antibody in the sample can be detected if the sample is diluted and retested. Dilution of the serum decreases the

concentration of antibody to a point where the optimal concentration of antigen and antibody occurs, and agglutination proceeds.

Another disadvantage is that coupled red cells are unstable and it is usually necessary to prepare fresh red cell labelled antigen/antibody reagent after 2-3 weeks storage at 4°C (Siddle *et al.*, 1984). To increase red cell stability, further chemical treatment with glutaraldehyde is often required (Cranage *et al.*, 1983a). Such treatment can alter cell membrane fluidity and thus decrease surface contact between erythrocytes which results in weaker agglutination. A sample pre-treatment step is also often required to reduce non-specific reactions. If red cells from a different species to that of the sample are used, the sample can be pre-adsorbed with uncoupled erythrocytes to remove sample constituents which may cause non-specific agglutination (Cranage *et al.*, 1983b).

Agglutination assays are easy to perform and require no wash procedures. The sole requirement is an initial dilution of serum, addition of coated red cells, mixing and incubation, usually at room temperature. However, the problems as mentioned above, have limited its widespread use.

1.12 ENDOGENOUS ERYTHROCYTE AGGLUTINATION ASSAYS

1.12.1 Principle

Rapid diagnostic assays were traditionally based on particle agglutination (Francis *et al.*, 1988), haemagglutination (Welsh *et al.*, 1987) and immunoblotting (Santos *et al.*, 1987). All these assays were comparable in terms of their sensitivity with enzyme immunoassays (EIA) but required the use of serum rather than whole blood. The quest for simpler methods led to the development of an autologous whole blood agglutination assay as reported by Hillyard *et al.*, in a patent application in 1988 and published later (Hillyard *et al.*, 1990). This patent described the use of a non-agglutinating anti-red cell monoclonal antibody raised against human glycophorin which is present on essentially all human erythrocytes (Rylatt *et al.*, 1990). The principle feature of the whole blood immunoassay system lies in a bispecific reagent consisting of a monoclonal antibody against red cells chemically coupled to an antigen (Figure 1.9). Upon addition to whole blood from the individual being tested, the bispecific reagent effectively coats the erythrocytes with antigen. In the presence of specifically reacting antibodies to the coupled antigen, cross-linking of the erythrocytes occurs which is seen as visible agglutination.

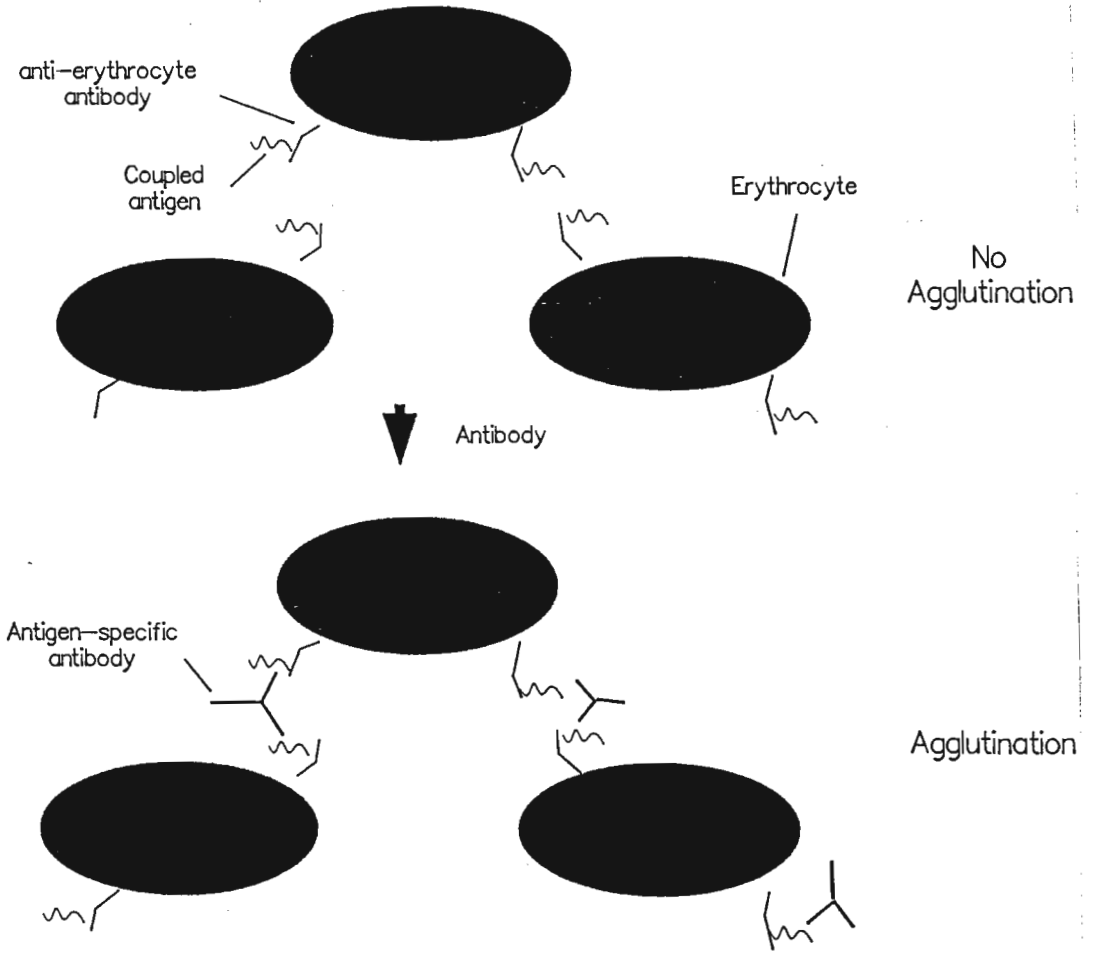

Figure 1.9 Principle of the autologous RBC agglutination assay.

Such an assay system was used for the detection of antibodies to the human immunodeficiency virus, HIV-1 (Kemp *et al.*, 1988), where the conjugate consisted of a peptide, representing an immunodominant portion of the virus, coupled to the intact

anti-red cell monoclonal antibody. The test detected 42 out of 43 seropositive patients and gave one false positive response out of 874 normal blood donors.

1.12.2 Advantages over other immunoassays

The endogenous erythrocyte test format has three major advantages.

1. The test is carried out using whole blood with minimal sample handling.
2. Performance of the assay requires no special training or equipment.
3. Results can be obtained within two minutes.

Most immunoassays are characterized by the necessity to separate red cells from serum or plasma. This usually requires the use of a centrifuge restricting the use of the test in the field. Thus the applicability of the autologous red blood cell agglutination assay in the field may be considered one of its main advantages. Furthermore, the simplicity of the assay - no sample pre-dilution or washing steps are necessary - means that highly trained staff are not required to perform the test.

1.13 THE PREPARATION OF ANTIBODY CONJUGATES

1.13.1 Monoclonal antibodies

The advent of monoclonal antibodies (Köhler and Milstein, 1975) opened opportunities for new assay configurations. This technique allowed for the growth of clonal cell populations secreting antibodies of defined specificity. The splenocytes of the immunized animal are allowed to fuse with immortal myeloma cells (Figure 1.10). These myeloma cells are derived from a mutant cell line of tumour B-lymphocytes unable to produce immunoglobulins. The resultant hybrid cells, or hybridomas, are

maintained *in vitro* and will continuously secrete monoclonal antibodies with a defined specificity. The different stages of hybridoma production are illustrated in Figure 1.10.

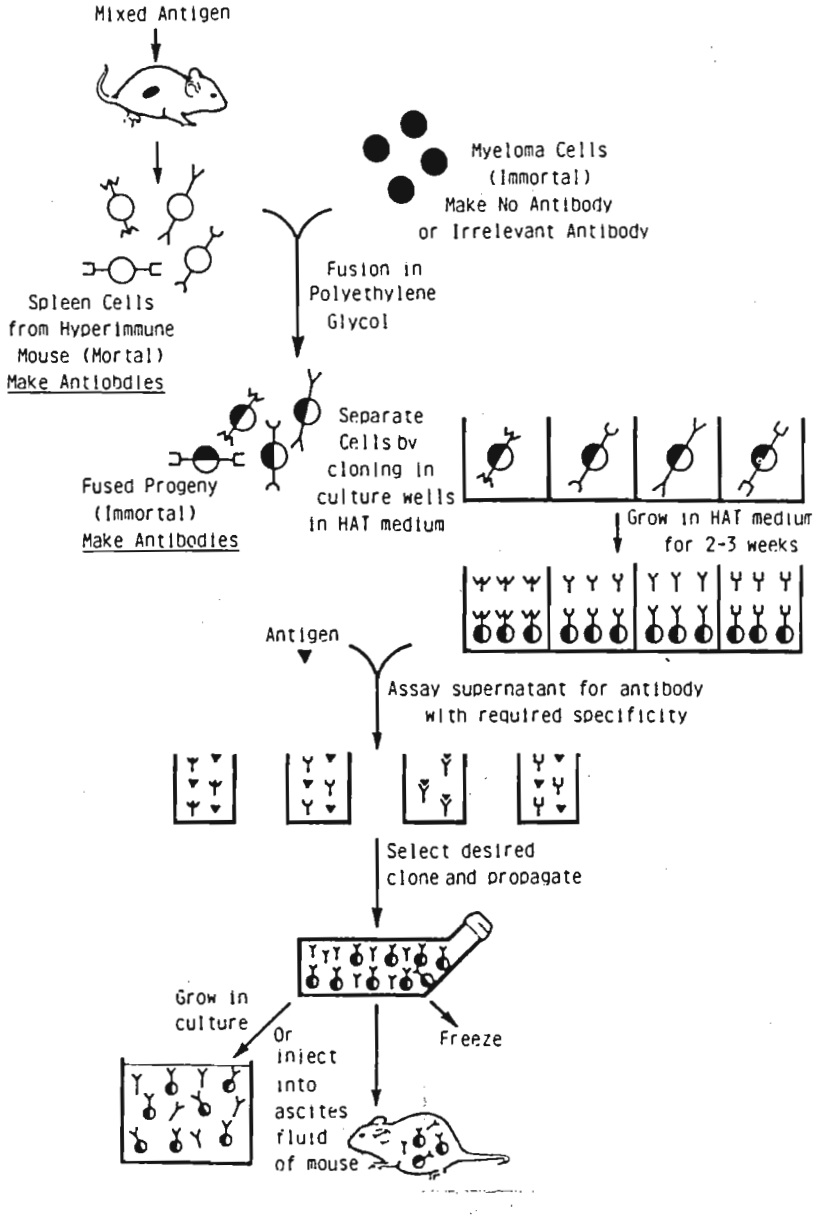

Figure 1.10 The general procedure of monoclonal antibody production in mice (Campbell, 1987).

The immortal nature of the myeloma cells gives hybrid cells the ability to multiply indefinitely in culture. The hybridomas are grown on a selective growth medium

known as hypoxanthine-aminopterin-thymidine (HAT) medium. Aminopterin is an inhibitor that blocks the normal biosynthetic pathways by which nucleotides are made. The myeloma cells used for hybridization lack a purine salvage enzyme, hypoxanthine phosphoribosyl transferase (HPRT). The absence of HPRT results in a defect of the alternate pathway to synthesize nucleic acids, so that the cells die in HAT medium. The fused cells survive in HAT medium, whereas the unfused spleen and myeloma cells die in HAT medium. Therefore, the B-lymphocytes provide the HPRT enzyme for metabolic bypass, and the myeloma cells the ability to grow indefinitely in culture (Campbell, 1987).

1.13.2 Structure and properties of an immunoglobulin molecule

Methods used in the construction of antibody conjugates, and their applications, have been greatly expanded by the availability of highly specific and purified monoclonal antibodies. Underlying all this is understanding and utilising the different properties of the antibody molecule. The general structure of antibodies was elucidated by several key studies (Porter, 1959; Edelman *et al.*, 1961, 1962, 1963, 1968). Immunoglobulin molecules are glycoproteins, each containing four polypeptide chains, two identical heavy chains (H), and two identical light chains (L) (Figure 1.11).

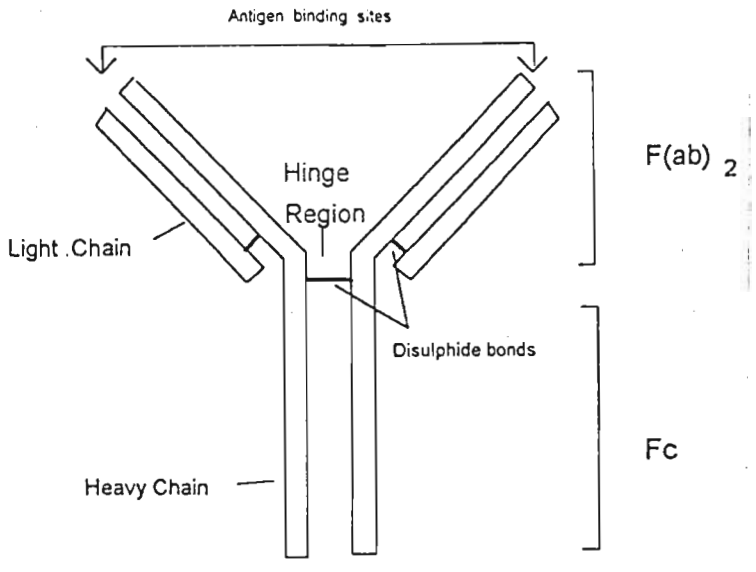

Figure 1.11 The immunoglobulin class G molecule

The amino terminal ends of each polypeptide chain show considerable variation in their amino acid composition and are referred to as the variable (V) regions to distinguish them from the relatively constant (C) regions. The chains are held together by a combination of noncovalent interactions and covalent interchain disulphide bonds, forming a bilateral symmetric structure. The V regions of the heavy (H) and light (L) chains comprise the antigen-binding sites of the immunoglobulin (Ig) molecules. Each Ig monomer contains two antigen-binding sites and is therefore bivalent. The hinge region is the area of the H chains which is held together by disulphide bonds. This flexible hinge region allows the distance between the two antigen-binding sites to vary. The portion of the antibody below the hinge region is known as the Fc region and is responsible for the effector functions (e.g. complement fixation) of the antibody molecule. The five primary classes of immunoglobulins are IgG, IgM, IgD, IgA and IgE and are distinguished by differences in the heavy chain. IgG, a monomer, is the

predominant Ig class present in human serum, and it is the antibody produced in the secondary immune response to an antigen. The subclass of an Ig molecule is also determined by its heavy chain type. The constant region of the heavy chain of each IgG subclass is an isotype that is present in all members of a particular species. Mouse derived monoclonal antibodies can be distinguished as four subclasses of IgG: IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3. Because of its relative abundance and excellent specificity toward antigens, IgG is the preferred antibody class used in immunological research and clinical diagnostics.

The immunoglobulin molecule can be selectively cleaved into fragments, each having discrete activities, by the enzymes papain and pepsin (Parham *et al.*, 1982; Mage and Lamoyi, 1987; Beale, 1987) (Figure 1.11). Papain is a non-specific, thiol-endopeptidase that has a sulfhydryl group in the active site, which must be in the reduced form for activity. When IgG molecules are incubated with papain in the presence of a reducing agent, one or more peptide bonds in the hinge region are cleaved, producing three fragments of similar size: two Fab' fragments and one Fc fragment (Coulter and Harris, 1983). Papain is primarily used to generate Fab' fragments, but it can also be used to generate $F(ab)_2$ fragments (Goding, 1983). To prepare $F(ab)_2$ fragments, the papain is first activated with cysteine. The excess cysteine is then removed by gel filtration. If no cysteine is present during papain digestion, $F(ab)_2$ fragments can be generated. Pepsin, on the other hand, is a non-specific endopeptidase that is active only at acid pH. It is irreversibly denatured at neutral or alkaline pH. Digestion by the enzyme pepsin normally produces one $F(ab)_2$

fragment and numerous small peptides of the Fc portion (Parham *et al.*, 1982). The resulting $F(ab)_2$ fragment is thus composed of two disulphide-connected Fab' units.

1.13.3 Intact antibody-protein conjugates

In general, intact antibody can be used to produce active conjugates provided the appropriate cross-linking reagents and reaction conditions are chosen. However, the use of intact antibody also has some drawbacks. One of the main disadvantages associated with cross-linking whole antibodies is inactivation of the antigen binding portion of the antibody by random coupling. Secondly, the hydrophobicity of the Fc region is undesirable in immunoassay systems as it is commonly implicated in non-specific binding (Gosling, 1990). It is for these reasons that many conjugation methods now employ antibody Fab' fragments, where the hinge sulfhydryls are an ideal coupling target distal to the antigen binding region. Also, the Fc region is removed prior to coupling with this reaction strategy.

1.13.4 Antibody fragment-protein conjugates

In order to produce active bispecific antibody a region specific coupling method which allows binding distal to the Fab' region is necessary. Brennan and colleagues (1985) reported a method which fulfilled these requirements. The method involved the use of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), a chemical compound which reacts quantitatively with free thiols to produce the 2-nitro-5-thiobenzoate (TNB) anion, which has a characteristic orange colour (Ellman, 1959), and TNB coupled to the sulfhydryl (Figure 1.12).

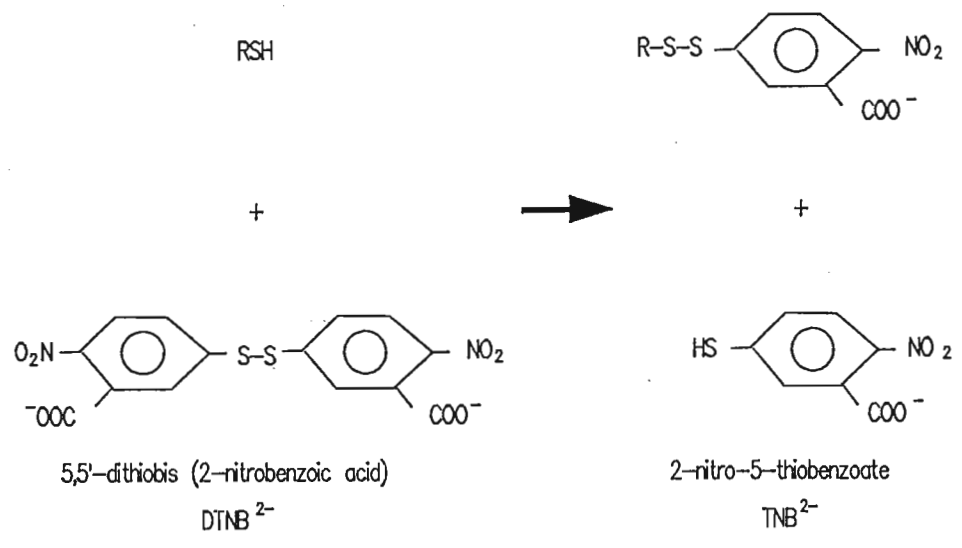

Figure 1.12 The reaction of DTNB with thiol groups. One mole of free thiol produces one mole of TNB^{2-}.

A mouse monoclonal antibody directed against a red blood cell epitope can be digested to produce $F(ab)_2$ fragments. The $F(ab)_2$ inter-heavy chain disulphides are reduced with 2-mercaptoethylamine (Figure 1.13), in the presence of EDTA to prevent heavy metal catalyzed disulphide formation, which would result in self-dimerisation of Fab' fragments.

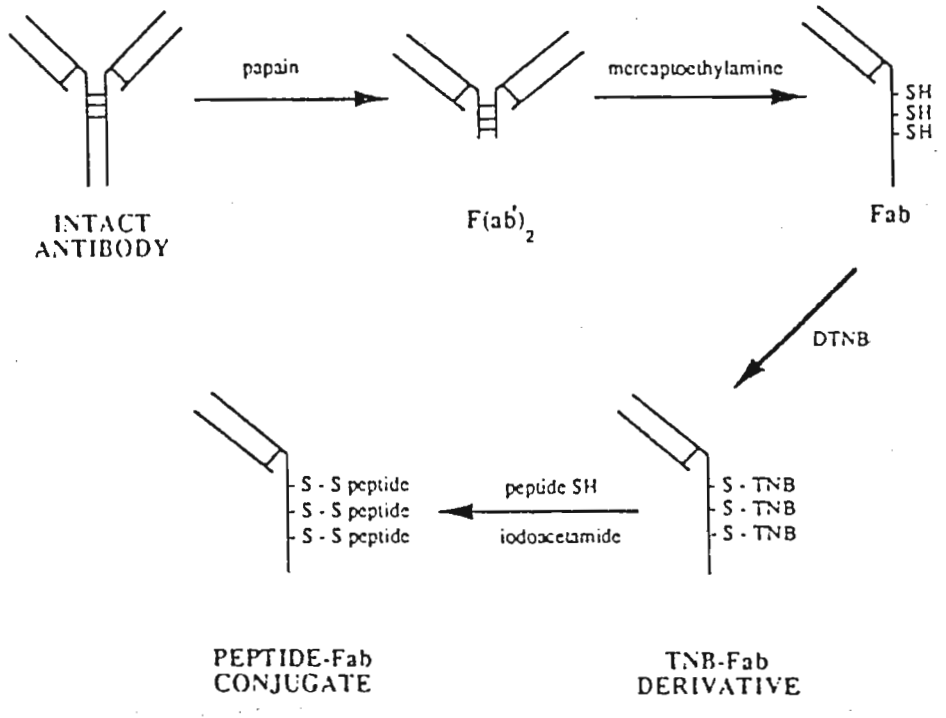

Figure 1.13 Flow diagram for the preparation of Fab'-peptide conjugate. Intact anti-sheep erythrocyte antibody is digested with papain to produce $F(ab)_2$ fragments, which are subsequently reduced with 2-mercaptoethylamine to Fab' fragments. The Fab' fragments are then stabilised with DTNB and the TNB substituted with reduced synthetic peptide. Any remaining free sulfhydryls were alkylated with iodoacetamide resulting in a stable Fab'-peptide conjugate.

Three TNB per Fab' are produced, corresponding to the three thiols in the hinge region of IgG_1. The resulting molecule, Fab'-TNB, is stable and can subsequently be allowed to react with an immunodominant peptide containing free reduced sulfhydryls (Figure 1.13). The reaction is stopped by alkylating the free sulfhydryls before purifying the Fab'-peptide conjugate.

1.14 AIMS OF THIS STUDY

The broad aim of this study was to clone and express antigenic epitopes from Maedi-Visna virus (MVV) and investigate their use in immunodiagnostic assays. The successful development of sensitive diagnostic methods for human AIDS lentiviruses stimulated the search for a reliable, sensitive and accountable ELISA for MVV. Thus, the initial step was to express such selected antigenic regions of MVV in a prokaryotic expression system. The diagnostic performance of each recombinant antigen was then evaluated by comparison with pathological evidence (Chapter 2).

Although prokaryotic expression systems are known to be versatile vehicles for the cloning and expression of foreign DNA, certain limitations do exist. In the past few years, many of these limitations were addressed by the construction of various eukaryotic gene expression systems. The methylotrophic yeast, *Pichia pastoris,* is known for high-level gene expression and its potential use as an alternative expression system for MVV epitopes, was investigated (Chapter 3).

The urgent need for simpler test methods has been highlighted by reports describing the development of rapid serological assays. Such an assay, performed on whole blood samples with a visible result in two minutes, was reported recently. This assay system has led to the development of diagnostic tests for a variety of diseases of both medical and veterinary importance. The use of such a whole blood diagnostic assay for the detection of MVV infection, was investigated in the present study (Chapter 4).

CHAPTER 2

CLONING AND EXPRESSION OF A SINGLE FUSION TRANSMEMBRANE-CORE p25 RECOMBINANT PROTEIN FOR USE IN A MVV ELISA

2.1 INTRODUCTION

Since MVV poses a world-wide threat to commercial sheep farming (Pritchard and Dawson, 1987; Cutlip *et al.*, 1992), various techniques have been developed for its serologic diagnosis. These include agar gel immunodiffusion (AGID) (Cutlip *et al.*, 1977), indirect enzyme-linked immunosorbent assay (i-ELISA) (Houwers *et al.*, 1982), ELISA based on a double antibody sandwich blocking procedure (Houwers and Schaake, 1987) and Western blot analysis (Houwers and Nauta, 1989).

Most current MVV antibody tests are based on an EIA format, with screening tests usually performed in a 96-well EIA plate. Recombinant viral proteins have proved to be of great value in the serodiagnosis of MVV. Studies using either the core p25 or TM proteins have been used in screening assays (Reyburn *et al.*, 1992; Kwang *et al.*, 1993; Keene *et al.*, 1995). However, these studies have shown that both the core p25 and TM epitopes are necessary to produce a sensitive and specific screening assay. This is due to the fact that the immune response tends to shift during the course of the disease from initially being p25 specific to being predominantly TM specific towards the end of the disease (Houwers and Nauta, 1989; Kajikawa *et al.*, 1990) (see Figure 1.5).

In this chapter, the following are described; i) cloning of selected fragments of MVV p25 and TM proteins in the same expression cassette, ii) expression of these two

distinct epitopes as a single fusion protein in *Escherichia coli*, and, iii) purification and use of this fusion protein in an MVV antibody detection ELISA. The cut-off value of this assay was determined using a two-graph receiver operating characteristic method (TG-ROC) recently reported by Greiner *et al.* (1995). The optimised assays using the GST-TM, GST-TM-p25 and a commercial assay based on the p25 protein were tested against 46 MVV positive and 46 MVV negative sheep sera.

2.2 MATERIALS AND METHODS

2.2.1 Polymerase Chain Reaction (PCR)

The genes coding for the TM and p25 proteins of MVV (obtained from a clone of SA-OMVV provided by R. Vigne, Marseilles, France) were amplified as a 273-bp TM and 748-bp p25 DNA fragment by PCR (Boshoff *et al.*, 1996a). The 5' primers were designed to amplify the TM and p25 regions so that they respectively contained *BamH1* and *EcoR1* restriction sites to facilitate cloning of the amplicons (Table 2.1). The sequences of the 3' primers are also given in Table 2.1.

Table 2.1
The nucleotide sequences and genomic locations of PCR primer pairs used to PCR amplify the MVV p25 and transmembrane (TM) genes.

Primer	Sequence (5' - 3')	Gene and location*
p25 5' primer	TCC CCC GAA TTC GTA TGA AAG AAG GGC TAC AC	*gag* (737-756)
p25 3' primer	TCC CCC GAA TTC TCC TTC TGA TCC TAC GTC	*gag* (1485-1468)
TM 5' primer	TCC CCC GGA TCC GAA GCT ATA GTC GAT AGA	*env* (8084-8101)
TM 3' primer	TCC CCC GGA TCC TGC CTC GTG TTG CTC TAT	*env* (8357-8340)

* See Querat *et al.* 1990.

The PCR mix contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl, 0.01% gelatin, 200 µM of each dNTP, 40 pmol of each of the TM and p25 primers and 20 ng of template DNA in a final volume of 100µl. The mixture was heated to 94°C for 5 min in a Perkin-Elmer 9600 thermocycler, cooled on ice for a further 5 min and 2.5 U of Taq DNA polymerase (Promega, Madison, WI) was added. The samples were amplified for 25 cycles. Each cycle consisted of a denaturation step at 94°C for 30 sec followed by annealing at 55°C for 30 sec and extension at 72°C for 30 sec. The final extension step was at 72°C for 5 min to allow complete extension of all DNA products. Verification of the correct size of each amplicon was established by running

each completed PCR reaction product through 2% (w/v) agarose (Sigma) containing 0.005% ethidium bromide in TAE-buffer

(40 mM Tris, 20 mM acetic acid and 50 mM EDTA, pH 7.2) as compared to a standard DNA marker VIII (Boehringer Mannheim) and visualised by using ultraviolet transillumination (Sambrook *et al.*, 1990). These PCR products were purified (Spin kit, Qiagen, Chatsworth, CA, U.S.A.) and subsequently digested with either *BamH1* or *EcoR1* restriction enzymes.

2.2.2 Restriction digestion and dephosphorylation of pGEX vector DNA

The pGEX vector DNA (Pharmacia, Uppsala, Sweden) was linearized by digesting maximally 5 μg of DNA in a total volume of 50 μl with 20 units of the appropriate restriction enzyme for 5 h at 37°C. The One-Phor-All buffer PLUS™ (OPA) from Pharmacia was used since it is compatible with virtually all restriction enzymes and many modifying enzymes. (See Appendix 1 for the composition of OPA.) A small aliquot of the reaction mixture was examined by agarose gel electrophoresis as described above, to ensure that the pGEX DNA had been digested to completion. Since the pGEX DNA was digested with a single restriction enzyme, the 5' phosphate groups had to be removed prior to ligation, to prevent recircularization of the vector. Calf intestinal alkaline phosphatase (Pharmacia) was diluted in OPA buffer before a total of 0.1 units (1-2 μl of diluted enzyme) was added to the digested pGEX vector DNA. Dephosphorylation took place for 30 min at 37°C before heat-inactivation at 85°C for 15 min. The vector DNA was subsequently purified by ethanol precipitation. Briefly, an equal volume of phenol-chloroform-isopropanol 25:24:1 (v/v) (Sigma) was added to the aqueous sample, mixed by vortexing for 1 min and then centrifuged at 12000 x g for 5 min to separate the phases. The upper aqueous phase was

subsequently transferred to a clean tube to which an equal volume of chloroform-isopropanol 24:1 (v/v) was added. Mixing and centrifugation was repeated as described above to separate the phases. The upper aqueous phase was then transferred to a clean tube to which 0.1 volume of 3 M sodium acetate (pH 5.4) and 2.5 volumes of absolute ethanol (Merck) was added. The solution was carefully mixed and kept at -20°C for 15 min. The vector DNA was pelleted by centrifugation at 4°C for 15 min at 12000 x g before being washed with 1 ml 70% ethanol. The subsequent DNA pellet was allowed to air dry at 37°C before being dissolved in 20 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The pGEX DNA was stored at -20°C for later use.

2.2.3 Ligation of insert DNA to pGEX DNA

The linearized pGEX vector DNA and insert DNA were mixed at a vector to insert molar ratio of 1:5. The molar number of ends of linear DNA were calculated using the following formula where one O.D. unit at 260 nm was equivalent to 50 µg/ml linear double-stranded pGEX vector DNA consisting of 4900 base pairs.

moles of ends = 2 x (g of DNA) ÷ [(number of bp) x (649 Daltons/bp)]

The final ligation reaction mix consisted of not more than 200 ng of DNA and contained 1 mM ATP and 5 units of FPLC*pure*™ T4 DNA ligase (Pharmacia). Incubation was overnight at 16°C. The reaction was terminated by heating the ligation mixture at 65°C for 10 min. Ligated DNA was used to transform competent cells.

2.2.4 Transformation of competent cells with pGEX DNA

The protocol used was based on the procedure described by Hanahan (1985). The composition of buffers and media used are described in Appendix 1. A single colony of the *Escherichia coli* JM105 strain was picked from a SOB agar plate and incubated in 10 ml of SOB medium at 37°C until the cells reached mid-logarithmic growth phase (A_{660} = 0.35-0.40). The cells were harvested by centrifugation (1000 x g, 15 min, 4°C) and resuspended in standard transformation buffer (TFB)

(see appendix 1), using 1/3 of the original cell culture volume. The cell culture was vortexed and incubated on ice for 15 min. The cells were centrifuged, as described above, before being resuspended in TFB using 1/25 of the original cell culture volume. This concentration gives a standard unit transformation volume. The competent *E. coli* JM105 cells (100 µl) were aliquoted in polypropylene tubes. For transformation, pGEX DNA in maximally 20 µl (10 - 50 ng) was added to the competent cells and incubated on ice for 30 min. The cells were heat-shocked by placing the tubes in a 42°C water bath for 45 sec before being chilled by returning the tubes immediately to ice. To this, SOC medium (800 µl) was added and incubated at 37°C for 60 min with moderate agitation. Incubated cells were spread onto 2 x YT-G agar plates (Appendix 1), containing 100 µg/ml ampicillin and incubated overnight at 37°C.

2.2.5 Small-scale isolation of pGEX DNA

The alkaline lysis plasmid mini-prep method allows for the rapid isolation of small amounts of plasmid DNA without the need for column purification or banding in CsCl gradients (Krieg and Melton, 1984). The procedure takes advantage of the rapid alkaline denaturation of plasmid and chromosomal DNA and the selective renaturation

of plasmid DNA following neutralization of the solution. Colonies appearing after incubation as described above, were picked and grown overnight in 1.5 ml 2 x YT-G medium containing 100 µg/ml ampicillin. Cells were harvested by centrifugation (12000 x g, 15 min, 4°C) and resuspended in 200 µl solution I (Appendix 1) by vortexing vigorously. Solution II (Appendix 1) (200 µl) was added and mixed by inverting the tube several times. The solution was incubated for 5 min at room temperature. Solution III (Appendix 1) (200 µl) was added, mixed by inverting the tube several times. The solution was incubated on ice for 5 min, centrifuged (12000 x g, 5 min, 4°C) and the DNA containing supernatant transferred to a clean tube. Ambient-temperature isopropanol (0.7 volume) was added and carefully but thoroughly mixed by inverting the tube. The plasmid DNA was pelleted by centrifugation (12000 x g, 10 min, RT), the supernatant decanted and the DNA resuspended in 200 µl TE buffer. Using this protocol, 1-4 µg of plasmid DNA can be obtained from 1.5 ml of culture. To verify the presence of the correct sized insert, 2 µl (± 200 ng DNA) was digested with the appropriate restriction enzyme (10 units) for 1 h at 37°C and analysed by agarose gel electrophoresis as described in Section 2.2.1.

2.2.6 Sequencing of cloned fragments

The plasmid containing the cloned fragments, pGEX-MVV/TM-p25, was purified using the Qiagen midi prep method, concentrated using a Microcon-30 filter (Amicon, Beverly, U.S.A.), and sequenced using an autoread sequencing kit (Pharmacia Biotech) with a few modifications. One microgram per kilobase of plasmid/insert DNA was sequenced using the pGEX 5' and 3' sequencing primers (2 pmol) and a Fluoro-dATP mix (Pharmacia Biotech). Sequencing was performed using the Automated Laser

Fluorescent (ALF) DNA sequencer. Sequencing data was analyzed using the DNASIS software (Hitachi Software Engineering, U.S.A.) and Genbank data bank sequences. Internal primers were used to confirm the sequence in both directions.

2.2.7 Expression and purification of fusion proteins

All recombinant proteins were expressed in *E. coli* JM105 as a fusion protein linked to glutathione S-transferase (GST) (Smith and Johnson, 1988). Expression and purification of the recombinant proteins was essentially as described by Grieco *et al.* (1992). Briefly, an overnight culture of transformed *E. coli* was diluted 1:10 in Luria-broth containing 50 μg/ml ampicillin and incubated at 37°C until the culture reached an $O.D._{600}$ of 0.6-0.8. Isopropyl β-D thiogalactoside (IPTG) (Sigma) was added to a final concentration of 0.1 mM to induce the expression of the recombinant proteins. The cultures were incubated at 20°C for 3 h, centrifuged (5000 x g, 10 min, 4°C) the supernatant discarded, and the pellet resuspended in 1/20 the original culture volume in PBS (0.15 M NaCl, 0.05 M phosphate) containing 0.2% N-lauroylsarcosine (Sigma), 2 mM EDTA and 1 mM triethanolamine. The solution was freeze-thawed twice and sonicated (Virtis instrument) on ice for five 10 s, 140 Watt, pulses with 1 min intervals. Triton X-100 was added to a final concentration of 1% before centrifugation (10 000 x g, 30 min, 4°C). The supernatant was added to a 50% preswollen slurry of glutathione agarose beads (Sigma). The suspension was gently agitated for 3 min and washed three times with 50 ml ice-cold PBS. The fusion proteins were eluted with 1 ml elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione) and gentle mixing for 2 min. The elution step was repeated a total of 4 times and the eluants pooled.

2.2.8 Electrophoresis of fusion proteins

The purity and relative molecular size of the recombinant proteins were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). This one-dimensional electrophoretic analysis was carried out in a 0.1% SDS-containing 12.5% polyacrylamide gel with a 5% stacking gel in a Bio-Rad slab-gel apparatus. The affinity purified proteins were suspended in an equal volume of 0.1 M Tris-HCl buffer, pH 6.8, containing 2% (w/v) SDS, 20% (w/v) sucrose, 1% (v/v) 2-mercaptoethanol, and 0.001% (w/v) bromophenol blue and heated in a 100°C water bath for 5 min. Samples (20 µl) were applied to the sample wells and subjected to electrophoresis at 50 mA/gel until the bromophenol blue migrated to 2 cm from the bottom of the gel. Proteins were stained overnight with 0.2% Coomassie Blue, in 45% methanol and 10% acetic acid. The gel was subsequently washed in a destaining solution consisting of methanol, acetic acid and water in a ratio of 5:1:5.

2.2.9 Western blot analysis of fusion proteins

The separated fusion proteins were electrophoretically transferred from the gels onto nitrocellulose membranes (Schleicher and Schuell, Keene, U.K.) as described previously by Zanoni *et al.* (1989). The transfer was performed at 100 V (constant voltage) for 1 h using a trans-blot electrophoretic transfer cell (Bio-Rad). The blotting buffer consisted of 20 mM Tris-HCl (pH 8.3), 150 mM glycine and 20% (v/v) methanol. After blotting, the membranes were equilibrated in TBS [50 mM Tris-HCl, 150 mM NaCl, pH 8.0] for 10 min and blocked for 1 h in 5% (w/v) skimmed milk in Tris-buffered saline containing 0.05% (v/v) Tween 20. The membrane was washed in Tris-buffered saline before being incubated with a hyperimmune MVV positive serum

diluted 1/1000 in Tris-buffered saline containing 0.05% (v/v) Tween 20 for 4 h on a rocking platform at room temperature. The membrane was washed (3 x 10 min) with TBS, and incubated for 4 h with protein G peroxidase conjugate (Zymed, CA, U.S.A.) diluted 1/2000 in Tris-buffered saline containing 0.05% (v/v) Tween 20 at room temperature. The membrane was washed (3 x 10 min) in TBS and developed in a freshly prepared developing solution of 60 mg 4-chloro-1-naphtol (Bio-Rad) in 20 ml methanol to which 100 ml Tris-buffered saline containing 60 µl of 30% hydrogen peroxide was added.

2.2.10 Detection of antibodies

The ELISA procedures were essentially as described by Kwang and workers (1993, 1995). Purified recombinant GST-TM and GST-TM-P25 fusion proteins were diluted 1 in 200 in 0.1 M sodium bicarbonate buffer, pH 9.6, and passively adsorbed onto the surface of 96-well microtitre plates (Greiner, Germany) (Wade-Evans *et al.*, 1993). Following an overnight incubation, at room temperature, the plates were washed three times with washing buffer [50 mM Tris-HCl, 150 mM NaCl, pH 8.0 containing 0.05% (v/v) Tween 20]. Glutathione S-transferase (GST) extracted from *E. coli* transformed with pGEX-2T served as the negative control antigen in the ELISA. The plates were dried under vacuum and stored at 4°C. On the day of use, plates were blocked for 1 h at 37°C with 200 µl of a 5% (w/v) skimmed milk solution in washing buffer. Test sera were added (100 µl per well) at a dilution of 1:100 in 5% (w/v) skimmed milk in washing buffer solution and incubated for 1 h at 37°C. After washing, protein G-peroxidase conjugate (Zymed, CA, U.S.A.) was added at a dilution of 1:5000 in 5% (w/v) skimmed milk solution in washing buffer (100 µl) and incubated at 37°C for 1 h. The plates were washed and the substrate [o-phenylenediamine (10 mg) and urea-hydrogen peroxide (8 mg) in 10 ml sodium citrate buffer 0.1 M, pH 4.5] added (50 µl/well) and incubated for 5 min at room temperature in the dark. The reaction was stopped by adding 100 µl of 0.5 M sulphuric acid per well. The absorbance of each well was read at two wavelengths (492 and 690 nm) using an automatic ELISA plate reader (Titertek Multiskan microplate reader, Flow Laboratories, McLean, Va.). A commercially available Maedi-Visna virus ELISA kit (Institut Pourquier, Montpellier, France) consisting of the p25 antigen only, was used as a reference assay. The ELISA procedure and cut-off value determination was performed according to the

manufacturer's instructions. All sera were tested in duplicate and mean absorbancies were determined for each specimen.

2.2.11 Selection of sera

Since no gold standard test for MVV infection exists, flock history and previous serological evidence had to be considered as criteria for catagorizing the sheep sera as MVV positive or negative. A group of 92 sheep sera was selected as follows: 46 serum samples were collected from animals originating from a flock with a history of being seropositive to MVV, with macroscopic and histological lesions for MVV infection present in some animals at slaughter. These 46 animals tested seropositive on a repeat MVV p25 ELISA performed 18 months previously. Another 46 sera were collected from sheep originating from flocks with no history of MVV and testing seronegative. These animals tested seronegative on more than one occasion during the previous 18 months. All sheep were between 3-4 years of age.

2.2.12 Evaluation of ELISA results

The user-defined template two-graph receiver operating characteristic (TG-ROC) was used as a Microsoft-EXCEL™ (version 5.0) spreadsheet which selects cut-off values in quantitative diagnostic tests (Greiner and Böhning, 1994). The underlying principle of this analysis uses a modification of the classical receiver operating characteristic (ROC) analysis (reviewed by Zweig and Campbell, 1993). For the construction of the TG-ROC plots, the variable range [$O.D._{min}$, $O.D._{max}$] as observed for the two reference populations is divided arithmetically into 250 intervals with the resulting limits termed "thresholds" (d_j), Se_j and Sp_j obtained with each threshold value are calculated as the proportion of positive results in the positive and negative results in the negative

reference population, respectively. The resulting matrix of d_j and the corresponding percentages Se_j and Sp_j (j = 1.....250) are displayed as a multiple *x-y* plot, representing the two observed parameters over the specified range of O.D. values. These graphs are equivalent to the empirical cumulative frequency distribution of the negative (CFD_{neg}) and the inverse positive ($1\text{-}CFD_{pos}$) sample. The cut-off (d_0) that realises equal test parameters $Se_j = Sp_j = \theta_0$ (theta-zero) can be read from the intersection point of the two graphs (point of equivalence) with the co-ordinates (d_0; θ_0). The intersection of the two graphs with the appropriate accuracy level (95 or 90%, respectively) indicates two alternative cut-off values, which are the "lower" and "upper limits" of the intermediate range (IR). Considering only results outside IR (for $\theta <$ accuracy level), the test's Se and Sp would be 95 or 90%, respectively. The valid range proportion (VRP) can be established using IR and the measurement range (MR), the latter being defined as the maximal minus the minimal observed test value (VRP = (MR - IR)/MR). TG-ROC provides both parametric and non-parametric methods for the estimation of measures described above. For the parametric approach the distribution of both reference populations should not deviate substantially from normal whereas in the non-parametric case the analysis does not require any assumptions concerning distribution types.

Using absorbancy data of negative and positive reference samples obtained with the GST-TM-p25 ELISA, the software plots the test sensitivity and specificity against the cut-off value as the independent variable. From the TG-ROC plots, cut-off values can be read for any combination of sensitivity and specificity (Greiner *et al.*, 1995). All measures established by TG-ROC are indicated with their appropriate 95% confidence

intervals. TG-ROC is public domain software and was obtained from Dr. M. Greiner, Free University of Berlin, FRG.

2.3 RESULTS

2.3.1 PCR amplification of TM and p25 coding DNA

A clone of SA-OMVV (obtained from R. Vigne, Marseilles, France), was used as template for the amplification of a 273-bp TM -and 748-bp p25 coding DNA fragment (Figure 2.1).

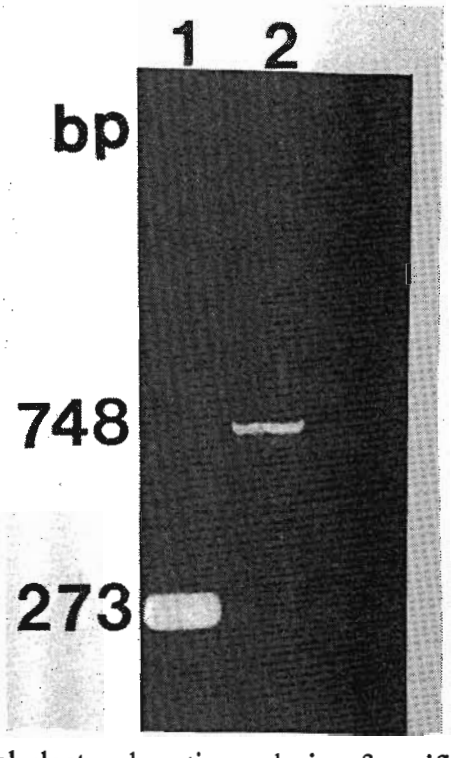

Figure 2.1 Agarose gel-electrophoretic analysis of purified TM and p25 amplicons. Lane1: 273-bp TM amplicon; Lane 2: 748-bp p25 amplicon.

2.3.2 Sequence analysis of pGEX-MVV/TM-p25

The recombinant plasmid pGEX-MVV/TM-p25 (Figure 2.2) containing the cloned MVV fragments coding for selected regions of the TM and p25 proteins of MVV was sequenced as described in Section 2.2.6.

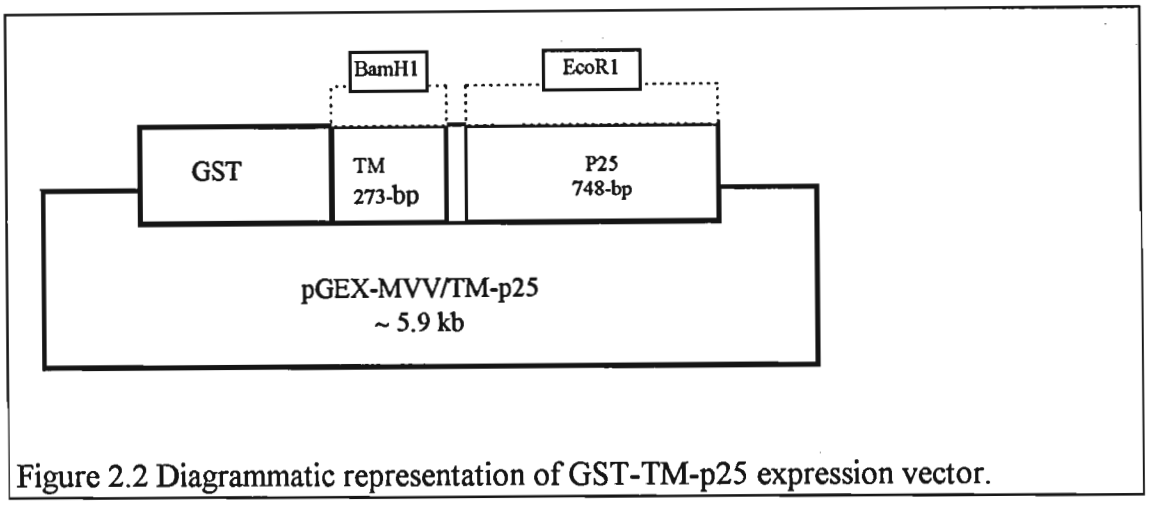

Figure 2.2 Diagrammatic representation of GST-TM-p25 expression vector.

Alignment of pGEX-MVV/TM-p25 sequenced and translated codons, compared with SA-OMVV (Querat *et al.*, 1990) sequencing data of corresponding genomic region, is shown in Appendix 2.

2.3.3 Expression, purification and analysis of recombinant proteins

The plasmid constructs pGEX-MVV/TM, pGEX-MVV/p25 and pGEX-MVV/TM-p25 expressed fusion proteins with molecular weights of 36, 56 and 62 kDa, respectively (Figure 2.3). The molecular weights of native GST is 26 kDa (Smith *et al.*, 1988), implying that the molecular weight of the cloned MVV TM, p25 and TM-p25 protein fragments are about 10, 30 and 36 kDa, respectively. The yield of purified fusion proteins varied between 1.5 and 2.0 mg per litre of culture.

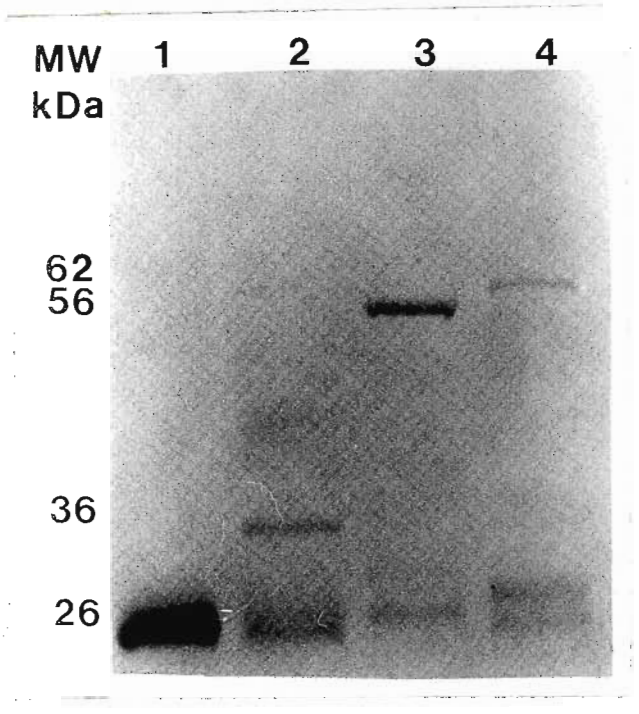

Figure 2.3 SDS-PAGE of purified recombinant proteins. Lane 1: GST; Lane 2: GST-TM; Lane 3: GST-p25; Lane 4: GST-TM-p25.

On Western blot analysis, positive MVV sera reacted with all three fusion proteins, but not with native GST (Figure 2.4). Negative control sera reacted with neither the fusion proteins nor the GST (results not shown).

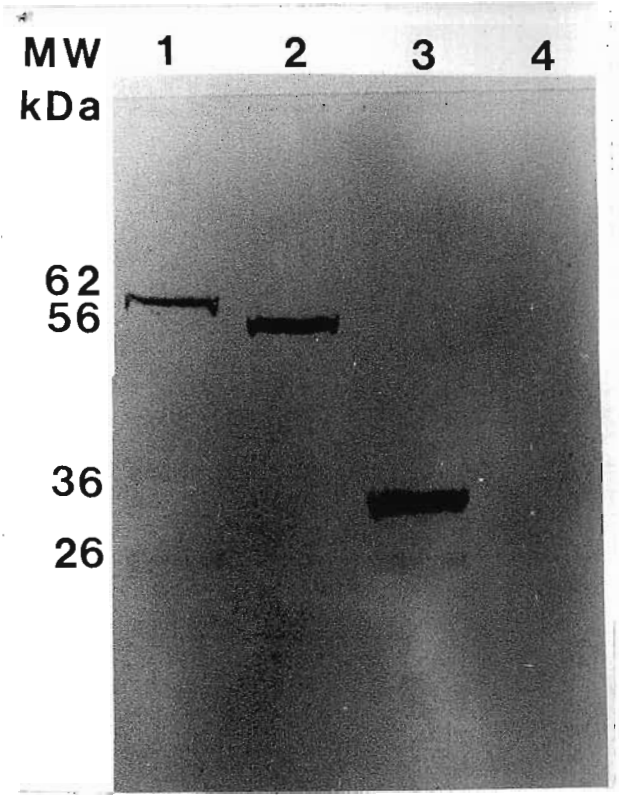

Figure 2.4 Western blot analysis using recombinant proteins as antigens. Lane 1: GST-TM-p25; Lane 2: GST-p25; Lane 3: GST-TM; Lane 4: GST.

2.3.4 ELISA evaluation

2.3.4.1 Two-graph-ROC analysis (TG-ROC)

Statistical variables of the negative and positive reference samples are summarised in Table 2.2.

Table 2.2
Statistical summary of the results of the GST-TM-p25 specific antibody ELISA against a positive and negative reference population

Measure [a]	Negative	Positive
Sample size	46	46
Mean	0.043	0.792
Median	0.038	0.779
Standard deviation	0.024	0.383
Minimum	0.003	0.117
Maximum	0.112	1.751

[a] Test variables are expressed as OD_{492} values

The cut-off (d_0) as determined by TG-ROC is 0.115 (Table 2.3). This cut-off yields equal sensitivity (Se) and specificity (Sp) as test parameters (θ_0 = 0.995) and was calculated using the non-parametric programme option. This option was chosen, because TG-ROC indicated a deviation from a normal distribution of the test results.

Table 2.3
Results of the TG-ROC analysis of the GST-TM-p25 specific ELISA

Measure [a, b]	Non-parametric
θ_0	0.995
d_0	0.115 (0.112,0.156)
IR	0.000
upper limit	0.092 (0.074, 0.111)
lower limit	0.211 (0.112, 0.294)
VRP	1.000

[a] The test variables are expressed as OD_{492} values, 95% confidence intervals of point estimates are given in brackets.
[b] Equality in test parameters ($Se=Sp=\theta_0$) can be realized when the cut-off d_0 is selected.

The cut-off (d_0) can be read directly from the TG-ROC graph (Figure 2.5).

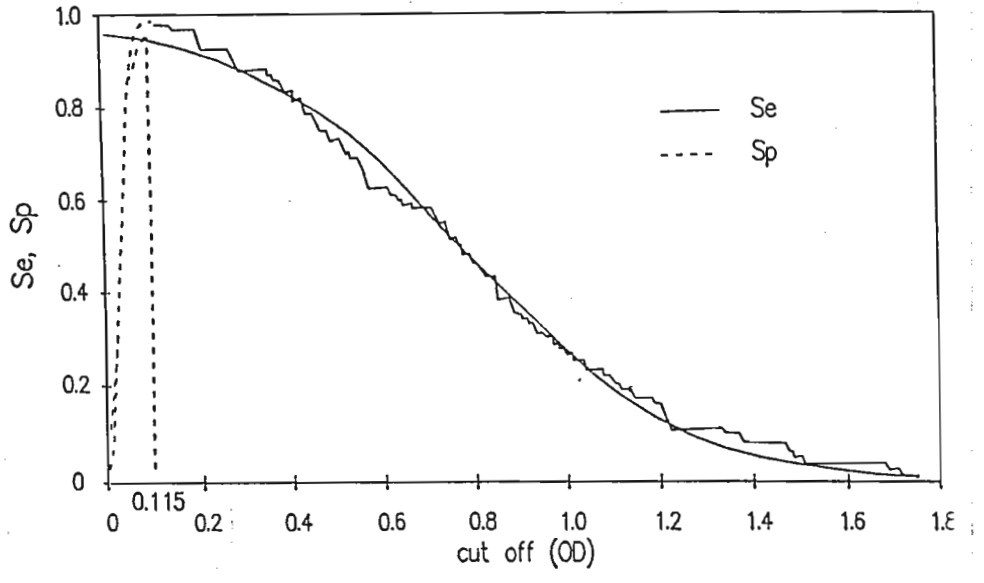

Figure 2.5 TG-ROC analysis of a MVV GST-TM-p25 specific antibody ELISA. The intersection point of the two graphs indicates the cut-off point d_0 at which equivalent $Se=Sp=\theta_0$ can be achieved.

The intersection of the two graphs with the appropriate accuracy level (95 or 90%, respectively) indicates two alternative cut-off values, which are the "lower" and "upper limits" of the intermediate range (IR). The valid range proportion (VRP) can be established using IR and the measurement range (MR), the latter being defined as the maximal minus the minimal observed test value (VRP = (MR - IR) / MR).

2.3.4.2 ROC plot

In Figure 2.6 a ROC plot of the GST-TM-p25 fusion protein ELISA was constructed using the software described above for the TG-ROC plot. In this graph the value of $1 - Sp_j$ is plotted on the x-axis (independent variable) and the value of Se_j is plotted on the y-axis (dependent variable).

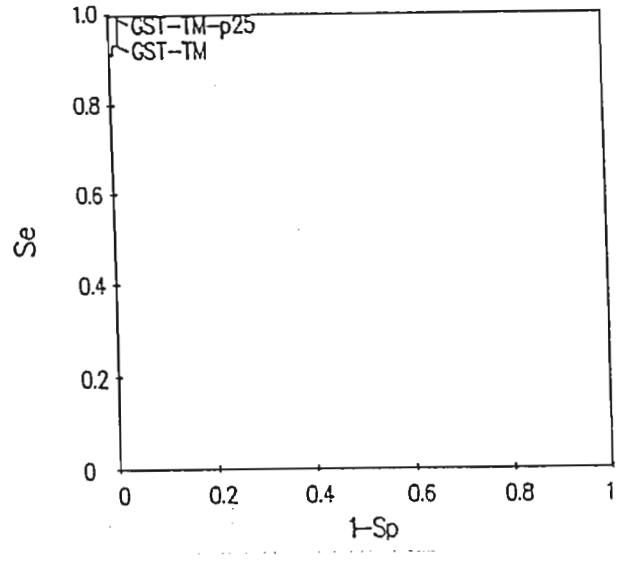

Figure 2.6 ROC plot of the GST-TM and GST-TM-p25 specific ELISA.

Thus the ROC graph is a plot of all the sensitivity/specificity pairs resulting from continuously varying the decision threshold over the entire range of results observed. In each case, the ROC plot depicts the overlap between the two distributions by plotting the sensitivity (or true positive fraction) versus 1- specificity (or false-positive fraction) for the complete range of decision thresholds. As shown in Figure 2.6 the GST-TM-p25 ELISA has a ROC plot that passes through the upper left corner, where the true-positive fraction is 1.0 or 100% (perfect sensitivity), and the false-positive fraction is 0 (perfect specificity).

2.3.4.3 Comparison of the GST-TM-p25 ELISA with a commercial p25 ELISA

Each of the GST-TM and GST-TM-p25 ELISA results for the 46 negative and positive reference sera was evaluated using the TG-ROC calculated cut-off value. These results were then statistically compared (Table 2.4) with the results obtained using the same reference sera and a commercial MVV p25 ELISA kit (Institut Porquier).

Table 2.4

Comparison of recombinant GST-TM-p25 and GST-TM with a commercial MVV p25 test kit. Characteristics are based on testing 46 negative and positive reference sera

Immunoassay	TP/PR	Sensitivity %	TN/NR	Specificity %
Commercial p25	40/46	87	46/46	100
GST-TM	46/46	100	45/46	98
GST-TM-p25	46/46	100	46/46	100

[a] TP, number of test-positive samples; PR, number of positive-reference sera tested; TN, number of test negative samples; NR, number of negative reference sera tested.

2.4 DISCUSSION

Several studies have shown that the agar gel immunodiffusion (AGID) test by itself is not sufficient for MVV diagnosis because it lacks sensitivity (Houwers *et al.*, 1982; Vitu *et al.*, 1982; Simard and Briscoe, 1990). Second-generation recombinant protein assays solved many of the problems associated with the previous generation assays which used viral lysates.

The core (p25) and transmembrane (gp40) proteins encoded for by the *gag* and *env* genes were selected as diagnostic proteins because collective data suggested that MVV infected sheep produce a strong immune response against the core protein early in infection which is followed by a strong transmembrane immune response in the later stages of disease (Kajikawa *et al.*, 1990). This is analogous to the situation in human beings infected with HIV-1 (Allain *et al.*, 1986). Thus, antibodies against *gag* and *env* protein products increase and decrease inversely through progression of the disease. Therefore, any individual serum may react with only one antigen during the course of MVV infection, but it is believed to be unlikely that serum from an infected sheep will not react with either of them. The conclusion can therefore be made that the ideal serologic assay for MVV should contain at least these two antigens to diagnose infected animals at all stages of disease. This was achieved by cloning both the p25 and TM genes in frame in the same expression cassette (Figure 2.2). Sequence analysis and expression of these two antigens as a combined fusion protein with glutathione S-transferase (GST) as fusion partner (Figure 2.3) confirmed that the correct genes had been cloned and that the codons were in frame.

The GST expression vector allows for inducible, high-level expression of proteins and has been used to produce recombinant caprine, equine, human, ovine and simian lentiviral fusion proteins (Zanoni *et al.*, 1991; Beatty *et al.*, 1992; Kwang and Cutlip, 1992a,b; Mills *et al.*, 1992; Thomas *et al.*, 1992; Rimstad *et al.*, 1994). However, certain foreign proteins often do not express well. Typically, problems encountered with protein expression in prokaryotic systems can be traced to over-expression of the foreign protein causing protein aggregation, toxicity of expressed protein or intracellular proteolytic degradation. Additionally, elevated temperatures lead to enhanced expression of the heat-shock proteins such as DnaK and GrpE which participate in the degradation of foreign and abnormal proteins in *E. coli* (Echols, 1990; Sherman and Goldberg, 1992). It is also our experience that such co-purified proteins often cause unacceptable ELISA background signals, making them unsuitable for the development of sensitive assays. Although overexpression of a protein at an elevated temperature leads to a higher yield, this advantage is often counteracted by aggregation and/or enhanced proteolysis of the protein. Most of the problems mentioned above could be circumvented by inducing the *E. coli* cell culture with IPTG at 20°C instead of 37°C (Ghosh *et al.*, 1995) and using an improved purification protocol as previously described (Grieco *et al.*, 1992).

The establishment of a reliable cut-off value is essential for a serologic test of diagnostic significance. This requires the selection of a cut-off value that will differentiate between subpopulations of infected and non-infected individuals with a stated diagnostic accuracy (Greiner and Böhning, 1994). Cut-off values in enzyme-linked immunosorbent assays (ELISA) have often been established as the mean plus two standard deviations of values from a non-infected population. Barajas-Rojas and

colleagues (1993) pointed out that this approach leads automatically to a specificity of 97.5% provided that the test variables are normally distributed. However, deviations from normality are often seen in serodiagnostic data which should be taken into account when selecting legitimate cut-off values (Vizard *et al.*, 1990). Furthermore, the sensitivity (Se) and specificity (Sp) of an assay always inversely influence each other so that only the entire spectrum of sensitivity/specificity pairs provides a complete picture of test accuracy (Zweig and Campbell, 1993).

These factors are all taken into consideration by using the two-graph receiver operating characteristic (TG-ROG) plot to establish a cut-off value. In TG-ROC, Se and Sp are plotted separately as dependent variables against the assumed cut-off value as the independent variable (Figure 2.3). The estimated cut-off d_0 then reflects equal Se and Sp with the resulting test parameter ($Se = Sp = \theta$) (Table 2.3).

TG-ROC can also be used to determine "intermediate" test results by establishing two alternate cut-off values. The intermediate range (IR) for an ideal test should be equal to zero because the upper limit of IR is less than the lower limit. In such a case a single cut-off value can be selected. The valid range proportion (VRP) would then be 1.0 and both Se and Sp would be greater than or equal to 95%. This is the case for the described GST-TM-p25 ELISA assay which shows a zero IR value and a resultant VRP value of 1.0 (Table 2.3). In Figure 2.4 a plot of all the sensitivity/specificity pairs resulting from continuously varying the decision threshold over the entire range of results is shown. Sensitivity, or the true-positive fraction [defined as (number of true-positive test results) / (number of true-positive + number of false-negative test results)] is plotted on the y-axis and the false-positive fraction, or 1 - specificity [defined as (number of false-positive results) / (number of true-negative + number of false-positive

results)] is plotted on the x-axis. The GST-TM-p25 ELISA showed perfect discrimination (no overlap in the two distributions) and has an ROC plot that passes through the upper left corner, where the true-positive fraction is 1.0 or 100% (perfect sensitivity) and the false-positive fraction is 0 (perfect specificity). Table 2.4 further illustrates these results and emphasises the increased sensitivity obtained with the inclusion of the TM antigen compared to a commercial MVV p25 ELISA kit.

All previously described recombinant MVV ELISA assays utilised either the p25 or TM proteins alone (Reyburn *et al.*, 1992; Kwang *et al.*, 1993) or combined (Keen *et al.*, 1995), but as individual proteins. In this chapter the cloning and expression of both proteins as a single fusion protein is described and demonstrated the need for both proteins to ensure a sensitive assay. The GST fusion protein was not cleaved from the antigens used in this study. All sera were tested against GST alone but none had antibodies to the GST. This was slightly unexpected as there have been unpublished reports that a low percentage of sheep have anti-GST antibodies. The next phase of this study will include a much larger population of animals. The anti-GST problem will be further investigated and the sensitivity and specificity of this assay will be compared with other confirmatory criteria such as molecular diagnosis and pathology.

CHAPTER 3

INVESTIGATING THE USE OF THE METHYLOTROPIC YEAST, *PICHIA PASTORIS*, FOR THE EXPRESSION OF A MVV TM-POLYPEPTIDE

3.1 INTRODUCTION

In HIV, the transmembrane (TM) glycoprotein portion of the envelope viral protein has been identified as the antigen most consistently recognised by antibodies (Schupbach *et al.*, 1984; Barin *et al.*, 1985). Recombinant polypeptides representing regions of the TM and synthetic oligopeptides corresponding to highly antigenic segments of the HIV TM have been used for serodiagnosis of the human AIDS viral infections (Wang *et al.*, 1986; Cabradilla *et al.*, 1986; Gnann *et al.*, 1987). Results presented in Chapter 2 (Table 2.4) seem to indicate that the same applies to MVV as the GST-TM fusion protein, expressed in *E. coli*, has comparable sensitivity to the GST-TM-p25 fusion protein, but lacks specificity. This is probably due to the fact that the MVV TM protein is known to be hydrophobic (Querat *et al.*,1990), and difficult to purify using conventional induction and purification protocols (Kwang *et al.*, 1993). Despite the fact that a truncated version of the TM protein was expressed (Figure 2.3), special induction and purification procedures had to be adopted (Section 2.2.7) in order to purify the fusion protein, free of contaminating *E. coli* proteins. The successful development of sensitive diagnostic methods for HIV based on TM-polypeptides stimulated the search for a reliable, sensitive and continuous source of pure MVV TM-polypeptide to be used in an ELISA.

The yeast *Pichia pastoris*, representing one of four different genera of methylotrophic yeasts, which also include *Candida*, *Hansenula*, and *Torulopsis*, was shown to be

suitable for the high-level expression of various heterologous proteins, either intracellularly localised (Cregg and Madden, 1988; Clare *et al.*, 1991) or secreted into the culture supernatant (Tschopp *et al.*, 1987b; Digan *et al.*, 1989). *P. pastoris* combines the features of a eukaryotic secretion machinery with the fast growth and simple growth media requirements of bacteria (Cregg *et al.*, 1993). Additionally, the induction can be efficiently regulated by the supplementation of methanol as the carbon source and high levels of heterologous proteins expression require only small scale production in shake flasks. These features made *P. pastoris* an attractive host for the expression of the hydrophobic transmembrane protein of MVV.

P. pastoris is capable of metabolising methanol as a sole carbon source (Veenhuis *et al.*, 1983). From an expression system perspective, the most interesting aspect of this metabolic pathway was alcohol oxidase (AOX), the first enzyme in the methanol-utilisation pathway (Ellis *et al.*, 1985; Koutz *et al.*, 1989) (see Figure 3.1).

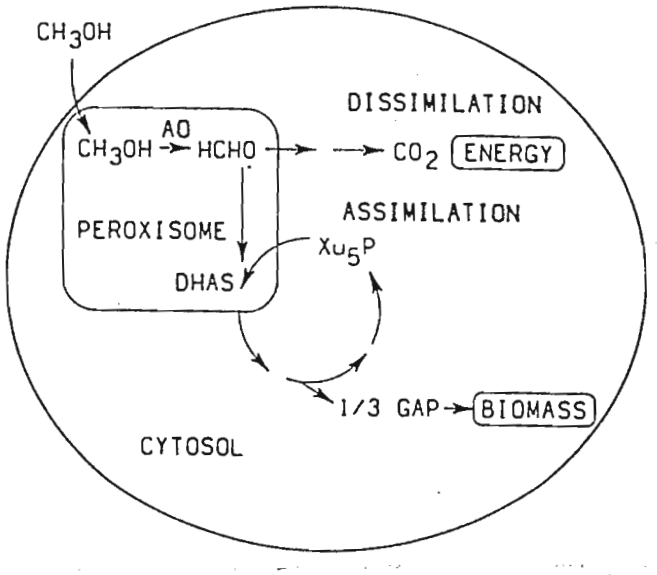

Figure 3.1 Overview of methanol metabolism in yeast.
AOX, alcohol oxidase; DHAS, dihydroxyacetone synthase; Xu_5P, xylulose 5-phosphate; GAP, glyceraldehyde 3-phosphate.

AOX is undetectable in cells cultured on carbon sources such as glucose, glycerol or ethanol, but constitutes up to 30% of total soluble protein in methanol-grown cells (Couderc and Baratti, 1980). It was anticipated that AOX synthesis would be regulated at the transcriptional level and that the promoter for this gene would be most useful for controlling the expression of foreign genes. Under control of an AOX promoter, foreign genes could be maintained in an "expression-off" mode on a non-methanolic carbon source to minimise selection for nonexpressing mutant strains during cell growth, then efficiently switched on by shifting to methanol. Another advantage of expressing secreted proteins is that *P. pastoris* secretes very low levels of native proteins. That, combined with the very low amount of protein in the growth medium, means that the secreted heterologous protein comprises the vast majority of the total protein in the medium and this simplifies the purification of the protein.

The *P. pastoris* expression system is based on a histidinol dehydrogenase-defective mutant host strain, G115. The expression plasmid which contains the complete *P. pastoris HIS4* gene can complement the mutant *P. pastoris* host defect and therefore be used as a selectable marker (Cregg *et al.*, 1985). The expression vector is also designed to be linearized with the restriction enzyme, *Bgl II*, such that His^+ recombinants can be generated by integration at the AOX1 locus. This integration can result in the complete removal of the AOX1 coding region (i.e. gene replacement) that in turn results in a recombinant phenotype of His^+ Mut^- (Mut^- refers to the methanol utilisation minus phenotype caused by the loss of alcohol oxidase activity encoded by the AOX1 gene that results in a no growth or slow growth phenotype on methanol media). The events as described above are illustrated in Figure 3.2.

Plasmid pPIC9 is an *Escherichia coli- P. pastoris* shuttle vector, with sequences required for selection in each host (Figure 3.2). The left half of the plasmid is a portion of pBR322, from *Cla I* site through the *Pvu II* site (modified to a *Bgl II* site). This segment of pBR322 contains the ampicillin resistance gene (Amp^r) and the *E. coli* origin of replication (*ori*). The *EcoR I* site in this segment has been eliminated. There is no f_1-bacteriophage origin of replication. The DNA elements comprising the rest of the plasmid are derived from the genome of *P. pastoris* with the exception of the *Saccharomyces* cerevisiae α-factor secretion signal and sequence (pre-pro sequence), the multiple cloning site and short regions of pBR322 used to link the yeast elements. The *P. pastoris* elements in the plasmid are as follows:

1) 5' AOX1, approximately 1000 bp segment of the alcohol oxidase promoter fused to the *Saccharomyces cerevisiae* α-factor secretion signal sequence with *Xho I*,

Hind III, *SnaB I*, *EcoR I*, *AvrII* and *Not I* cloning sites.

2) 3' AOX1, approximately 256 bp segment of the alcohol oxidase terminating sequence.

3) *P. pastoris* histidinol dehydrogenase gene, *HIS4*, contained on a 2.4 Kb fragment to complement the defective *His4* gene in host GS115.

4) Region of 3' AOX1 DNA approximately 650 bp in size, which together with the 5' AOX1 region is necessary for site-specific integration by homologous recombination.

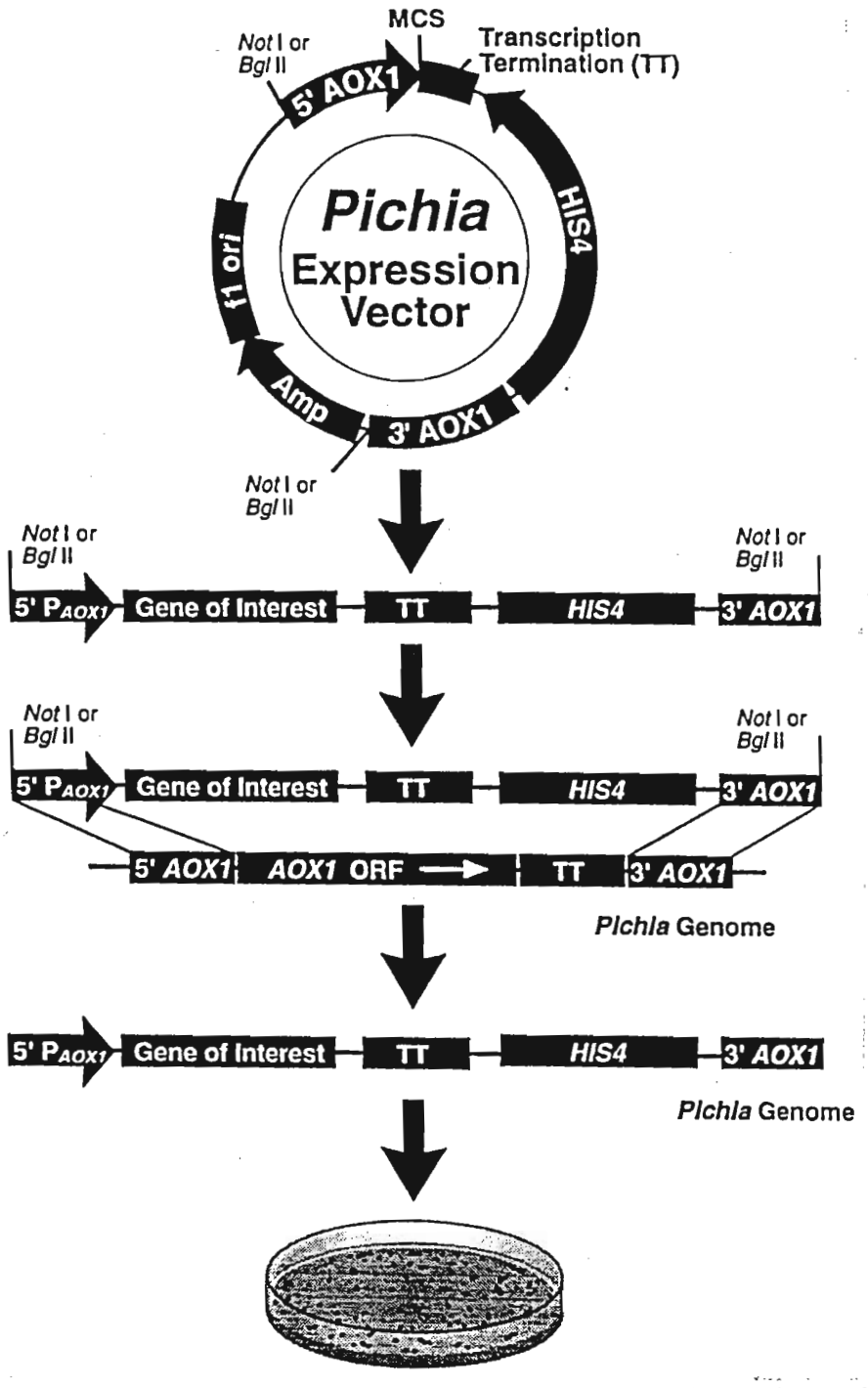

Figure 3.2 Outline of the cloning, transformation, recombination and selection of *P. pastoris* clones expressing a specific gene of interest.

Expression of the foreign DNA is achieved by integration into the *P. pastoris* genome which ensures stable recombinant cell lines (Schiestl and Gietz, 1989). The site of

integration however, also determines the selection of resultant transformants. Transformation of GS115 with *Bgl II*-linearized pPIC9 constructs resulted in recombination with the AOX1 locus (see Figure 3.2). Displacement of the alcohol oxidase (AOX1) structural gene occurs at a frequency of 5-35% of the His^+ transformants. Because these transformants are not producing alcohol oxidase, they can not efficiently metabolise methanol (see Figure 3.1) as a carbon source, and therefore grow poorly on media containing methanol as sole carbon source. This slow growth on methanol phenotype can be used to distinguish His^+ transformants in which the AOX1 gene has been disrupted (His^+ Mut^-) from His^+ transformants with an intact AOX1 gene (His^+ Mut^+). Protein producing clones can easily be scaled up from shake flask to fermentor (Cregg and Madden, 1988). This expression system has also been shown to successfully express a wide variety of proteins ranging in size from 6 KDa to 135 KDa (Tschopp *et al.*, 1987a; Vedvick *et al.*, 1991). To conclude, *P. pastoris* has proven to be a powerful tool for the production of foreign proteins of both academic and commercial interest (Wegner, 1990). Advantages of the system include: (1) the AOX1 promoter which has transcription characteristics useful for regulating heterologous protein expression; (2) well-developed methods for classical- and molecular-genetic manipulation of the organism; and (3) technology for the growth of expression strains in large high-density fermentor cultures. These advantages prompted investigating the use of the *P. pastoris* expression system for the expression of the MVV TM-polyprotein as discussed above.

3.2 MATERIALS AND METHODS

3.2.1 Polymerase Chain Reaction (PCR)

A coding region from the TM gene of MVV was amplified as a 117-bp DNA fragment by PCR essentially as described in Section 2.2.1. The 5' primer was designed to amplify the TM region with an *EcoRI* restriction site and the 3' primer with a *Not I* restriction site (Table 3.1).

Table 3.1
The nucleotide sequences and genomic locations of PCR primer pairs used to PCR amplify the MVV TM coding region to be expressed in *P. pastoris*.

Primer	Sequence (5'-3')	Gene and location*
TM-His 5' primer	TCC AGG GAA TTC CAT CAT CAT CAT CAT CAT GAA GCT ATA GTC GAT AGA	*env* (8084-8101)
TM 3' primer	TCC CGG GCG GCC GCA TAT CTT GTC CAA TTT AC	*env* (8201-8183)

* See Querat *et al.*, 1990.

The TM-His 5' primer contains six consecutive histidine codons designed to be in frame with the pPIC9 *P. pastoris* expression vector (see Section 3.2.2). The resultant PCR product (Figure 3.3) was purified (Spin kit, Qiagen, Chatsworth, CA, U.S.A.) and subsequently digested with *EcoRI* and *NotI* restriction enzymes.

3.2.2 Construction of yeast expression vector for MVV TM-polypeptide

The pPIC9 vector DNA was linearized by digesting with *EcoRI* and *Not I* restriction enzymes as described in Section 2.2.2. Since two restriction enzymes were used no dephosphorylation of the 5' phosphate groups, to prevent recircularization, was necessary. Ligation of the 117-bp TM-polypeptide coding DNA insert to the linearized pPIC9 vector was done as described in Section 2.2.3. Subsequent transformation of

ligated pPIC9 DNA using competent *E. coli* JM109 cells was performed as described in Section 2.2.4. Small-scale isolation of pPIC9 DNA to verify the presence of the correct size insert was done as described in Section 2.2.5 using *EcoRI* and *Not I* restriction enzymes. Sequencing of the cloned fragment to confirm that it was in the correct reading frame was performed as described in Section 2.2.6 using the sequencing primers included in the *Pichia* expression kit (Invitrogen). The nucleotide sequences of the above mentioned sequencing primers are given in Appendix 1.

3.2.3 Growth and storage of yeast strains

The growth temperature for *P. pastoris* is 30°C for liquid cultures (with shaking), plates and slants. Since the *P. pastoris* GS115 strain is defective in its histidinol dehydrogenase activity coded for by the *HIS4* gene, GS115 will only grow on complex media such as YPD or on minimal media supplemented with histidine, such as MMH, MDH or MGYH. Until transformed, GS115 will not grow on minimal media such as RD, MM, MD or MGY (see Appendix 1 for media descriptions). Yeast strains can be stored at -70°C in 15% glycerol (viable for >5 years) or at 4°C on YPD agar slants (viable for 1 to 2 years). The GS115 *His*$^+$ transformants for storage are initially cultured in selective medium (MGY or MD) harvested and suspended in YPD containing 15% glycerol, frozen and stored at -70°C.

3.2.4 Preparation of transforming DNA

Large-scale preparation of recombinant pPIC9 vector DNA was done using a maxiprep plasmid isolation kit from Qiagen (Chatsworth, CA, U.S.A.). The plasmid DNA was digested with *Bgl II* restriction enzyme (Pharmacia Biotech. U.S.A.), to obtain His^+ Mut^- transformants (see Figure 3.2). After digestion, two plasmid fragments were generated. This was confirmed by agarose gel electrophoretic analysis (Section 2.2.1). A non-recombinant pPIC9 plasmid digestion was also included as a negative protein expression control.

3.2.5 Preparation of spheroplasts

The host strain GS115 was streaked onto a YPD plate and incubated at 30°C for 2 days. A 10 ml solution of YPD was inoculated with a single colony and grown overnight at 30°C with vigorous shaking. This culture was used as inoculum in the subsequent steps and could be stored at 4°C for several days. A 200 ml YPD solution was inoculated with 5 µl of inoculum solution and incubated overnight with vigorous shaking at 30°C. Once an $O.D._{600}$ of $0.2 \rightarrow 0.3$ was reached, cells were harvested by centrifugation (1500 x g, 10 min, RT), washed with freshly prepared SED (1 M sorbitol, 25 mM EDTA, 50 mM DTT, pH 8.0) and with 1 M sorbitol, with intermediate centrifugation as before. The cells were resuspended in 20 ml SCE (1 M sorbitol, 1 mM EDTA and 10 mM sodium citrate buffer, pH 5.8) and divided into two tubes containing 10 ml each. Zymolyase (3 mg/ml in water) which is provided as a slurry was mixed thoroughly before 8 µl (24 µg) was added to one tube of cells, mixed and incubated at 30°C. Monitoring of the percentage spheroplasting was done by adding 200 µl of the Zymolyase containing cell suspension to 800 µl of 5% SDS at

time t=0, 2, 4, 5, 6, 7, 8, 9, 10, 15, 20 and 30 min after Zymolyase addition and measuring the $O.D._{800}$. Determination of the percent of spheroplasting for each time point was calculated using the equation:

% Spheroplasting = 100 - [(OD_{800} at time t/OD_{800} at time 0) x 100]

The time needed to obtain 70% spheroplasting was determined and the spheroplasting step repeated on the second tube of cells. The spheroplasts were harvested by centrifugation (750 x g, 10 min, RT) and washed with 10 ml 1 M sorbitol and 10 ml CaS (1 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$) with intermediate centrifugation (750 x g, 10 min, RT). The spheroplasts were gently resuspended in 0.6 ml of CaS and immediately used for transformation.

3.2.6 Transformation

For each transformation performed, a 100 µl solution of spheroplasts from Section 3.2.5, was dispensed into a sterile 6 ml snap-top microcentrifuge tube. The linearized recombinant and non-recombinant pPIC9 vector DNA (2 - 5 µg), prepared as described in Section 3.2.4, was added to each of the spheroplast containing tubes and incubated at room temperature for 10 min. *Bgl II* digested non-recombinant pPIC9 vector DNA was used to obtain negative expression control clones. No DNA was added in the negative control transformation tube. A freshly prepared PEG/CaT [1:1 mixture of 40% PEG and CaT (20 mM Tris, pH 7.5, 20 mM $CaCl_2$)] solution (1 ml) was added, gently mixed and incubated at room temperature for 10 min. Each transformation tube was subsequently centrifuged (750 x g, 10 min, RT) the PEG/CaT solution aspirated and the pellet of transformed cells was resuspended in 150 µl SOS

medium (1 M sorbitol, 0.3X YPD, 10 mM $CaCl_2$) and incubated at room temperature for 20 min. Before plating, 1 M sorbitol (850 µl), was added.

3.2.7 Plating for transformants

A 200 µl aliquot of freshly transformed spheroplasts was added to 10 ml of molten RD which was held at 45°C, and poured onto corresponding RDB base plates (see Appendix 1 for composition of media). The top agar was allowed to harden and the plates inverted and incubated at 30°C. Transformants appeared within 5-7 days. The negative controls should not form colonies.

3.2.8 Screening for AOX1-disrupted transformants

Using a sterile toothpick, His^+ transformed colonies were picked and used to inoculate a test tube containing 2 ml YPD for each colony. After an overnight shaking incubation at 30°C, separate test tubes containing 2 ml BMGY and 2 ml BMMY each, were inoculated with 10 µl of the individual colonies, respectively. The cultures were allowed to grow for 24 h shaking at 30°C. Visual comparison of any growth difference observed for each colony could be established after allowing the cells to settle overnight at 4°C. The GS115/His^+ Mut^- albumin secreting and GS115 host strain, supplied with the *P. pastoris* Expression Kit (Invitrogen), were used as positive and negative AOX1 disrupted controls, respectively.

3.2.9 Expression of recombinant MVV TM-polypeptide

Fifty two colonies displaying poor growth on BMMY medium (His^+ Mut^- phenotype) were identified as described in Section 3.2.8. Selective MD agar plates were used to maintain the individual clones before each colony was inoculated into 10 ml BMGY medium and grown for two days at 30°C with vigorous shaking until the cells reached an $O.D._{600}$ > 10.0. The cells were harvested by centrifugation 4000 x g, 10 min, RT). The supernatants were discarded and the individual pellets resuspended in 2 ml BMMY medium and grown as before for another two days. The MVV TM-polypeptide containing supernatants were obtained by centrifugation as described and used in subsequent steps.

3.2.10 ELISA evaluation of expression supernatants

The 52 putative MVV TM-polypeptide containing supernatants were tested for reactivity towards MVV positive sera using the ELISA technique. To each supernatant, PMSF and EDTA were added to give the final concentrations of 1.0 mM and 5 mM, respectively, diluted 1:1 with 100 mM sodium bicarbonate pH 9.6, and dispensed at 100 µl per microtitre well (Greiner, Germany). The solution was dried by evaporation overnight at 37°C. The plates were washed three times with washing buffer [50 mM Tris-HCl, 150 mM NaCl, pH 8.0 containing 0.05% (v/v) Tween 20]. The ELISA procedure was performed as described in Section 2.2.10 and the selection of positive and negative MVV serum based on the criteria described in Section 2.2.11. Control *P. pastoris* expression supernatants obtained as described in Sections 3.2.4, 3.2.6 and 3.2.8 were used as negative control supernatants.

3.2.11 PCR analysis of MVV TM-polypeptide expressing clones

Total DNA was isolated from clones identified to express the MVV TM-polypeptide as determined above by ELISA. The individual clones were grown to an $O.D._{600} > 5.0$ in 10 ml MD medium at 30°C. The cells were centrifuged at 1500 x g, washed with 10 ml sterile water before being resuspended in 2 ml SCE buffer, pH 7.5, containing DTT (1 M sorbitol, 10 mM sodium citrate, 10 mM EDTA, 10 mM DTT). Zymolyase (0.3 mg) was added to each cell suspension and incubated at 37°C to achieve < 80% spheroplasting (monitored as described in Section 3.2.5.) Two ml of 1% SDS was added, gently mixed and set on ice for 5 min, before 1.5 ml of 5 M potassium acetate, pH 8.9, was added. The lysed cell suspensions were centrifuged at 10 000 x g for 10 min and the supernatants retained. Two volumes of ethanol were added to each of the supernatants and incubated at room temperature for 15 min. The solutions were subsequently centrifuged at 10 000 x g for 10 min and the resultant DNA pellets gently resuspended in 0.7 ml TE buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0) and transferred to a microcentrifuge tube. Contaminating protein was extracted with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) followed by an equal volume of chloroform. The purified genomic DNA was precipitated by adding 1/2 a volume of 7.5 M ammonium acetate, pH 7.5, and 2 volumes of ethanol to each tube. The tubes were incubated at -20°C overnight before being centrifuged at 10 000 x g for 20 min. The DNA pellets were each washed once with 1 ml 70% ethanol, dried and then resuspended in 50 µl of TE buffer, pH 7.4. PCR amplification of the 117-bp MVV TM-polypeptide was carried out with the TM-His 5' and TM 3' primers (Table 3.1), using the isolated genomic DNA as template. The PCR was essentially as described in Section 2.2.1, except that the samples were amplified for 30 cycles, each cycle

consisted of a denaturation step at 94°C for 1 min, followed by annealing at 55°C for 1 min and extension at 72°C for 30 seconds. DNA isolated from the host strain, GS115 and a His^+ Mut^- clone obtained by transforming with non-recombinant linearized pPIC9 vector (see Section 3.2.4) were used as negative control template DNA. Visualisation of the amplicons was as described in Section 2.2.1.

3.2.12 Large-scale expression and purification of the secreted MVV TM-polypeptide

Expression of the MVV TM-polypeptide was performed in shake flasks in 100 ml of BMGY medium at 30°C to an $O.D._{600} > 15$. The cells were subsequently harvested and resuspended in 50 ml of BMMY (methanol induction), containing 2 mM Pefabloc® SC (Boehringer Mannheim), and incubated at 30°C for 96 h to induce expression. Expression supernatant aliquots were taken every 24 h and tested by ELISA (see Section 3.2.10) to determine the optimum time of TM-polypeptide expression as reflected by ELISA reactivity. Separation of protein by SDS-PAGE and subsequent transfer to a nitrocellulose membrane for Western-blot analysis was performed as described in Sections 2.2.8 and 2.2.9. The histidine-tagged MVV TM-polypeptide was purified using immobilised metal affinity chromatography (IMAC) (Porath and Olin, 1983). The principle behind IMAC lies in the fact that many transition metal ions, i.e. nickel, can co-ordinate to histidine via electron donor groups (Sulkowski, 1985). In order to utilise this interaction for chromatographic purposes, the metal ion must be immobilised onto an insoluble chromatographic matrix. It is coupled to a matrix such as Sepharose 6B, via a long hydrophilic spacer arm. The spacer arm ensures that the chelating metal is fully accessible to all available binding sites on a protein. The basic

methodology of IMAC consist of three steps: (1) charging the gel, (2) binding the proteins, and (3) eluting the proteins. A Ni-NTA (nickel-nitrilotriacetic acid) (Qiagen) resin for proteins with an affinity tag of six consecutive histidine residues (6x His tag) was used. It is supplied pre-swollen and charged with Ni^{2+} as an aqueous suspension (50%). The Ni-NTA resin contains 8-12 µmol Ni^{2+}/ml gel and has a binding capacity of approximately 5-10 mg protein per ml resin, however, the capacity of the resin may vary to some extent with the size and shape of the recombinant protein. After optimum time of induction was reached the TM-polypeptide containing supernatant was adjusted to pH 8.0 with 0.1 M NaOH and PMSF and β-mercaptoethanol were added to a final concentration of 1 mM and 10 mM, respectively. No EDTA was added as this will inhibit binding of the 6x His tagged TM-polypeptide to the Ni-NTA resin. The supernatant was passed twice through the Ni-NTA column before washing the resin with washing buffer (0.1 M sodium phosphate, 0.01 M Tris-HCl, pH 8.0 containing 10 mM imidazole) until the A_{280} of the effluent was <0.01. The MVV TM-polypeptide was eluted with 10 ml washing buffer containing 200 mM imidazole as competitor and collected in 1 ml fractions. Expressed protein was precipitated as follows: The sample to be concentrated was transferred to an Eppendorf tube and 1/10 volume of 5% SDS added. The tube was inverted a few times and 1/10 volume 3 M KCl added. After a few more inversions the sample was centrifuged (12 000 x g, 5 min, RT). The resultant protein pellet was resuspended in 1/10 the original volume of 100 mM sodium carbonate pH 9.6, ELISA coating buffer. Optimum coating concentration was determined by titration of the purified MVV TM-polypeptide and adsorption onto ELISA plates (Greiner, Germany) performed as described in Section 3.2.10, at the determined optimum dilution.

3.2.13 Two-graph receiver operating characteristic (TG-ROC) analysis

The user-defined template two-graph receiver operating characteristic (TG-ROC) was used as a Microsoft-EXCEL™ (version 5.0) spreadsheet which selects cut-off values in quantitative diagnostic tests (Greiner and Böhning, 1994) as described in Section 2.2.12. Absorbancy data of negative and positive reference samples, obtained with the *P. pastoris* expressed MVV TM-polypeptide ELISA, were used to plot test sensitivity and specificity against the cut-off value as the independent variable. All measures established by TG-ROC are indicated with their appropriate 95% confidence intervals.

3.3 RESULTS

3.3.1 PCR amplification of TM-polypeptide coding DNA

A clone of SA-OMVV (obtained from R. Vigne, Marseilles, France), was used as template for the amplification of a 117-bp DNA fragment (Figure 3.3). Primer sequences are shown in Table 3.1.

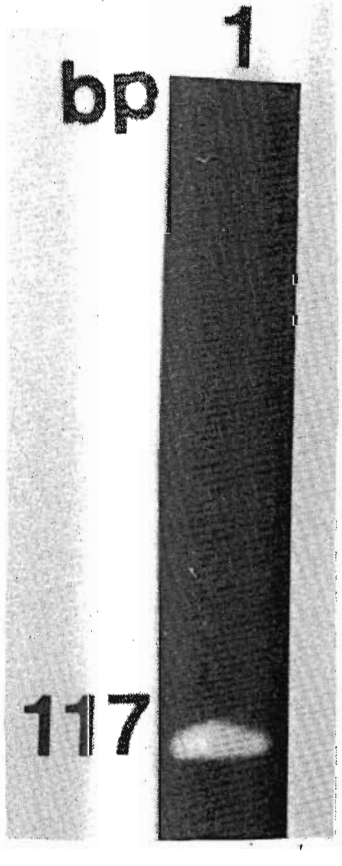

Figure 3.3 Agarose (2% w/v) gel-electrophoretic analysis of purified TM-polypeptide coding DNA produced by PCR. Lane 1: 117-bp amplicon.

3.3.2. Sequence analysis of pPIC9-TM recombinant plasmid

The recombinant plasmid pPIC9-TM containing the cloned 117-bp TM-polypeptide coding DNA was sequenced as described in 2.2.6. Sequences of the sequencing primers are shown in Appendix 1. Alignment of the pPIC9-TM sequenced and translated codons compared with SA-OMVV (Querat *et al.*, 1990) sequence data of the corresponding genomic region are shown in Appendix 3. The 117-bp TM-polypeptide coding DNA fragment has been cloned in frame with the *P. pastoris* pPIC9 vector to be expressed as a secreted polypeptide with six consecutive N-terminal histidine amino acid residues. However, a nine nucleotide deletion occurred, which might be due to a PCR artefact, without altering the reading frame of the cloned MVV-TM DNA (see Appendix 3).

3.3.3 PCR analysis of putative positive *P. pastoris* integrants

Two clones designated as A2 and B11 were identified as both AOX1-disrupted (Section 3.2.8) as well as TM-polypeptide expressing (Section 3.2.9) transformants as identified by ELISA evaluation of expression supernatants (Section 3.2.10). Genomic DNA from the above mentioned transformants was analyzed to determine if the gene of interest was integrated into the *P. pastoris* genome (Figure 3.4).

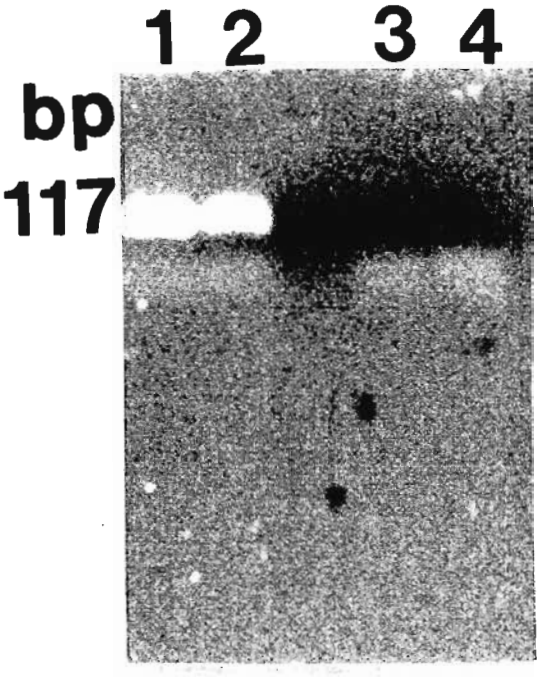

Figure 3.4 Agarose (2% w/v) gel-electrophoretic analysis of PCR products of genomic DNA isolated from clones A2 (lane 1), B11 (lane 2), host cell GS115 (lane 3) and pPIC9 plasmid-only transformant negative control transformant (lane 4).

PCR amplification of the gene of interest was carried out with the TM-His 5' primer and TM 3' primer as shown in Table 3.1. Confirmation of integration of the TM-polypeptide coding gene was established for both transformants A2 and B11 (Figure 3.5, lanes 1 and 2, respectively). Genomic DNA isolated from the host cell line GS115 and the pPIC9 plasmid-only transformant negative expression control transformant, obtained as described in Section 3.2.4, served as negative controls (Figure 3.5, lanes 3 and 4, respectively).

3.3.4 Purification of the secreted TM-polypeptide

Seventy two h of growth on 0.5% methanol, was found to be the optimum time of expression, whereafter the supernatant was collected and the histidine-tagged MVV TM-polypeptide purified using immobilised metal chelate affinity chromatography as described in Section 3.2.12.

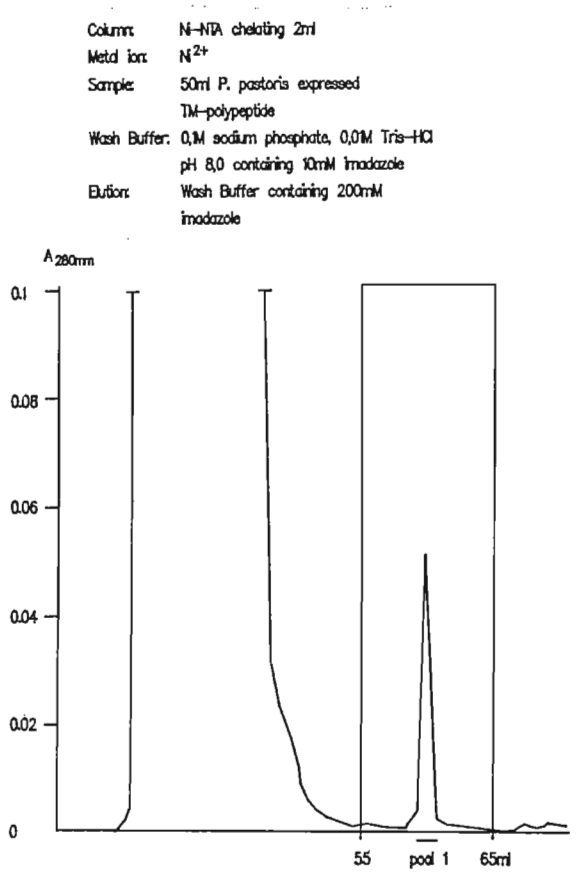

Figure 3.5 Purification of the *P. pastoris* expressed histidine-tagged MVV TM-polypeptide by metal chelate affinity chromatography. Pool 1 contains the histidine-tagged MVV TM-polypeptide.

Protein yield, determined using the micro-Lowry assay, revealed a concentration of 40 µg TM-polypeptide from 50 ml of culture supernatant. This implied that the supernatant had a concentration of less than 1 µg/ml TM-polypeptide.

3.3.5 SDS-PAGE and Western-blot analysis of MVV TM-polypeptide

As a result of the low concentration of the secreted MVV TM-polypeptide, visualisation after electrophoretic separation with either Coomassie or silver staining (data not shown) of the expected 5 kDa protein was difficult. Furthermore, Western blot analysis of the concentrated TM-polypeptide using nitrocellose membranes, yielded no positive reaction. ELISA, as described in 3.2.10, remained the only way to detect and evaluate the diagnostic value of the *P. pastoris* expressed MVV TM-polypeptide.

3.3.6 Evaluation of the *P. pastoris* expressed TM-polypeptide ELISA

3.3.6.1 Two-graph-ROC analysis (TG-ROC)

Statistical variables of the negative and positive reference samples are summarized in Table 3.2.

Table 3.2
Statistical summary of the results of the *P. pastoris* expressed MVV TM-polypeptide ELISA against a positive and negative reference population

Measure[a]	Negative	Positive
Sample size	36	36
Mean	0.210	0.487
Median	0.168	0.381
Standard deviation	0.175	0.312
Minimum	0.009	0.032
Maximum	0.898	1.631

[a]Test variables are expressed as $O.D._{492}$ values

The cut-off (d_0) as determined by TG-ROC is 0.256 (Table 3.3). This cut-off yields equal sensitivity (Se) and specificity (Sp) as test parameters ($\theta = 0.833$) and was calculated using the non-parametric programme option. This option was chosen, because TG-ROC indicated a deviation from a normal distribution of the test results.

Table 3.3
Results of the TG-ROC analysis of the *P. pastoris* expressed MVV TM-polypeptide specific ELISA

Measure[a, b]	Non-parametric
θ_0	0.833
d_0	0.256 (0.225, 0.276)
IR	0.493 (0.237, 0.861)
upper limit	0.566 (0.44, 0.893)
lower limit	0.073 (0.032, 0.203)
VRP	0.696 (0.469, 0.854)

[a] The test variables are expressed as $O.D._{492}$ values, 95% confidence intervals of point estimates are given in brackets.
[b] Equality in test parameters ($Se=Sp=\theta_0$) can be realised when the cut-off d_0 is selected. In order to meet the accuracy criteria (90 or 95%, respectively), two cut-off values are selected, to determine the intermediate range (IR) and the valid range proportion (VRP).

The cut-off (d_0) can be read directly from the TG-ROC graph (Figure 3.6).

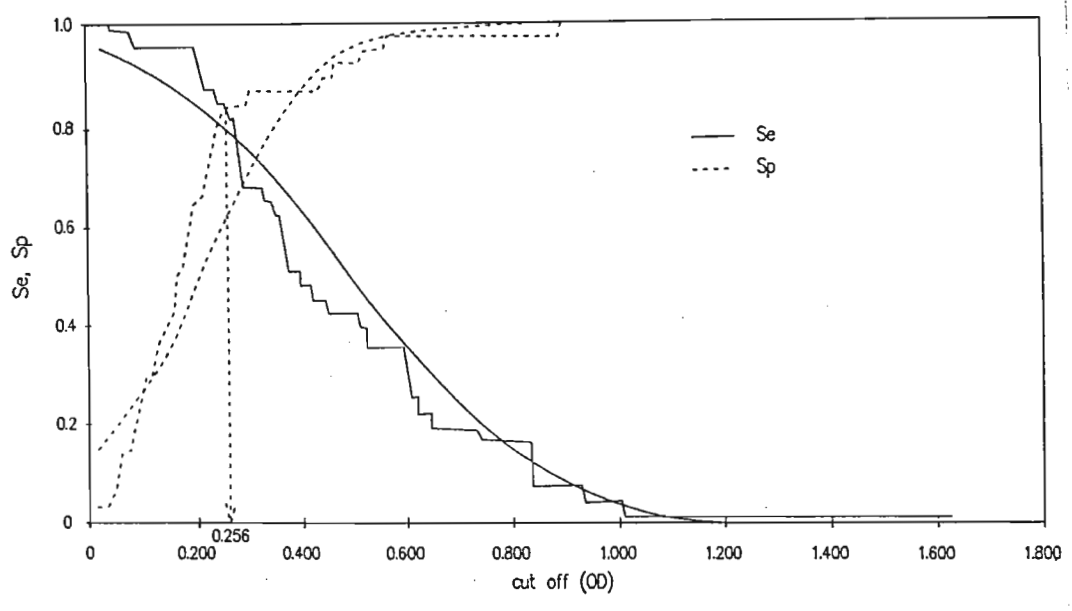

Figure 3.6 TG-ROC analysis of the *P. pastoris* expressed MVV TM-polypeptide specific antibody ELISA. The interSection point of the two graph indicates the cut-off point d_0 at which equivalent $Se=Sp=\theta_0$ can be achieved.

The interSection of the two graph with the appropriate accuracy level (95 or 90%, respectively) indicate two alternative cut-off values, which are the "lower" and "upper limits" of the intermediate range (IR). The valid range proportion (VRP) can be established using IR and the measurement range (MR), the latter being defined as the maximal minus the minimal observed test value (VRP = (MR - IR) / MR).

3.3.6.2 Sensitivity and specificity of the *P. pastoris* expressed MVV TM-polypeptide ELISA.

Results obtained using the *P. pastoris* expressed MVV TM-polypeptide ELISA for the 36 negative and positive reference sera were evaluated using the TG-ROC calculated cut-off value (Table 3.4).

Table 3.4
Sensitivity and specificity values obtained of the MVV TM-polypeptide ELISA using the TG-ROC calculated cut-off value.

Antigen	TP/PR	Sensitivity %	TN/NR	Specificity %
Recombinant MVV-TM polypeptide	30/36	83	30/36	83

[a] TP, number of test-positive samples; PR, number of positive reference sera tested; TN, number of test negative samples; NR, number of negative reference sera tested.

Equality of test parameters ($Se=Sp=\theta_0$) is thus obtained when the cut-off d_θ (0.256) is selected (Table 3.4).

3.3.6.3 ROC plot

In Figure 3.7 a ROC plot of the *P. pastoris* expressed MVV TM-polypeptide ELISA was constructed using the TG-ROC software as described in Chapter 2. In this graph the value of $1\text{-}Sp_j$ is plotted on the x-axis (independent variable) and the value of Se_j is plotted on the y-axis (dependent variable).

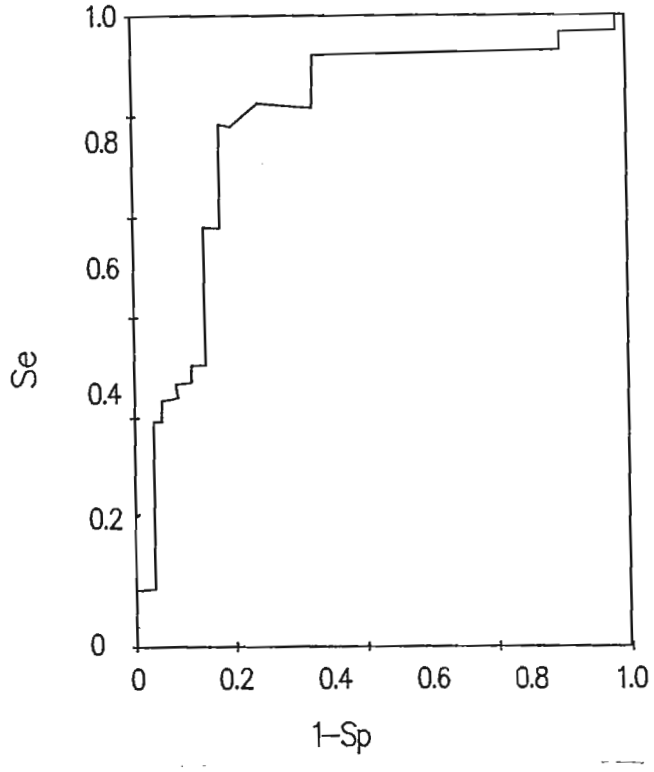

Figure 3.7 ROC plot of the *P. pastoris* expressed MVV TM-polypeptide specific ELISA.

The ROC graph is a plot of all the sensitivity/specificity pairs resulting from continuously varying the decision threshold over the entire range of results observed. In each case, the ROC plot depicts the overlap between the two distributions by plotting the sensitivity (or true positive fraction) versus 1- specificity (or false-positive fraction) for the complete range of decision thresholds.

3.4 DISCUSSION

Early successes in the production of heterologous proteins were achieved using the well-studied bacterium *Escherichia coli* as a host (Itakura *et al.*, 1977). However, as the nature of the recombinant proteins being expressed became more complex, other potential host systems were investigated. Yeast offers a number of advantages as an expression system. As an unicellular micro-organism, it retains the advantages of the bacterial systems in ease of manipulation and growth. On the other hand, yeasts possess a eukaryotic subcellular organisation capable of accurately processing and secreting many different proteins (Buckholz and Gleeson, 1991). Thus, the expression of a recombinant protein in yeast is often a suitable choice that combines a cost-effective method of production with the ability to post-translationally modify proteins in an appropriate manner.

In this chapter the construction of a MVV TM coding *P. pastoris* expression vector, and its use in the production of a MVV TM-polypeptide antigen. A 117-bp DNA fragment (Figure 3.3), coding for an antigenic TM-polypeptide region, was cloned in frame with the *S. cerevisiae* α-mating factor secretion signal (Figure 3.4). The 89 amino acid *S. cerevisiae* prepro alpha mating factor leader sequence (Figure 3.2) has been effective at directing secretion of several proteins from *P. pastoris* (Vedvick *et al.*, 1991; Clare *et al.*, 1991). To maximise the stability of foreign protein production clones, expression vectors are integrated into the *P. pastoris* genome. A major factor which determines the state (autonomous versus integrated) as well as location of a plasmid in *P. pastoris* transformed cells is whether sequences homologous to the *P. pastoris* genome were present on the plasmid (Cregg *et al.*, 1985; Cregg and Madden,

1987). The recombinant pPIC9 vector was linearized at the AOX1 5' sequences to stimulate single-crossover type integration events. A subset of resulting His^+ colonies are deleted at AOX1 which forces them to rely on the transcriptionally weak AOX2 gene (Cregg *et al.*, 1993). These clones metabolise methanol at a reduced rate which can be used to selectively screen for clones with such a specific phenotype (see Section 3.2.8).

These AOX disrupted clones were also shown to express higher levels of foreign protein than wild-type hosts, e.g. β-galactosidase (Tschopp *et al.*, 1987a), invertase (Tschopp *et al.*, 1987b) and hepatitis B surface antigen (Cregg *et al.*, 1987). However, despite the fact that both clones A2 and B11 exhibited reduced growth on methanol containing medium, poor expressed protein concentrations were obtained. Furthermore, the presence of the integrated 117-bp MVV TM-polypeptide coding DNA fragment has been confirmed by PCR analysis (Figure 3.5), excluding the possibility of non-recombinant mutant clones. *P. pastoris* clones with multiple copies of heterologous gene expression cassettes have been described (Romanos *et al.*, 1991). Clones harbouring multiple copies often, but not always, synthesise significantly higher levels of protein (Sreekrishna *et al.*, 1989; Clare *et al.*, 1991; Barr *et al.*, 1992). Thus the possibility exists that both clones A2 and B11 contain only a single gene copy of the MVV TM-polypeptide coding sequence which could explain the low expression levels obtained.

Large-scale expression of the MVV TM-polypeptide was performed in the presence of Pefabloc® SC, a non-toxic serine protease inhibitor (Section 3.2.12). Omitting such a protease inhibitor has not led to an increase in expressed protein (data not shown), indicating that the low expression levels were not due to the presence of proteases in the medium. However, optimum expression levels were repeatedly obtained after 72 h of methanol induction (Section 3.3.4) for both clones A2 and B11, as revealed by ELISA reactivity (Section 3.2.10).

Since the expected molecular weight of the secreted MVV TM-polypeptide is only about 5 kDa, an N-terminal histidine-coding tag was incorporated during the PCR amplification (Table 3.1). Subsequent protein purification was based on the selectivity of the Ni-NTA resin for proteins with an affinity tag of six consecutive histidine residues- the 6x His tag. This technology allowed for one-step purification of the recombinant protein under mild elution conditions. Furthermore, the 6x His affinity tag is much smaller than most other affinity tags e.g. GST, and is known to be poorly immunogenic in most species and therefore did not require removal by protease cleavage. The short 6x His tag has also been shown to be readily expressed in yeast (Bush *et al.*, 1991; Johnson *et al.*, 1993; Ridder *et al.*, 1995), and also in other host systems such as: bacteria (Le Grice and Grüninger-Leitch, 1990), mammalian cells (Sporeno *et al.*, 1994) and baculovirus (Waeber *et al.*, 1993).

Purification of the MVV TM-polypeptide was done under non-denaturing conditions in the presence of a protease inhibitor (PMSF) and the reducing agent mercaptoethanol (Section 3.2.12). The addition of mercaptoethanol to the loading buffer reduced background due to cross-linked proteins. This has been reported to be important especially when purifying proteins which contain cysteine residues (Janknecht *et al.*, 1991), as is the case with the expressed MVV TM-polypeptide. Background contamination can arise from proteins that contain multiple consecutive histidines, and thus have some affinity for the Ni-NTA resin or proteins that associate non-specifically with the 6x His tagged protein.

As a result of the low expression levels and small expected molecular weight of the *P. pastoris* expressed MVV TM-polypeptide, visualisation of the recombinant protein using conventional electrophoretic and Western blot analysis proved to be unsuccessful. ELISA remained the only means of detecting and arbitrarily quantifying the MVV TM-polypeptide.

Evaluation of the diagnostic performance of the MVV TM-polypeptide was accomplished using a panel of 36 confirmed negative and positive sera, respectively, selected on the criteria as described in Section 2.2.11 and evaluated using the two-graph operating characteristic (TG-ROC) as described in Section 2.2.12. The receiver operating characteristic (ROC) analysis is the standard method to demonstrate the co-variation of Se and Sp. ROC plots for diagnostic tests with perfect discrimination

between negative and positive reference samples would pass through the co-ordinates (0; 1) (Se = Sp = 100%). This was the situation using the *E. coli* GST-TM-p25 expressed antigen (Figure 2.3.3.2). Consequently, the area under such ROC curves would be 1.0, irrespective of the difference between the greatest observation in the negative and the lowest observation in the positive sample. The ROC curve for the *P. pastoris* expressed TM-polypeptide (Figure 3.3.6.3) demonstrates all valid pairs of Se and Sp which can be obtained when the cut-off value is changed systematically over the complete measurement range. In TG-ROC, Se and Sp are plotted as dependent variables separately against the assumed cut-off value as the independent variable (Figure 3.6). Numerically, one obtains estimates for the cut-off d_0 which can be used if an equal Se and Sp is desired and the resulting test parameter (Se = Sp = θ_0) is indicated. Therefore, a selected cut-off (d_0 = 0.225) (Table 3.3) yielded equal Se and Sp of 83%, (Table 3.4), respectively. This cut-off was obtained using the non-parametric programme option as the distribution of the negative and positive reference data (Table 3.2) showed a deviation from normal (see Section 2.2.12 for criteria of a normal distribution). If a conventional cut-off value (mean plus two-fold standard deviation of the negative reference sample) was considered, a Se of about 36% and an Sp of about 97% would result.

TG-ROC can also be used to define "intermediate" test results by establishing two alternative cut-off values. The TG-ROC algorithm warrants an Se and Sp of at least 95% (as preselected), for results outside the IR. The IR for an ideal test can be assumed to be equal to zero because the upper limit of IR is less than the lower limit as

was the case with the GST-TM-p25 ELISA (Table 2.3). The valid range proportion (VRP) would then be 1.0 and both Se and Sp would be greater than or equal to 95%. For the TM-polypeptide ELISA, in contrast, TG-ROC indicates a positive IR (Table 3.3) and VRP = 0.696. It can therefore be concluded that only those individual test results that fall within 69.6% of the variable range (MR) can be diagnosed with an accuracy of greater than 95%.

A further significant aspect of TG-ROC is that it can be used to compare diagnostic tests. VRP and θ_0 are independent of any selected cut-off value and, therefore, ideal parameters for test comparison. Comparison of the above two parameters for the GST-TM-p25 ELISA (VRP = 1.0; θ_0 = 0.995) (Table 2.3) and the TM-polypeptide ELISA (VRP = 0.696; θ_0 = 0.833) (Table 3.3) confirmed the superior diagnostic ability of the GST-TM-p25 antigen compared to the TM-polypeptide.

CHAPTER 4

DEVELOPMENT OF A RAPID WHOLE BLOOD AGGLUTINATION TEST FOR MAEDI-VISNA VIRUS

4.1 INTRODUCTION

An accurate assessment of the animal health situation of the livestock in a country is a necessary condition for implementing disease eradication or control programmes. In many developing countries, it is difficult for veterinary services to establish a full list of the diseases prevalent in the country, due to constraints such as insufficient numbers of field veterinarians and a lack of well-equipped diagnostic laboratories. As a result, developing countries are often aware of only a small proportion of disease outbreaks. It is therefore difficult to make an accurate assessment of the geographical distribution and relative importance of diseases.

Recent advances in biotechnology may help to resolve these problems through the development of rapid field diagnostic assays. These field diagnostic assays could help diagnosticians to make rapid and appropriate decisions in the event of a disease outbreak. The ideal field diagnostic assay should be sensitive and specific but should also be easy to use by unskilled persons and require minimal equipment.

A novel rapid immunoassay system has been described (see Figure 1.13), which allows the detection of circulating antibodies in whole blood (Kemp *et al.*, 1988; Wilson *et al.*, 1991). This test uses endogenous erythrocytes as indicator cells and a monoclonal anti-erythrocyte Fab' fragment conjugated via the inter heavy chain cysteines to a

synthetic peptide epitope. The erythrocytes are coated with the peptide antigen by adding Fab'-peptide conjugate to a drop of blood. If circulating antibodies to the peptide are present the erythrocytes visibly agglutinate. This test format is carried out in whole blood with minimal sample handling and a result is obtained within two min.

The central feature of this whole blood immunoassay system lies in a monoclonal antibody against a glycophorin epitope that is present on all red blood cells of the particular species being tested. In this chapter the isolation and characterization of a monoclonal antibody that recognises all sheep erythrocytes but does not cross-react with other mammalian erythrocytes (Boshoff *et al.*, 1996b), its coupling to MVV specific antigen and its use in the rapid diagnosis of Maedi-Visna virus in sheep (Boshoff *et al.*, 1996c).

4.2 MATERIALS AND METHODS

4.2.1 Preparation of whole sheep blood cells for immunisation

Freshly drawn sheep blood was washed three times in 0.15 M NaCl by centrifugation at 1000 x g. The washed red blood cells were diluted in 0.15 M NaCl to a final 10% (v/v) suspension.

4.2.2 Immunisation of mice

Balb/c mice were immunised intraperitoneally with 100 µl of a 10% (v/v) sheep red blood cell suspension in 0.15 M NaCl. Two weeks later, a booster immunisation of similar dose was given followed by a further two immunisations at three-weekly intervals, the last one, three days prior to fusion.

4.2.3 Monoclonal antibody production

Hybridomas were produced by fusing the splenocytes of an immune mouse with NS0 myeloma cells using PEG 1500 essentially following the method of Köhler and Milstein (1975). Briefly, the spleen of an immune mouse was aseptically removed and prepared as described by Galfre and Milstein (1981). Splenocytes were obtained by homogenisation of the spleen with a scalpel and forceps followed by trituration in a sterile syringe as described before (Boshoff *et al.*, 1992). After sedimentation of spleen debris, the cells were transferred to sterile 10 ml centrifuge tubes, washed twice with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with $NaHCO_3$ (3.7 g/l),

sodium pyruvate (1 mM), amphotericin B (1 μg/ml) and penicillin streptomycin mixture (10 IU/ml and 10 μg/ml, respectively) and finally resuspended in the same medium. NS0 cells were harvested, washed once and resuspended in DMEM with supplements. The cells were fused by adding a total of 1 ml 40% polyethylene glycol (PEG) (Boehringer Mannheim MW 1500) dropwise over a period of 60 seconds. The suspension was incubated for 90 sec at 37°C, and 5 ml serum free DMEM was added dropwise over a period of 5 min with constant swirling. The suspension was left at room temperature for 10 min before transfer to 195 ml DMEM containing supplements as above, 10% fetal calf serum [Highveld Biological (Pty) Ltd], 5% horse serum [Highveld Biological (Pty) Ltd] and 1 x HAT [hypoxanthine (100 μM), aminopterin (0.4 μM) and thymidine (16 μM)] (Flow Laboratories). The mixture was distributed into the wells of 96-well microtitre plates (Cel-Cult, Sterilin, Middlesex, U.K.) at 100 μl per well and incubated in a humidified incubator at 5% CO_2 air ventilation. Medium was changed every 3 days, aminopterin was excluded after one week and after the second week hypoxanthine and thymidine were omitted as well. The wells were monitored microscopically for colony growth and culture supernatants of the hybridomas were screened according to their ability to agglutinate sheep erythrocytes. Hybridomas secreting reactive antibodies were subcloned by limiting dilution. This process was repeated until all of the subclones secreted the desired antibody ensuring monoclonality.

4.2.4 Screening of hybridoma supernatants

Erythrocyte suspensions were prepared by washing as described above. The washed erythrocytes were diluted in 0.15 M NaCl to give a 2-5% (v/v) suspension. Fifty µl of suspension was placed in a small test tube to which 50 µl of hybridoma supernatant was added. The contents of the tube were mixed well and centrifuged immediately at (1000 x g, 15 sec, RT). Following centrifugation, 20 µl of a 30% (w/v) BSA solution (Miles, Il, U.S.A.), was added to the tube and gently shaken to resuspend the cells and agglutination recorded. The degree of agglutination was scored on a scale of 1+ to 4+ by evaluation of agglutination with the naked eye. The weakest agglutination detected in this manner was scored as 1+ and strong complete clumping of erythrocytes as 4+.

4.2.5 Ascites production

Ascitic fluid was produced by injecting 10^6 viable hybridoma cells per mouse into the peritoneal cavity of pristane (tetramethylpentadecane) (Aldrich Chemical Company, Milwaukee, WI) primed Balb/c mice (0.5 ml/mouse, 7 days prior to hybridoma inoculation). After a few days, when swelling of the abdomen could be observed, the ascites fluid was tapped off using a wide gauge needle. The fluid was clarified by centrifugation (3000 x g, 10 min, RT) and stored at -20°C.

4.2.6 Purification of mAb JC6 using protein A affinity chromatography

Chromatography of antibody solutions over a protein A bead column is one of the most effective and widely used methods for purifying antibodies from many types of crude preparations (Goding, 1978; Langone, 1982). Protein A coupled to preswollen agarose (5 ml) (Boehringer Mannheim), was packed into a plastic column and washed with five column volumes of TS-buffer (50 mM Tris-HCl, 150 mM NaCl, pH 8.0). The pH of the ascites preparation was adjusted to 8.0 by adding 1/10 volume of 1.0 M Tris (pH 8.0) before being passed through the protein A agarose column. Any unbound material was removed by washing the agarose beads with ten column volumes of TS-buffer. IgG_1 was subsequently eluted with 5 ml 0.1 M sodium citrate pH 6.0, followed by IgG_{2a} elution with 5 ml 0.1 M sodium citrate pH 5.0 and finally IgG_{2b} and IgG_3 with 5 ml 0.1 M sodium citrate pH 3.0.

4.2.7 CHARACTERIZATION OF AN ANTI-SHEEP GLYCOPHORIN SPECIFIC MONOCLONAL ANTIBODY, JC6

4.2.7.1 Epitope characterization of mAb JC6

The nature of the epitope recognised by mAb JC6 was examined by selective chemical and enzymatic treatment of erythrocytes immobilized on polyethyleneimine (PEI) coated 96-well microtitre plates (Greiner, Germany). Microtitre plates were prepared by coating PEI (MW 50000, Sigma) at a concentration of 20 μg/ml in PBS pH 7.2 for 30 min (F. Green, personal communication). The plates were washed with 50 mM Tris-HCl, 150 mM NaCl pH 8.0 containing 0.05% Tween 20 (TST) buffer, rinsed with water and vacuum dried. Washed sheep erythrocytes were diluted to a 0.2% (v/v)

suspension in 0.15% NaCl and dispensed at 100 µl/well onto the PEI coated microtitre plates. After 30 min at room temperature the erythrocyte suspension was flicked out and the microtitre plate wells washed three times with TST. Plates prepared in this manner were coated with a visible monolayer of sheep erythrocytes which were lysed by inverting the plate and centrifuging (1000 x g, 30 sec, RT). Stroma prepared in this manner was free of haemoglobin when tested for pseudo-peroxidase activity.

4.2.7.2 Trypsin treatment of immobilized sheep erythrocytes

The microtitre ELISA plates were coated as described above. The immobilized sheep erythrocytes were incubated with trypsin EC 3.4.21.4 (Sigma) at 100 µg/ml in PBS pH 7.2 (5 µg/well) (Arya *et al.*, 1994). In the controls, no trypsin was added to antigen-coated wells. The plates were incubated for 1 h at 37°C. After washing three times with TST, the non-specific sites were blocked for 1 h at room temperature using TS containing 5% (w/v) skimmed milk powder. Blocked wells were filled with a 1/1000 dilution of ascitic fluid in 5% (w/v) skimmed milk powder in TS (50 µl/well) and incubated for another h at room temperature. The plates were washed with TST and subsequently incubated with rabbit anti-mouse IgG (H+L)-peroxidase conjugate, diluted 1/200 in TS, followed by a final TST washing and colour development with 50 µl/well substrate solution of o-phenylenediamine (10 mg) and urea-hydrogen peroxide (8 mg) in 10 ml sodium citrate buffer (0.1 M, pH 4.5). The reaction was stopped with 0.5 M sulphuric acid (100 µl/well) and read at 492 nm in an automatic ELISA plate reader (Titertek Multiskan, Flow laboratories, McLean, Va.).

4.2.7.3 Periodate treatment of immobilized sheep erythrocytes

Sodium metaperiodate-mediated oxidation of erythrocyte carbohydrate residues was performed using 20 mM sodium periodate in 50 mM sodium acetate (pH 4.5) for 1 h as described before (Woodward *et al.*, 1985). Wells without sodium periodate treatment served as controls. The effect of periodate oxidation on the epitope recognised by mAb JC6 was tested by ELISA as described above.

4.2.7.4 Neuraminidase treatment of immobilized sheep erythrocytes

Neuraminidase EC 3.2.1.18 from *Clostridium perfringens* (Boehringer Mannheim) was dispensed at 0.1 units/100 µl/well in PBS pH 7.2 supplemented with 100 µg/ml BSA. Wells were incubated at 37°C for 1 h and washed with TST. Untreated wells were used as controls. The specificity of mAb JC6 for sialic acid was examined by ELISA as described before.

4.2.7.5 Isotyping

The heavy-chain subclass of monoclonal antibody JC6 was determined by double immunodiffusion (Ouchterlony) and ELISA using goat anti-mouse IgM, IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 specific unlabeled and HRP-labeled antibodies (Sigma Chemical Company, St. Louis, MO).

4.2.7.6 Western Blot Analysis

a) Epitopes detected by mAb, JC6

Sheep erythrocyte membranes were prepared by cold hypotonic lysis according to the procedure of Dodge *et al.* (1963). The membranes were repeatedly washed with cold 5 mM phosphate buffer until all haemoglobin was removed. The washed membranes were solubilized in an equal volume of 0.1 M Tris-HCl (pH 6.8) containing 5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 20% (w/v) sucrose, 2 mM phenylmethylsulphonylfluoride (PMSF) and 0.001% (w/v) bromophenol blue. SDS-PAGE was performed on the solubilized erythrocyte membranes using the discontinuous buffer system of Laemmli (1970), with a 4% (w/v) acrylamide stacking gel on a 12.5% (w/v) acrylamide separating gel using a Bio-Rad slab-gel apparatus. One hundred micrograms of membrane protein was applied per lane. Electrophoresis was performed for 2 h at 25 mA/gel. Separated sheep erythrocyte proteins were electrophoretically transferred from the gel onto nitrocellulose membranes (Schleicher and Schuell, Keene, U.K.) using the method of Hampson *et al.* (1989). The nitrocellulose membranes were blocked for 1 h in 5% (w/v) skimmed milk/TS solution, washed in TST, then incubated for 4 h at room temperature with mAb JC6 ascitic fluid diluted 1/1000 in 5% skimmed milk/TS. The membranes were then washed three times with TST and incubated for 1 h at room temperature with rabbit anti-mouse IgG (HRP)-labelled conjugate diluted 1/200 in 5% skimmed milk/TS. After washing twice with TST and a final wash with TS, the nitrocellulose membranes were immersed in freshly prepared substrate (60 mg 4-chloro-1-naphtol, Bio Rad) in 20 ml cold methanol mixed with 100 ml cold TS buffer containing 60 µl of 30% hydrogen peroxide.

b)Total carbohydrate and protein detection

To detect the carbohydrate and protein moieties of the separated membrane proteins blotted onto the nitrocellulose membranes, a Digoxigenin-glycan/protein Double Labelling Kit from Boehringer Mannheim was used. This involved the specific oxidation of the polysaccharides of a glycoprotein with periodate followed by covalent coupling of the Digoxigenin hydrazide hapten to the newly created aldehyde groups. The protein fraction of the glycoprotein and nonglycosylated proteins were labelled according to the principle of the Digoxigenin-Glycan/ protein Detection Kit in which a succinimide ester conjugated fluorescein hapten is coupled to all free amino groups. The specifically introduced haptens were detected in a reaction using anti-digoxigenin-POD and anti-fluorescein-AP antibody conjugates, respectively.

4.2.8 $F(ab)_2$ fragment production

The monoclonal antibody, JC6, directed specifically against sheep erythrocytes was purified as described in 4.2.5. The purified antibody was subsequently dialyzed against 100 mM phosphate buffer, pH 6.9. $F(ab)_2$ fragment production was essentially as described by Parham *et al.* (1982). Papain (EC 3.4.22.2) (Boehringer Mannheim) was diluted to 30 units/ml (activity was taken to be that stated by the manufacturer) with 100 mM phosphate pH 6.9 consisting of 50 mM L-cysteine. The enzyme was allowed to activate for 30 min at 37°C prior to desalting on a PD-10 (Sephadex G-25) desalting column (Pharmacia) equilibrated in 100 mM phosphate, pH 6.9. The papain containing fractions free of cysteine, as determined by reaction with 5,5'- dithiobis (2-nitrobenzoic acid) DTNB (Riddles *et al.*, 1983), were pooled and the concentration of papain

determined using an A_{280} for a 0.1% w/v solution as 2.5 (determined by diluting a solution of papain of known concentration and measuring the absorbancy at 280 nm). To each 1 mg of antibody, 1 unit of papain was added and the digestion allowed to proceed for 2 h whilst shaking at 37°C. The reaction was stopped by the addition of iodoacetamide to a final concentration of 30 mM. The digested antibody was dialyzed against a 50 mM Tris-HCl, 150 mM NaCl, pH 8.0 (TS-buffer), solution and the $F(ab)_2$ fragment separated from residual intact IgG and Fc fragments using a protein A column. The $F(ab)_2$ fragments were concentrated using a Microcon-100 filter (Amicon, Beverly, U.S.A.) as prescribed by the manufacturers.

4.2.9 $F(ab)_2$ fragment reduction

The purified $F(ab)_2$ fragments were suspended in 1 ml, 100mM phosphate, pH 6.0 containing 5mM EDTA. This solution was added to a vial containing 6 mg of 2-mercaptoethylamine-HCl (2-MEA, 50 mM final concentration) (Pierce Chemical Company, U.S.A.) and incubated for 90 min at 37°C. The solution was allowed to cool to room temperature and desalted using a PD-10 desalting column (Pharmacia Biotech, U.S.A.) equilibrated in 100 mM phosphate, 150 mM NaCl, 0.05% w/v NaN_3, pH 6.8, buffer. The void volume fraction containing the reduced Fab' fragments was added to solid DTNB to a final concentration of 25 mM and after mixing for 15 min, a final concentration of 30 mM iodoacetamide was added and allowed to react in the dark for a further 15 min. The Fab'-TNB-Ac (acetamide blocked Fab'-TNB) was desalted as described above in 100 mM phosphate, 150 mM NaCl, 0.05% w/v NaN_3 pH 6.8 buffer before any unreacted $F(ab)_2$ fragments were removed using a microcon-100 filter (Amicon, Beverly, U.S.A.) and collecting the Fab'-TNB-Ac flow through.

4.2.10 Peptide reduction

A 16-mer synthetic peptide analogue of gp40 (785-800) RFKDNCTWQQWEEEIE designed from the published SA-OMVV sequence (Querat *et al.*, 1990) was purchased from Neosystem, France. The peptide was solubilized at a final concentration of 10 mg/ml in 0.18 M acetic acid by pre-wetting the lyophilized peptide with 20 µl of 0.9 M acetic acid, then making up to volume with de-ionised, distilled water. The solution was aliquoted and stored at -70°C. Peptide gp40 (785-800) was reduced as described by Kemp *et al.* (1988) with some modifications. The solubilized peptide was diluted to 2 mg/ml with 0.1 M Tris-HCl, 1 mM EDTA, 4 M guanidine hydrochloride, 50 mM DTT, pH 8.0 and reduced for 30 min at room temperature. The peptide was maintained in reduced form by the addition of an equal volume of 10 mM HCl and desalted using a Biogel P2 (Bio-Rad, Richmond, CA) column, eluted with 10 mM HCl. The reduced peptide was recovered by lyophilization.

4.2.11 Quantification of antibody and peptide concentrations

The concentrations of antibody and peptide solutions were determined by comparing the absorbancy of the solution at 280 nm with the absorbancy of a standard 0.1% w/v solution at 280 nm [A_{280}(0.1% w/v)]. For antibodies a relative [A_{280}(0.1% w/v)] value of 1.4 was calculated, by accurately weighing and making up a standard solution of lyophilised antibody which was extensively dialyzed against water and using comparative colour development with the Lowry protein assay (Peterson, 1983). In the case of the peptide solution, the relative concentration was obtained by making up a standard peptide solution in 180 mM acetic acid, making up several dilutions so that the absorbencies at 280 nm were between 0.1 and 1.0, and calculating the standard

[A_{280}(0.1% w/v)]. Free sulfhydryls present on the peptide were determined by Ellman's test, diluting the peptide in excess DTNB, measuring the absorbancy at 412 nm, and calculating the moles of TNB^{2-} released using $\varepsilon_{412\,nm}$ = 14 150 M^{-1} cm^{-1} (Riddles *et al.*, 1983). Similarly, the amount of TNB in modified antibody fragments was determined as described for the peptide by the addition of excess DTT to the antibody fragments and calculating the increase in absorbancy measured at 412nm.

4.2.12 Peptide conjugation and conjugate purification

The reduced peptide was added in molar TNB^{2-} ratios of 10:1 peptide to antibody fragment. The reaction was stopped after 1 min by adding iodoacetamide to a final concentration of 30 mM and incubation for 15 min at room temperature. Any reformed $F(ab)_2$ fragments were removed using a Microcon-100 filter. The Fab'-peptide conjugate flow-through was concentrated using a Microcon-30 filter. The conjugate was suspended at 10 µl/ml in PBS/azide pH 7.4, containing 0.5% fish gelatin (Sigma) and stored at 4°C.

4.2.13 Peptide ELISA

The synthetic gp40 peptide was dissolved in 100 mM $NaHCO_3$ buffer pH 9.6 and coated on 96-well microtitre plates (Greiner, Germany) at 9 µg per well (Kwang and Torres, 1994). The peptide was dried by evaporation overnight at 37°C. The peptide was tested for reactivity against a panel of confirmed negative and positive anti-MVV sera.

4.2.14 Red cell agglutination assay

For the agglutination assay, Fab'-peptide conjugate solution (30 µl) was added to a well of a plastic tray containing 10 µl confirmed negative or positive serum containing 10 µl of packed erythrocytes from a MVV negative sheep. The agglutination mixture was swirled for 2 min and agglutination assessed visually according to a scale of 0 (negative) to 4+ (strong positive) (Wilson *et al.*, 1991).

4.2.15 Electrophoresis and Western blot analysis of antibody fragments

Antibody fragments and conjugate were analyzed using SDS-PAGE (Laemmli, 1970) under non-reducing conditions with Coomassie blue G-250 staining and by immunoblotting (Towbin *et al.*, 1979; Wilson *et al.*, 1994).

4.2.16 Data analysis

For the 10 negative and 10 positive serum samples tested the following were calculated (Griner *et al.*, 1981; Hardy, 1991):

Sensitivity: the ratio of true positives divided by total positives (sum of true positives and false negatives)

Specificity: the ratio of true negatives divided by total negative results (sum of true negatives and false positives).

Predictive value-positive: the ratio of true positives divided by total positives (sum of true and false positives).

Predictive value-negative: the ratio of true negatives divided by the total negatives (sum of true and false negatives).

False positive rate: 100 minus specificity.

False negative rate: 100 minus sensitivity.

4.3 RESULTS

4.3.1 Selection of monoclonal antibodies

Hybridomas producing anti-sheep erythrocyte antibodies were screened for agglutination. Selection was on the basis of their ability to strongly agglutinate all sheep erythrocytes. Clone JC6 gave a convincing 4+ agglutination score and was found to be of the IgG_1 isotype.

4.3.2 Reactivity of mAb JC6 to erythrocytes from different sheep breeds

Erythrocytes from six different sheep breeds were screened by agglutination with mAb JC6. All blood samples from the different breeds reacted with mAb JC6 (Table 4.1), suggesting a conserved and ubiquitous nature of the epitope recognised by mAb JC6.

Table 4.1 Agglutination strength of anti-sheep red blood cell monoclonal antibody tested against various sheep breeds.

BREED TESTED	AGGLUTINATION STRENGTH
Merino	4+
Döhne	4+
Dorper	4+
Finn	4+
Cross-breeds	4+

[a]Total number tested > 200

4.3.3 Nature of the epitope recognised by mAb JC6

The chemical nature of the epitope recognised by mAb JC6 was investigated by treating the immobilized sheep erythrocytes with trypsin, sodium meta-periodate and neuraminidase (Figure 4.1).

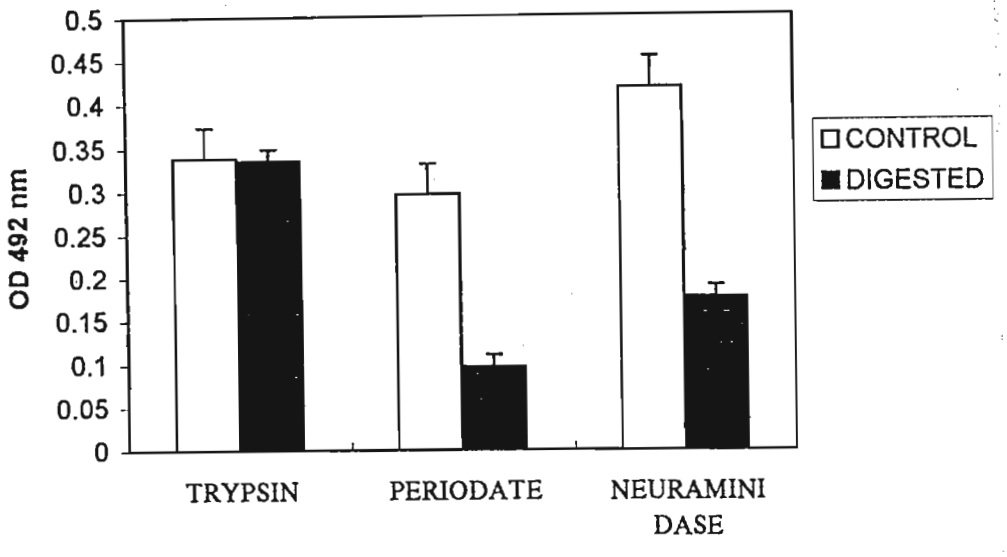

Figure 4.1 Effect of trypsin, sodium periodate and neuraminidase treated immobilized sheep erythrocytes on the reactivity of mAb JC6. ELISA values were obtained from two repeats, each comprising 32 measured ELISA plate wells per data point (i.e. n=64).

Trypsin treatment had little effect on the reactivity of mAb JC6 whereas periodate treatment significantly reduced the affinity of mAb JC6 for the immobilized sheep erythrocytes, suggesting a carbohydrate nature of the epitope recognised by mAb JC6. Immobilized sheep erythrocytes were subsequently treated with neuraminidase as described in 4.2.7.4. Neuraminidase treatment significantly reduced the reactivity of mAb JC6 for the immobilized sheep erythrocytes. Neuraminidase is known to specifically remove sialic acid residues present on glycoproteins (Suttajit and Winzler, 1971), hence the suggestion that the epitope recognised by mAb JC6 consists of sialic acid dependent moieties. Figure 4.2, lane 2 shows the reactivity of mAb JC6 to the immobilized sheep erythrocyte membrane.

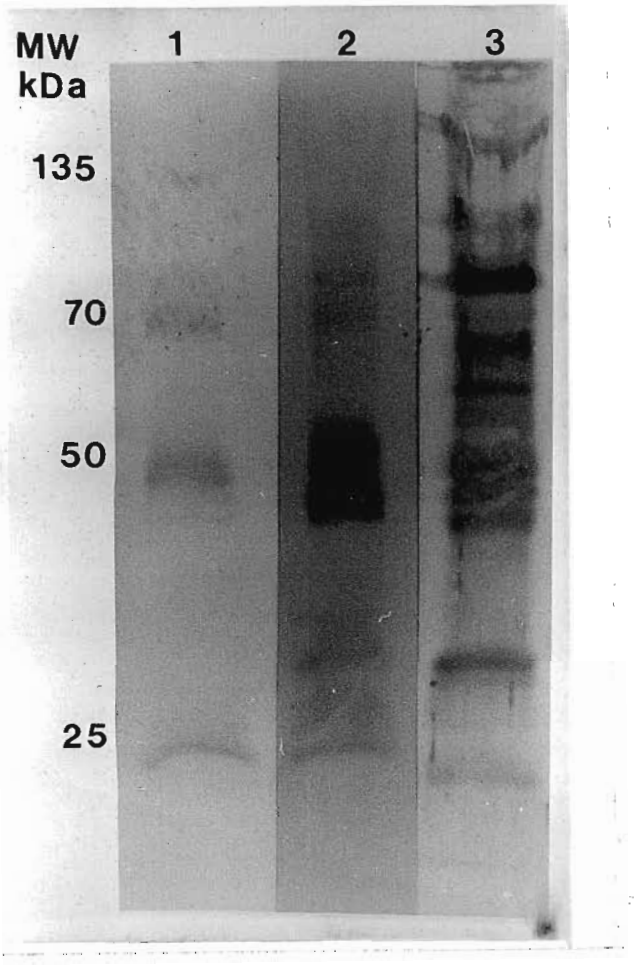

Figure 4.2 Western blot analysis of sheep erythrocyte membrane proteins immobilized onto nitrocellulose membranes. Lane 1: Western blot profile using mAb JC6; Lane 2: Total carbohydrate profile labelled with Dig-hydrazide hapten after periodate oxidation of the immobilized sheep erythrocyte membranes; Lane 3: Total protein profile of the immobilized sheep erythrocyte membranes following reaction with a succinimide ester conjugated fluorescein hapten.

Four distinct bands can be identified at 135 kDa, 70 kDa, 50 kDa and 25 kDa, respectively. Lane 3 is a total carbohydrate stain of sheep erythrocyte membranes clearly indicating that the majority of the carbohydrate is in the region of 50 kDa. In addition, the bands recognised by mAb JC6 (lane 2) tend to correlate with

carbohydrate stained bands (lane 3). Lane 4 shows a total protein profile of sheep erythrocyte membranes.

4.3.4 Fab'-peptide conjugate preparation

The preparation of the anti-erythrocyte Fab'-peptide conjugate was illustrated in Figure 1.13. It is based on the procedure developed by Brennan *et al.* (1985) for the preparation of bispecific antibodies. The SDS-PAGE gel analysis of the product at each stage of the anti-sheep erythrocyte Fab'-peptide conjugate preparation is shown in Figure 4.3.

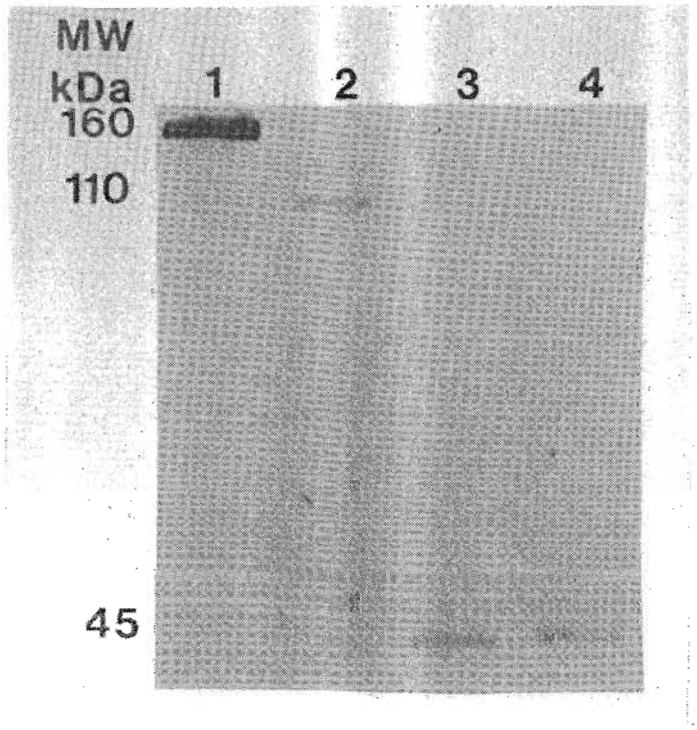

Figure 4.3 12.5% SDS-PAGE of samples taken at various stages of conjugate preparation. Lane 1: Intact antibody; Lane 2: $F(ab)_2$ fragment; Lane 3: Fab'-TNB-Ac; Lane 4: Fab'-peptide conjugate.

The intact anti-sheep erythrocyte antibody (lane 1) was subjected to pre-activated papain digestion to produce $F(ab)_2$ fragments (lane 2) (Parham *et al.*, 1982). This was

necessary as pepsin digestion was found to produce a variety of intermediate products due to indiscriminate digestion of the intact antibody (results not shown). The $F(ab)_2$ fragments were reduced to Fab' fragments (lane 3) using 2-mercaptoethylamine (2-MEA). The reduced hinge sulfhydryls were protected with 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Brennan *et al.*, 1985). Addition of reduced gp40 (785-800) peptide displayed the TNB to give an Fab'-peptide conjugate. Any residual sulfhydryl groups were blocked with iodoacetamide. This is an important step as it prevents disulfhide exchange and the rapid loss of peptide from the Fab' fragment. Purification of each resultant product indicated in Figure 4.3 described in 4.2.12 eliminated all non-reacted or chemically reformed protein fragments. These purification steps proved to be vital to prevent any non-specific agglutination reactions.

4.3.5 Evaluation of the test

The agglutination reaction was scored visually on a scale of 0 (negative) to 4+ (strong positive) (Wilson *et al.*, 1991). The test was performed using the conjugate at 10 μg/ml in PBS/azide supplemented with 0.5% fish gelatin. The choice of peptide used was based on ELISA results published earlier (Kwang and Torres, 1994) showing the antigenic nature of this particular peptide which represents an immunodominant region of the envelope gp40 protein of the Maedi-Visna virus. The Fab'-peptide conjugate was tested against 10 MVV seropositive and 10 seronegative serum samples as ascertained using the gp40 (785-800) peptide ELISA as reference assay. The agglutination strength observed for each sample is shown in Table 4.2.

Table 4.2 Red cell agglutination results and peptide inhibition tests on 10 seronegative and 10 seropositive MVV serum samples.

Negative sample	Agglutination Result		Positive sample	Agglutination Result	
	No peptide	Added peptide		No peptide	Added peptide
1	0	0	11	2+	0
2	0	0	12	2+	0
3	1+	1+	13	3+	1+
4	0	0	14	2+	0
5	0	0	15	1+	0
6	0	0	16	2+	0
7	0	0	17	0	0
8	1+	1+	18	2+	0
9	0	0	19	2+	1+
10	0	0	20	2+	0

The specificity of each agglutination reaction was assessed by the addition of 50 μg free gp40 (785-800) peptide to each serum sample and compared with the agglutination strength observed without the presence of free peptide (Table 4.2). Negative samples 3 and 8 showed false positive agglutination reactions which could not be inhibited by the addition of free peptide. Positive sample 17 showed a false negative agglutination reaction. Furthermore, positive samples 13 and 19 could not be totally inhibited by the presence of free peptide indicating that some of the agglutination observed is due to peptide non-specific reactions. Although the 10 positive serum samples were shown to be highly reactive towards the gp40 (785-800) peptide on ELISA, only relatively weak agglutination reactions were observed with the Fab'-peptide conjugate. Sensitivity, specificity and predictive value positive/negative and false positive/negative rate values for the gp40 (785-800) peptide ELISA and Fab'-peptide agglutination assay are summarized in Table 4.3.

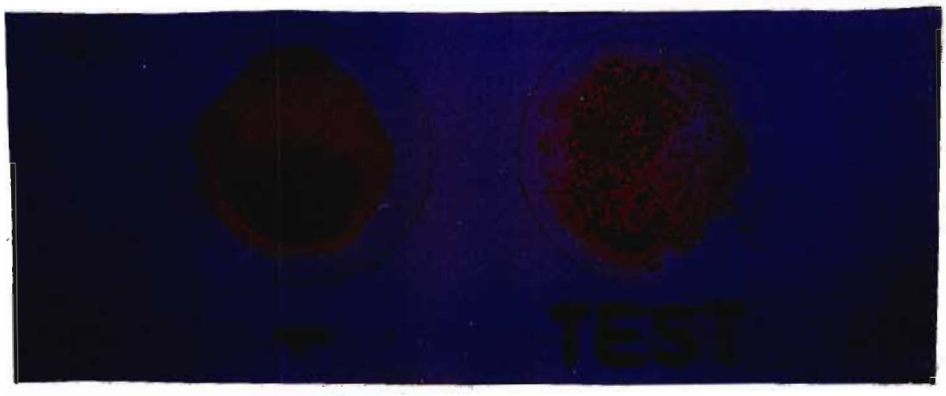

Figure 4.4 Visual agglutination of a MVV seronegative and seropositive whole blood sample, respectively. The Fab'-peptide conjugate was added to each sample and scored after 2 min.

Table 4.3: Sensitivity, specificity, predictive value positive/negative and false positive/negative rate values for the gp40 (785-800) peptide ELISA and the whole blood agglutination test.

Assay	Sensitivity	Specificity	PVP	PVN	FPR	FNR
gp40 peptide ELISA	100%	100%	100%	100%	0%	0%
whole blood agglutination	90%	80%	83%	90%	20%	10%

PVP: Predictive value positive; PVN: Predictive value negative; FPR: False positive rate; FNR: False negative rate.

4.3.6 Western blot analysis of discrepant samples

The two false positive samples (3 and 8) and the false negative sample (17) (see Table 4.2) were analyzed by Western blot for reactivity towards the Fab'-peptide conjugate (Figure 4.5).

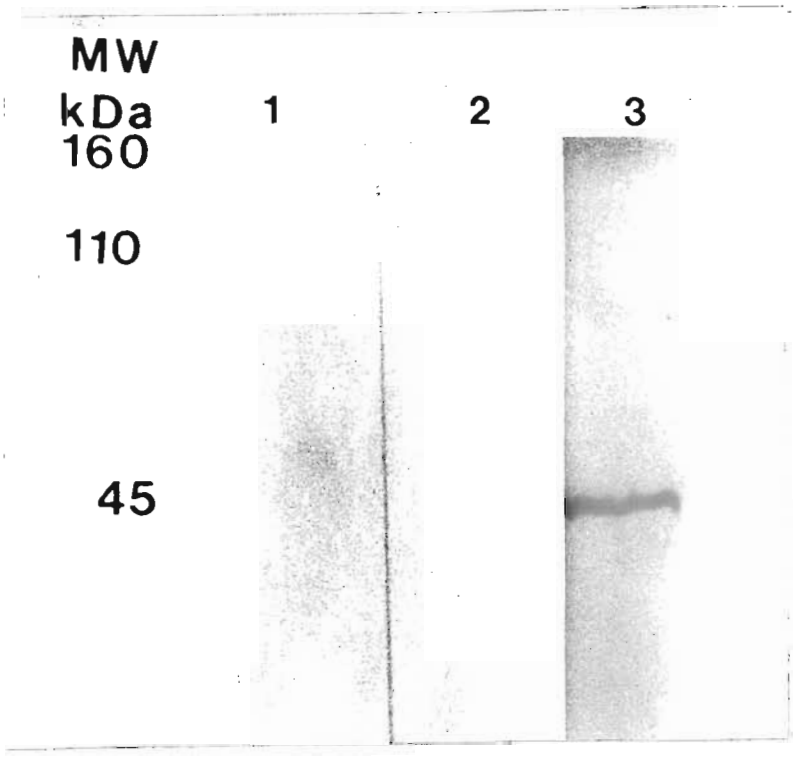

Figure 4.5 Western blot analysis of discrepant agglutination samples: False positive samples [3 and 8] (lanes 1 and 2, respectively), and false negative sample [17] (lane 3) as revealed in Table 4.2.

Figure 4.5 shows that neither sample 3 (lane 1) nor 8 (lane 2) reacted with the Fab'-peptide conjugate, further substantiating the notion that the false agglutination that occurred was due to peptide non-specific reactions. However, sample 17 reacted strongly with the Fab'-peptide conjugate (Figure 4.5, lane 3) confirming its specificity towards the coupled peptide. The reason for its inability to produce agglutination, however, is not clear.

4.4 DISCUSSION

Mammalian erythrocyte membrane proteins carry numerous cell surface antigens that have been studied extensively (Hamaguchi and Cleve, 1972). The proteins of erythrocyte membranes from various species have been compared by SDS-PAGE followed by protein and carbohydrate staining. Although a marked similarity between the protein profiles is evident amongst the various animal species, differences in periodic acid Schiff (PAS) staining bands corresponding to glycophorin have been noted (Tillack *et al.*, 1972; Fujita and Cleve, 1975). Glycophorins are present in abundance and carry a large portion of the total carbohydrate on the erythrocyte membranes. Comparison of the complete amino acid sequences of human glycophorin A (Tomita *et al.*, 1978), porcine glycophorin (Honma *et al.*, 1980) and horse glycophorin (Murayama *et al.*, 1981) show that there is no significant homology between the sequences of the extracellular amino-terminal glycosylated domains. This is in line with the contention that glycophorin is a species-specific antigen.

We have described the isolation and characterization of a mAb, JC6, which recognises a sheep specific membrane protein, possibly glycophorin, that shows no reactivity towards human, bovine, rabbit and goat erythrocytes (data not shown). The epitope is present on all sheep breeds tested (Table 4.1).

Selective chemical and enzymatic treatments revealed that trypsin treatment of the epitope had little effect on its recognition by mAb JC6 (Figure 4.1). This was surprising but could be due to the absence of a trypsin site or more likely the

inaccessibility of such a cleavage site as a result of attached polysaccharide chains (Furthmayr and Marchesi, 1983; Wasniowska *et al.*, 1992; Wasniowska *et al.*, 1993). In contrast, periodate oxidation reduced the ability of mAb JC6 to bind to the immobilized sheep erythrocytes emphasising the carbohydrate nature of the recognised epitope (Figure 4.1). In addition, neuraminidase treatment of the immobilized sheep erythrocytes destroyed the recognition of mAb JC6 indicating that sialic acid residues are required to constitute the epitope recognised by mAb JC6 (Figure 4.1). Western immunoblot analysis, Figure 4.2 (lane 1), showed four separate bands appearing at 25 kDa, 50 kDa, 70 kDa and 135 kDa, respectively. This might indicate that two distinct monomeric forms of glycophorin are present at 25 kDa and 70 kDa, respectively, or that they can exist as dimers. The majority of carbohydrate is associated with a 50 kDa protein (Figure 4.2, lane 2), although different protein bands were visualised over a wide range of molecular masses (Figure 4.2, lane 3). It is possible that the major sialoglycoprotein of sheep erythrocytes exists as a dimer configuration at 50 kDa.

Current immunoassays available for the diagnosis of MVV infection are complex, require several washing steps and expensive instrumentation. Technician error and subject variability further limit the utility of these techniques as field diagnostic assays. In addition, it has been shown that stored sera may give occasional false positive results in ELISAs (Gruffydd-Jones *et al.*, 1988).

The simplicity of the whole blood agglutination assay, which requires no technical manipulation, is an appealing veterinary field test as previously described for the feline

immunodeficiency virus (Wilson *et al.*, 1994; del Fierro *et al.*, 1995). In this chapter the development and evaluation of a similar assay format for the detection of Maedi-Visna virus in sheep is described. The principle of the endogenous agglutination assay is illustrated in Figure 1.13.

Possible deactivation of the antigen binding portion of an antibody that occurs when cross-linking whole antibodies, is avoided by selective enzymatic cleavage to obtain $F(ab)_2$ fragments and subsequent coupling of the antigenic peptide at the antibody hinge region. This approach, first applied by Nisonoff and Rivers (1961), also has the advantage of removing the hydrophobic Fc region which is implicated in non-specific binding (Boscato and Stuart, 1988) and numerous effector functions including complement binding and interactions with rheumatoid factor and heterophile antibodies (Gosling, 1990; Kato *et al.*, 1979). By removing the Fc region and using Fab' fragments the likelihood of obtaining a false positive agglutination through non-specific interactions is reduced.

The MVV agglutination test was assessed by direct comparison with the corresponding MVV gp40 (785-800) peptide ELISA (Table 4.2). Kwang and Torres (1994), showed that the above mentioned synthetic peptide is able to detect the presence of MVV antibodies in 96% of infected sheep. Only previously tested MVV seropositive and seronegative samples as confirmed by the gp40 (785-800) peptide ELISA were used to evaluate the performance of the agglutination test. The purpose of this study was to demonstrate the feasibility of the autologous red cell agglutination test for antibodies to MVV and not to attempt a comprehensive trial. Thus, the results are a preliminary

assessment of the sensitivity and specificity of the MVV agglutination test. Although the number of sheep used for the study was limited by the amount of prepared conjugate and free peptide used to inhibit specific agglutination, 10 seronegative and 10 seropositive samples were tested. The red cell agglutination test had a sensitivity of 90% and a specificity of 80% (Table 4.3). The calculated predictive value positive and predictive value negative (Table 4.3), which are a reflection of the reliability of a test, indicate limitations in the use of the agglutination assay compared to the gp40 peptide ELISA.

The principal contributor to false positive results was the autoagglutinating nature of monoclonal antibody JC6. The whole blood agglutination test system is a multi-step, expensive and error-prone process based on the chemical conjugation of Fab' fragments to peptides. Mixing the anti-red cell Fab' fragment with peptide could produce a number of theoretical reaction products, some of which are bivalent and cause autoagglutination of the red blood cells. This is because mouse IgG_1 contain three inter-heavy chain cysteines (Adetugbo, 1978; Sakano *et al.*, 1979) at the hinge region of the molecule. Thus, up to three TNB^{2-} per Fab' may have been substituted with three possible reaction products, namely Fab'-monopeptide, Fab'-dipeptide and Fab'-tripeptide. If it is assumed that the coupling process is random then the presence of Fab'-peptide-Fab', Fab' dimer- or $F(ab)_2$ and dipeptide cannot be excluded. The filtration purification method employed after each step to remove such unwanted by-products proved to be unsuccessful to fully block background agglutination. Western blot analysis of samples 3 and 8 (Figure 4.5) further substantiated the notion that the

false agglutination observed was more likely due to agglutinating antibody fragments than other non-specific reactions such as heterophile antibodies.

This by-product formation also emphasizes the need for a suitable purification method not only to separate Fab' from $F(ab)_2$ fragments but also Fab'-TNB from Fab'-peptide fragments. Since the Fab' and Fab'-peptide fragments are chemically closely related, affinity chromatography using a monoclonal antibody which is specific for the peptide can be used to separate the species. The inability of the filtration purification method to separate Fab' from Fab'-peptide might explain the overall low agglutination strength observed for the 10 seropositive samples (Table 4.2). The presence of contaminating Fab'-TNB fragments with the Fab'-peptide conjugate solution led to an underestimation of the actual Fab'-peptide conjugate concentration present. A final concentration of 10 µg/ml conjugate was used which implies that the actual working concentration of the conjugate was less than 10 µg/ml. Increasing the conjugate concentration, however, led to an increase in the percentage of false positive results obtained (results not shown). The inability of sample 17 to produce a positive agglutination result can possibly be attributed to an insufficient conjugate concentration to detect all positive samples. Sample 17, however gave a strong positive reaction with the Fab'-peptide conjugate using Western blot analysis (Figure 4.5) confirming its reactivity towards the conjugated peptide.

The current format for whole blood diagnostic reagent relies on the use of single-chain Fv fragments (scFv) with equivalent binding specificity to the parent monoclonal antibody (Lilley *et al.*, 1995). The possibility of producing a stable scFv fragment of

monoclonal antibody JC6 holds promise for developing a rapid immunoassay in ovine diagnostics similar to that developed for HIV in humans.

CHAPTER 5

CONCLUDING DISCUSSION

Several serological diagnostic tests have been developed to detect Maedi-Visna virus (MVV) specific antibodies, but only the agar gel immunodiffusion (AGID) test and the whole-virus (WV) ELISA proved to be of practical value. In addition to poorly defined sensitivity and specificity, a major problem for both the AGID test and WV ELISA is the general low quality and high expense associated with viral test antigen production. Recently, recombinant transmembrane protein (TM) and major capsid protein p25 were used as solid phase antigens in enzyme-linked immunosorbent assays. It has also been reported that antibody against p25 appears prior to the TM protein, although the TM immunoassay has proved to be the most accurate and sensitive at the subclinical and overt clinical disease stages. The implication at the practical level is that a MVV serological assay should include both the p25 and TM proteins to be able to detect early infected animals as well.

In this thesis the cloning and expression of antigenic regions of the Maedi-Visna virus and their use as diagnostic reagents is described. Recent advances in biotechnology have made available many options for expressing and producing protein products. Historically, the bacterial expression system has been the most extensively used heterologous expression system and has resulted in the accumulation of a large body of literature. Therefore, gene fragments coding for the major core p25 protein and TM protein were cloned in frame with the pGEX-2 prokaryotic expression vector. The pGEX series of vectors permit the expression of recombinant proteins in *Escherichia*

coli as fusion proteins with glutathione S-transferase. Unique high affinity binding sites incorporated into the fusion partner enables rapid one-step purification of the GST fusion protein. The p25 and TM protein products were shown to be antigenically authentic, both as separate proteins or as combined expressed fusion protein. Since no absolute method ("gold standard") for the diagnosis of subclinical MVV infection exists, authenticity of the above mentioned proteins could only be defined by its reactivity with control antisera from pathologically negative and positive animals, respectively.

Comparative analysis of the ELISA performance of the expressed GST-p25, GST-TM and combined expressed GST-TM-p25 proteins has shown the superior diagnostic ability of the combined expressed GST-TM-p25 fusion protein. Since only sheep older than three years of age were included in the study, a predominantly anti-TM antibody titre was expected. This was reflected by the GST-TM and p25 commercial assay giving a 100% and 87% sensitivity, respectively. The conclusion can therefore be made that the ideal serologic assay for MVV should contain at least these two antigens since any individual serum may react with only one of them during the course of infection, but is believed to be unlikely not to react with either of them.

Often, bacterial cells tend to accumulate the heterologous protein as an insoluble product, concentrating the protein within the cell in inclusion bodies. Subsequent use of denaturing conditions to solubilize such fusion proteins may render the fusion protein resistant to affinity purification. Furthermore, the MVV TM protein is known to be hydrophobic, causing protein aggregation despite the fact that a truncated

version of the TM protein was expressed. As a result, modification of the induction conditions and solubilization procedures of the expressed fusion proteins were adopted. Induction of protein expression at 20°C and subsequent solubilization of the expressed protein with the non-denaturing detergent N-lauroylsarcosine circumvented the protein aggregation problem. However, the possibility of non-specific reactions remained, as reflected by the slight drop in specificity observed for the GST-TM assay compared with that of the commercial p25 and GST-TM-p25 assay. Nevertheless, the recombinant pGEX expressed fusion protein ELISAs had cost and performance advantages over the conventional methods for anti-MVV antibody detection.

The solubility problems encountered with the pGEX expression system prompted the search for an alternative expression system. The *Pichia pastoris* yeast expression system offered several advantages in the production of recombinant proteins. Among these advantages, the high-level secretion of the recombinant protein into the culture medium promised to simplify product recovery and purification. However, limited success was achieved in an attempt to express the MVV TM-polypeptide in high yields. A possible explanation might be that only a single gene copy of the TM-polypeptide was integrated into the *P. pastoris* genome. In addition, the small expected size of the expressed TM-polypeptide made detection by SDS-PAGE and Western-blot analysis even more problematic. Although the above mentioned attempts proved to be in vain, evaluation of the expressed and purified TM-polypeptide as a diagnostic reagent, could be effected by ELISA.

No diagnostic test exhibits perfect operating characteristics under all conditions. The traditional approach to improve the accuracy of diagnosis is to develop new tests with superior sensitivity and specificity. An alternative, yet rarely used approach, is to improve existing tests by altering the cut-off value that distinguishes infected individuals from uninfected individuals. For any given serological test, sensitivity and specificity are determined by the cut-off value. Two steps are essential for the selection of cut-off values in any quantitative diagnostic test. Firstly, a statistically appropriate method has to be chosen that permits valid extrapolations from observed test results of reference samples to the situation when the test is applied to the target population. Secondly, a cut-off value has to be selected which differentiates the two subpopulations of infected and non-infected controls with defined operating characteristics. In many applications of serodiagnostic tests it may be appropriate to select cut-off values with equal weights on sensitivity and specificity. This thesis presented the use of a modified receiver operating characteristic (ROC) analysis programme which depicted sensitivity and specificity separately versus the selected cut-off value. The construction and application of this method, including the definition of "intermediate" test results and the "valid range proportion" are exemplified using data from the pGEX and the *P. pastoris* expressed recombinant antigens, respectively.

New diagnostic techniques are evolving rapidly and each provides new opportunities which can be beneficial to the study and treatment of veterinary diseases. In many cases these techniques are developed in human medicine, but can easily be adapted to veterinary disease problems. One such recent advance has been the preparation of antibody conjugates and their application to autologous red blood cell agglutination

assays. The conjugate consisted of a peptide, representing an immunodominant portion of MVV, coupled to the anti-sheep red blood cell monoclonal antibody. When added to whole blood, red blood cells are bound by the antibody domain, effectively coating the erythrocytes with antigen. In the absence of antibodies to the coupled antigen, no agglutination is expected, but cross-linking can occur if antibodies are present. Within a limited number of 10 seronegative and 10 seropositive samples the MVV autologous whole blood agglutination test had a sensitivity of 90% and a specificity of 80%. The principal contribution to the false positive agglutination reactions was attributed to the autoagglutinating nature of monoclonal antibody JC6. This is due to the fact that despite the fact that monovalent Fab' fragments were used, reformation of some Fab' fragments still occurred. A possible improvement in the manufacturing of antibody conjugates may result from recent explorations in the genetic engineering of antibody molecules (Winter and Milstein, 1991). Bacterial expression products representing antibody fragments linked to a protein or peptide have significant advantages over chemical methodologies for the formation of such antibody-based reagents (Hudson *et al.*, 1992).

Molecular biology, in its relatively short history, has dramatically altered our perception of infectious diseases and the paths that should be followed to improve the health of animals. Steady progress has resulted in a number of practical applications to the study and diagnosis of infectious diseases of veterinary importance. Work presented in this thesis illustrates the use of recombinant DNA and monoclonal antibody technology as applied to the serological diagnosis of MVV. Undoubtedly, these technologies will present many new opportunities for diagnosis and the

prevention of a wide range of diseases and as such their impact is certain to grow over the years to come.

REFERENCES

Adetugbo, K. (1978) Evolution of immunoglobulin subclasses, primary structure of a murine myeloma gamma 1 chain. J. Biol. Chem. 253, 6068-6075.

Allain, J.P., Laurian, Y., Paul, D.A. and Senn, D. (1986) Serologic markers in early stages of human immunodeficiency virus infection in haemophiliacs. Lancet II, 1233-1236.

Arya, A., Batra, H.V., Sahai, P. and Mukherjee, R. (1994) Production and characterisation of new murine monoclonal antibodies reactive to *Mycobacterium tuberculosis*. Hybridoma 13(1), 21-30.

Audoly, G., Sauze, N., Harkiss, G., Vitu, C., Russo, P., Querat, G., Suzan, M. and Vigne, R. (1992) Identification and subcellular localisation of the Q gene product of visna virus. Virology 189, 734-739.

August, M.J., Harter, D.H. and Compans, R.W. (1977) Characterisation of visna virus envelope neuraminic acid. J. Virol. 22, 832-834.

Banks, K.L., Adams, D.S., McGuire, T.C. and Carlson, J. (1983) Experimental infection of sheep by caprine-arthritis encephalitis virus and goats by progressive pneumonia virus. Am. J. Vet. Res. 44, 2307-2311.

Barajas-Rojas, J.A., Riemann, H.P. and Franti, C.E. (1993) Notes about determining the cut-off value in enzyme-linked immunosorbent assay (ELISA) [letter to the editor]. Prev. Vet. Med. 15, 231-233.

Barin, F., McLane, M.F., Allan, J.S., Lee, T.H., Groopman, J.E. and Essex, M. (1985) Virus envelope protein of HTLV-III represents major target antigen for antibodies in AIDS patients. Science 228(4703), 1094-1096.

Barr, K.A., Hopkins, S.A. and Sreekrishna, K. (1992) Protocol for efficient secretion of HSA developed from *Pichia pastoris*. Pharm. Eng. 12(2), 48-51.

Beatty, J.A., Reid, G., Rigby, M.A., Neil, J.C., Jarret, O. and Browning, M.J. (1992) Recombinant feline immunodeficiency virus envelope fusion protein stimulates peripheral lymphocytes from naive cats to proliferate in vitro. Vet. Immunol. Immunopathol. 35, 143-153.

Bironf, P. and Deluyker, H. (1985) Control programme for maedi/visna in Belgium. In: Slow Viruses in Sheep, Goats and Cattle. pp.123-1126. Commission of the European Communities.

Boscato, I.M. and Stuart, M.C. (1988) Heterophile antibodies: a problem for all immunoassays. Clin. Chem. 34, 27-33.

Boshoff, C.H., Coetzee, L., Visser, L. and Verschoor, J.A. (1992) Spontaneous hybridoma formation induced by immunisation with *Haemophilus paragallinarum*: evidence for a lipopolysaccharide fusion inducer. Hybridoma 11, 257-266.

Boshoff, C.H., Dungu, B., Williams, R., Vorster, J., Conradie, J.D., Verwoerd, D.W. and York, D.F. (1996a) Detection of Maedi-Visna virus antibodies using a single fusion transmembrane-core p25 recombinant protein ELISA and a modified receiver-operating characteristic analysis to determine cut-off values. Submitted to J. Virol. Methods.

Boshoff, C.H., Conradie, M., Green, F.A., York, D.F. and Conradie, J.D. (1996b) Preparation and characterisation of a monoclonal antibody against a species-specific antigen on sheep erythrocytes. Submitted to Hybridoma.

Boshoff, C.H., York, D.F. and Conradie, J.D. (1996c) Development of a rapid whole blood agglutination test for Maedi-Visna virus. Submitted to Onderstepoort J. Vet. Res.

Boyden, S.V. (1951) The adsorption of proteins on erythrocytes treated with tannic acid and subsequent haemagglutination by antiprotein sera. J. Exp. Med. 93, 107-120.

Brahic, M., Filippi, P., Vigne, R. and Haase, A.T. (1977) Visna virus RNA synthesis. J. Virol. 24, 74-81.

Brennan, M., Davidson, P.F., Paulus, H. (1985) Preparation of bispecific antibodies by chemical recombination of monoclonal immunoglobulin G_1 fragments. Science 229, 81-83.

Buckholz, R.G. and Gleeson, M.A.G. (1991) Yeast systems for the commercial production of heterologous proteins. Bio/Technology 9, 1067-1072.

Bulgin, M.S. (1990) Ovine progressive pneumonia, caprine arthritis-encephalitis, and related lentiviral diseases of sheep and goats. Vet. Clin. N. Am.: Food Anim. Pract. 6(3), 691-703.

Bush, G.L., Tassin, A-M., Friden, H. and Meyer, D.I. (1991) Secretion in yeast. Purification and *in vitro* translocation of chemical amounts of prepro-α-factor. J. Biol.Chem. 266, 13811-13814.

Campbell, A.M. (1987) Monoclonal Antibody Technology. Elsevier.

Carey, N. and Dalziel, R.G. (1993) The biology of Maedi-Visna virus- an overview. Br. Vet. J. 149, 437-454.

Clare, J.J., Romanos, M.A., Rayment, F.B., Rowedder, J.E., Smith, M.A., Payne, M.M., Sreekrishna, K. and Henwood, C.A. (1991) Production of mouse epidermal

growth factor in yeast: high-level secretion using *Pichia pastoris* strains containing multiple gene copies. Gene 105, 205-212.

Clements, J.E., Gdovin, S.L., Montelaro, R. and Narayan, O. (1988) Antigenic variation in lentiviral diseases. Annu. Rev. Immunol. 6, 139-159.

Constantine, N.T., Callahan, J.D. and Watts, D.M. (1992) Retroviral testing. Essentials for quality control and laboratory diagnosis. CRC Press.

Coombs, R.R.A. (1981) Assays utilising red cells as markers. In: Immunoassays for the 80's, pp. 17-34. Edited by A. Voller, A. Bartlett, D. Bidwell. Lancaster: MTP Press Ltd.

Coombs, R.R.A. (1987) Harnessing the red cell for immunoassays. Med. Lab. Sci. 44, 66-72.

Coombs, R.R.A. (1989) Viewpoint of a general immunologist. Vet. Rec. 124, 553-557.

Coulter, A. and Harris, R. (1983) Simplified preparation of rabbit Fab fragments. J. Immunol. Meth. 59, 199-203.

Cranage, M.P., Gurner, B.W., Coombs, R.R.A. (1983a) Gluteraldehyde stabilisation of antibody-linked erythrocytes for use in reverse passive and related haemagglutination assays. J. Immunol. Methods 64, 7-16.

Cranage, M.P., McLean, C.S., Buckmaster, E.A., Minson, A.C., Wildy, P., Coombs, R.R.A. (1983b) The use of monoclonal antibodies in (reverse) passive haemagglutination tests for herpes simplex virus antigens and antibodies. J. Med. Virol. 11, 295-306.

Crawford, T.B., Adams, D.S., Cheevers, W.P. and Cork, L.C. (1980) Chronic arthritis in goats caused by a retrovirus. Science 207, 997-999.

Cregg, J.M., Barringer, K.J., Hessler, A.Y. and Madden, K.R. (1985) *Pichia pastoris* as a host system for transformations. Mol. Cell. Biol. 5(12), 3376-3385.

Cregg, J.M. and Madden, K.R. (1987) Development of the methylotrophic yeast, *Pichia pastoris*, as a host system for the production of foreign proteins. Dev. Ind. Microbiol. 29, 33-41.

Cregg, J.M., Tschopp, J.F., Stillman, C., Siegel, R., Akong, M., Craig, W.S., Buckholz, R.G., Madden, K.R., Kellaris, P.A., Davis, G.R., Smiley, B.L., Cruze, J., Torregrossa, R., Velicelebi, G. and Thill, G.P. (1987) High-level expression and efficient assembly of hepatitis b surface antigen in the methylotrophic yeast *Pichia pastoris*. Bio/Technology 5, 479-485.

Cregg, J.M., Vedvick, T.S. and Raschke, W.C. (1993) Recent advances in the expression of foreign genes in *Pichia pastoris*. Bio/Technology 11, 905-910.

Cutlip, R.C. and Laird, G.A. (1976) Isolation and characterisation of a virus associated with progressive pneumonia (maedi) of sheep. Am. J. Vet. Res. 37, 1377-1382.

Cutlip, R.C., Jackson, T.A. and Lehmkuhl, H.D. (1977) Immunodiffusion test for ovine progressive pneumonia. Am. J. Vet. Res. 38, 1081-1084.

Cutlip, R.C., Howard, D., Lehmkuhl, H.D., Brogden, K.A. and Bolin, S.R. (1985) Mastitis associated with ovine progressive pneumonia virus infection in sheep. Am. J. Vet. Res. 46 (2), 326-328.

Cutlip, R.C., Lehmkuhl, H.D., Sacks, J.M. and Weaver, A.L. (1992) Seroprevalence of ovine progressive pneumonia virus in sheep in the United States as assessed by analysis of voluntarily submitted samples. Am. J. Vet. Res. 56(6), 976-979.

Daniel, M.D., Letvin, N.L., King, N.W., Kannagi, M., Sehgal, P.K., Hunt, R.D., Kanki, P.J., Essex, M. and Desrosiers, R.C. (1985) Isolation of T-cell tropic HTLV-III-like retrovirus from Macaques. Science 228, 1201-1204.

Davis, J.L. and Clements, J.E. (1988) Complex gene expression of lentiviruses. Microb. Pathogen. 4, 239-245.

Davis, J.L. and Clements, J.E. (1989) Characterisation of a cDNA clone encoding the visna virus transactivating protein. Proc. Natl. Acad. Sci., U.S.A. 86, 414-418.

Dawson, M., Done, S.H., Venables, C. and Jenkins, C.E. (1990) Maedi-Visna and sheep pulmonary adenomatosis: a study of concurrent infection. Brit. Vet. J. 146, 531-538.

De Boer, G.F. (1970) Antibody formation in zwoegerziekte, a slow infection in sheep. J. Immunol. 104, 414-422.

De Boer, G.F., Terpstra, C. and Houwers, D.J. (1979) Studies in epidemiology of maedi/visna in sheep. Res. Vet. Sci. 26, 202-208.

de la Concha-Bermejillo, A., Brodie, S.J., Magnus-Corral, S., Bowen, R.A. and DeMartini, J.C. (1995) Pathological and serologic responses of isogenic twin lambs to phenotypically distinct lentiviruses. J. Acq. Imm. Def. Syn. Hum. Ret. 8, 116-123.

del Fierro, G.M., Bundesen, P., Martin, S., Jones, S., Beetson, S. and Robinson, W.F. (1995) Evaluation of an autologous red cell agglutination test, VetRED FIV™, for the presence of FIV antibody in cats. Vet. Immunol. Immunopath. 46, 93-101.

DeMartini, J.C., Rosadio, R.H., Sharp, J.M., Russel, H.I. and Lairmore, M.D. (1987) Experimental coinduction of type D retrovirus-associated pulmonary carcinoma and

lentivirus-associated lymphoid interstitial pneumonia in lambs. J. Natl. Cancer. Instit. 79, 167-177.

Digan, M.E., Lair, S.V., Brierley, R.A., Siegel, R.S., Williams, M.E., Ellis, S.B., Kellaris, P.A., Provow, S.A., Craig, W.S., Velicelebi, G., Harpold, M.M. and Thill, G.P. (1989) Continuous production of a novel lysozyme via secretion from the yeast, *Pichia pastoris*. Bio/Technology 7, 160-164.

Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) The preparation and chemical characteristics of haemoglobin-free ghosts of human erythrocytes. Arch. Biochem. Biophys. 100, 119-123.

Echols, H. (1990) Nucleoprotein structures initiating DNA replication, transcription and site-specific recombination. J. Biol. Chem. 265, 14697-14700.

Edelman, G.M., Benacerraf, B., Ovary, Z., Poulik, M.D. (1961) Structural differences among antibodies of different specificities. Proc. Natl. Acad. Sci. U.S.A. 47, 1751-1758.

Edelman, G.M., Benacerraf, B. (1962) On structural and functional relations between antibodies and proteins of the gamma-system. Proc. Natl. Acad. Sci. U.S.A. 48, 1035-1042.

Edelman, G.M., Olins, D.E., Gally, J.A., Zinder, N.D. (1963) Reconstitution of immunologic activity by interaction of polypeptide chains of antibodies. Proc. Natl. Acad. Sci. U.S.A. 50, 753-760.

Edelman, G.M., Gall, W.E., Waxdal, M.J., Konigsberg, W.H. (1968). The covalent structure of a human gammaG-immunoglobulin. Isolation and characterisation of the whole molecule, the polypeptide chains, and the tryptic fragments. Biochemistry 7, 1950-1958.

Ellis, T., Robinson, W. and Wilcox, G. (1983) Effect of colostrum deprivation of goat kids on the natural transmission of caprine retrovirus infection. Aust. Vet. J. 60, 326-329.

Ellis, S.B., Brust, P.F., Koutz, P.J., Waters, A.F., Harpold, M.M. and Gingeras, T.R. (1985) Isolation of alcohol oxidase and two other methanol regulatable genes from the yeast *Pichia pastoris*. Mol. Cell. Biol. 5(5), 1111-1121.

Ellman, G.L. (1959) Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82, 70-77.

Emini, E.A., Hughes, J.V., Perlow, D.S. and Boger, J. (1985) Induction of a hepatitis A virus neutralising antibody by a virus specific synthetic peptide. J. Virol. 55, 836-839.

Engvall, E. and Perlman, P. (1971) Enzyme-linked immunosorbent assay (ELISA). Quantitative assay for immunoglobulin G. Immunochemistry 8, 871-874.

Francis, H.L., Kabeya, M., Kafuama, N., Riggins, C., Colebunders, R., Ryder, R., Curran, J., Izaley, L., Quinn, T.C. (1988) Comparison of sensitivities and specificities of latex agglutination and an enzyme-linked immunosorbent assay for detection of antibodies to the human immunodeficiency virus in African sera. J. Clin. Microbiol. 26, 2462-2464.

Fujita, S. and Cleve, H. (1975) Isolation and partial characterisation of the major glycoproteins of horse and swine erythrocyte membranes. Biochem. Biophys. Acta. 406, 206-213.

Furthmayr, H. and Marchesi, V.T. (1983) Glycophorins- isolation, orientation and localisation of specific domains. Meth. Enzymol. 96, 268-280.

Galfre, G. and Milstein, C. (1981) Preparation of monoclonal antibodies: strategies and procedures. Meth. Enzymol. Ed. J.J. Langone and H. Van Vunakis, 73, 3-41, Academic Press.

Gallaher, W.R., Ball, J.M., Garry, R.F., Griffin, M. and Montelaro, R.C. (1989) A general model for the surface glycoproteins of HIV and other retroviruses. AIDS Res. Human Retroviruses 11, 191-202.

Gendelman, H.E., Narayan, O., Molineaux, S., Clements, J.E. and Ghotbi, Z. (1985) Slow, persistent replication of lentiviruses: role of tissue macrophages and macrophage precursors in bone marrow. Proc. Natl. Acad. Sci., U.S.A. 82, 7086-7090.

Ghosh, S., Basu, S., Strum, J.C., Basu, S. and Bell, R.M. (1995) Identification of conditions that facilitate the expression of GST fusions as soluble, full-length proteins. Anal. Biochem. 225, 376-378.

Gnann, J.W., Nelson, J.A. and Oldstone, M.B. (1987) Fine mapping of an immunodominant domain in the transmembrane glycoprotein of human immunodeficiency virus. J. Virol. 61(8), 2639-2641.

Goding, J.W. (1983) Use of staphylococcal protein A as an immunological reagent. J. Immunol. Methods 20, 241-253.

Gonda, M.A., Wong-Staal, F., Gallo, R.C., Clements, J.E., Narayan, O. and Gilden, R.V. (1985) Sequence homology and morphologic similarity of HTLV-III and visna virus, a pathogenic lentivirus. Science 227, 173-177.

Gonda, M.A., Braun, M.J., Carter, S.G., Kost, T.A., Bess, J.W., Arthur, L.O. and Van der Maaten, M. (1988) Characterisation and molecular cloning of a bovine lentivirus related to human immunodeficiency virus. Nature 330, 388-391.

Gosling, J.P. (1990) A decade of development in immunoassay methodology. Clin. Chem. 36, 1408-1427.

Gourdou, I., Mazarin, V., Querat, G., Sauze, N. and Vigne, R. (1989) The open reading frame S of visna virus is a trans-activating gene. Virology 171, 170-178.

Greiner, M. and Böhning, D. (1994) Notes about determining the cut-off value in enzyme-linked immunosorbent assay (ELISA). Reply Prev. Vet. Med. 20, 307-310.

Greiner, M., Sohr, D. and Göbel, P. (1995) A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests. J. Immunol. Methods 185, 123-132.

Grieco, F., Hay, J.M. and Hull, R. (1992) An improved procedure for the purification of protein fused with glutathione S-transferase. Biotechniques 13, 856-857.

Griffin, D.E., Narayan, O. and Adams, R.J. (1978) Early immune responses in visna, a slow viral disease of sheep. J. Infect. Dis. 138, 340-350.

Griner, P.F., Mayewski, R.J., Mushlin, A.I. and Greenland, P. (1981) Selection and interpretation of diagnostic tests and procedures. Ann. Intern. Med. 94, 553-591.

Gruffydd-Jones, T.J., Hopper, C.D., Harbour, D.A. and Lutz, H. (1988) Serologic evidence of feline immunodeficiency virus infection in UK cats from 1975-76. Vet. Rec. 123, 569-570.

Gudnadottir, M. and Kristindottir, K. (1967) Complement-fixing antibodies in sera of sheep affected with visna and maedi. J. Immunol. 98, 663-667.

Gudnadottir, M. and Palsson, P.A. (1967) Transmission of maedi by inoculation of a virus grown in tissue culture from maedi-affected lungs. J. Infect. Dis. 117, 1-6.

Haase, A.T. and Varmus, H.E. (1973) Demonstration of a DNA provirus in the lytic growth of visna virus. Nature New Biol. 245, 237-239.

Hamaguchi, H. and Cleve, H. (1972) Solubilization and comparative analysis of mammalian erythrocyte membrane glycoproteins. Biochem. Biophys. Res. Commun. 47, 459-464.

Hampson, D.J., Mhoma, J.R.L. and Combs, B. (1989) Analysis of lipopolysaccharide antigens of *Treponema hyodysenteriae*. Epidem. Inf. 103, 275-284.

Hanahan, D. (1985) In: DNA Cloning Vol 1 (Glover, D.M., ed.), IRL Press, Oxford, pp.109-135 (1985).

Hardy, W.D. (1991) General principles of retrovirus immunodetection tests. J. Am. Vet. Med. Assoc. 199, 1282-1287.

Harris, J.D., Blum, H., Scott, J.V., Traynor, B., Ventura, P. and Haase, A. (1984) Slow virus visna: Reproduction *in vitro* of virus from extrachromosomal DNA. Proc. Natl. Acad. Sci., U.S.A. 81, 7212-7215.

Hess, J.L., Clements, J.E. and Narayan, O. (1985). *Cis* and *trans*-acting transcriptional regulation of visna virus. Science 229, 482-485.

Hillyard, C.J., Rylatt, D.B., Kemp, B.E., Bundesen, P.G. (1990) Erythrocyte agglutination assay. US Patent No. 4 894 347.

Honma, K., Tomita, M. and Hamada, A. (1980) Amino acid sequence and attachment sites of oligosaccharide units of porcine erythrocyte glycophorin. J. Biochem. 88, 1679-1691.

Hopp, T.P. and Woods, K.R. (1981) Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. U.S.A. 78, 3824-3828.

Houwers, D.J. and Gielkens, A.L.J. (1979) An ELISA for the detection of maedi-visna antibody. Vet. Rec. 104, 611-615.

Houwers, D.J., Gielkens, A.L.J. and Schaake, J. (1982) An indirect enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to Maedi-Visna virus. Vet. Microbiol. 7, 209-219.

Houwers, D.J. and Schaake, J. (1987) An improved ELISA for the detection of antibodies to ovine and caprine lentiviruses, employing monoclonal antibodies in a one-step assay. J. Immunol. Methods 98, 151-154.

Houwers, D.J. and van der Molen, E.J. (1987) A five-year serological study of natural transmission of maedi-visna virus in a flock of sheep, completed with post-mortem investigation. J. Vet. Med. Biol. 34, 421-431.

Houwers, D.J. and Nauta, I.M. (1989) Immunoblot analysis of the antibody response to ovine lentivirus infections. Vet. Microbiol. 19, 127-139.

Houwers, D.J., Visscher, A.H. and Defize, P.R. (1989) Importance of ewe/lamb relationship and breed in the epidemiology of maedi-visna virus infections. Res. Vet. Sci. 46, 5-8.

Hudson, P., Irving, R., Hoogenraad, N. and Hillyard, C.J. (1992) A new era in immunodiagnostics. Today's Life Science 4, 44-49.

Huso, D.L., Narayan, O. and Hart, G.W. (1988) Sialic acids on the surface of caprine arthritis-encephalitis virus define the biological properties of the virus. J. Virol. 62, 1974-1980.

Itakura, K., Hirose, T., Crea, R., Riggs, A.D., Heynecker, H., Bolivar, F. and Boyer, H.W. (1977) Expression in *Escherichia coli* of a chemically synthesised gene for the hormone somatostatin. Science 198, 1056-1063.

Janknecht, R., de Martynoff, G., Lou, J., Hipskind, R.A., Nordheim, A. and Stunnenberg, H.G. (1991) Rapid and efficient purification of native histidine-tagged

protein expressed by recombinant vaccinia virus. Proc. Natl. Acad. Sci. U.S.A. 88(20), 8972-8976.

Johnson, C.B., Donzella, G.A. and Roth, M.J. (1993) Characterisation of the forward and reverse integration reactions of the Maloney Murine Leukemia Virus integrase protein purified from *Escherichia coli*. J. Biol. Chem. 268, 1462-1469.

Kajikawa, O., Lairmore, M.D. and DeMartini, J.C. (1990) Analysis of antibody responses to phenotypically distinct lentiviruses. J. Clin. Microbiol. 28, 764-770.

Kato, K., Umeda, U., Suzuki, F., Hayashi, D., Kosaka, A. (1979) Use of antibody Fab' fragments to remove interference by rheumatoid factors with the enzyme-linked sandwich immunoassay. FEBS Letters 102, 253-256.

Keen, J., Kwang, J. and Rosati, S. (1995) Comparison of ovine lentivirus detection by conventional and recombinant serological methods. Vet. Immunol. Immunopath. 47, 295-309.

Kemp, B.E., Rylatt, D.B., Bundesen, P.G., Doherty, R.R., McPhee, D.A., Stapleton, D., Cottis, L.E., Wilson, K., John, M.A., Khan, J.M., Dinh, D.P., Miles, S. and Hillyard, C.J. (1988) Autologous red cell agglutination assay for HIV-1 antibodies. Science 241, 1352-1354.

Kennedy-Stoskopf, S. and Narayan, O. (1986) Neutralising antibodies to visna lentivirus: mechanism of action and possible role in virus persistence. J. Virol. 59, 37-44.

Knowles, D., Cheevers, W., McGuire, T., Stem, T. and Gorham, J. (1990) Severity of arthritis is predicted by antibody responses to gp135 in chronic infection with caprine arthritis-encephalitis virus. J. Virol. 64, 2396-2398.

Koutz, P.J., Davis, G.R., Stillman, C., Barringer, K., Cregg, J.M. and Thill, G. (1989) Structural comparison of the *Pichia pastoris* alcohol oxidase genes. Yeast 5, 167-177.

Köhler, G. and Milstein, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495-497.

Krieg, P. and Melton, D. (1984) Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs. Nucl. Acids Res. 12, 7057-7070.

Kwang, J. and Cutlip, R.C. (1992a) Analysis of antibody response to ovine lentivirus by using viral gene products expressed in a prokaryotic system. Biochem. Biophys. Res. Comm. 188(1), 20-27.

Kwang, J. and Cutlip, R.C. (1992b) Detection of antibodies to ovine lentivirus using a recombinant antigen derived from the envgene. Biochem. Biophys. Res. Comm. 183(3), 1040-1046.

Kwang, J., Keen, J., Cutlip, R.C. and Littledike, E.T. (1993) Evaluation of an ELISA for detection of ovine progressive pneumonia antibodies using a recombinant transmembrane envelope protein. J. Vet. Diagn. Invest. 5, 189-193.

Kwang, J. and Torres, J.V. (1994) Oligopeptide-based enzyme immunoassay for ovine lentivirus antibody detection. J. Clin. Microbiol. 32, 1813-1815.

Kwang, J., Kim, H.S., Rosati, S. and Lehmkuhl, H.D. (1995) Characterization of ovine lentivirus envelope glycoprotein expressed in *Escherichia coli* cell and baculovirus systems. Vet. Immunol. Immupath. 45, 185-193.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

Langone, J.J. (1982) Protein A of *Staphylococcus aureus* and related immunoglobulin receptors produced by streptococci and pneumonococci. Adv. Immunol. 32, 157-251.

Le Grice, S.F.J. and Gruninger-Leitch, F. (1990) Rapid purification of homodimer HIV-1 reverse transcriptase by metal chelate affinity chromatography. Eur. J. Biochem. 187, 307-314.

Lilley, G.G., Dolezal, O., Hillyard, C.J., Bernard, C. and Hudson, P.J. (1995) Recombinant single-chain antibody peptide conjugates expressed in *Escherichia coli* for the rapid diagnosis of HIV. J. Immunol. Methods 171, 211-226.

Lin, F.H. and Thormar, H. (1970) Ribonucleic acid-dependent deoxyribonucleic acid polymerase in visna virus. J.Virol. 6, 702-704.

Lutley, R., Petursson, G., Palsson, P.A., Georgsson, G., Klein, J. and Nathanson, N. (1983) Antigenic drift in visna. J. Gen. Virol. 64, 1433-1440.

Mage, M.G. and Lamoyi, E. (1987) Preparation of Fab and $F(ab')_2$ fragments from monoclonal antibodies. Immunol. Ser. 33, 79-97.

Marsh, H. (1923). Progressive pneumonia in sheep. J. Am. Vet. Med. Assoc. 62, 458-473.

Mazarin, V., Gourdou, I., Querat, G., Sauze, N. and Vigne, R. (1988) Genetic structure and function of an early transcript of visna virus. J. Virol. 62, 4813-4818.

Mazarin, V., Gourdou, I., Querat, G., Sauze, N., Audoly, G., Vitu, C., Russo, P., Rousselot, C., Filippi, P. and Vigne, R. (1990) Subcellular localisation of *rev*-gene product in visna virus-infected cells. Virology 178, 305-310.

McClure, M.A., Johnson, M.S. and Doolittle, R.F. (1987) Relocation of a protease-like gene segment between two retroviruses. Proc. Natl. Acad. Sci. U.S.A. 84, 2693-2697.

McGrath, M., Witte, O., Pincus, T. and Weissmann, L. (1978) Retrovirus purifications: Method that conserves envelope glycoprotein and maximizes infectivity. J. Virol. 25, 923-927.

Meric, C. and Goff, S.P. (1989) Characterization of Moloney Murine Leukemia Virus mutants with single-amino-acid substitutions in the Cys-His box of the nucleocapsid protein. J. Virol. 63, 1558-1568.

Mills, H.R., Berry, N., Burns, N.R. and Jones, I.M. (1992) Simple and efficient production of the core antigens of HIV-1, HIV-2 and simian immunodeficiency virus using pGEX expression vectors in *Escherichia coli*. AIDS (correspondence) 6(4), 437-439.

Murayama, J-I, Takeshita, K., Tomita, M. and Hamada, A. (1981) Isolation and characterization of two glycophorins from horse erythrocyte membranes. J. Biochem. 89, 1593-1598.

Myer, M.S., Huchzermeyer, H.F., York, D.F., Hunter, P., Verwoerd, D.W. and Garnet, H.M. (1988). The possible involvement of immunosuppression caused by a lentivirus in the aetiology of Jaagsiekte and Pasteurellosis in sheep. Onderstepoort J. Vet. Res. 55(3), 127-133.

Narayan, O., Griffin, D.E. and Clements, J.E. (1978) Virus mutation during "slow infection": temporal development and characterization of mutants of visna virus recovered from sheep. J. Gen. Virol. 41, 343-352.

Narayan, O., Wolinsky, J.S., Clements, J.E., Strandberg, J. D., Griffin, D. E. and Cork, L.C. (1982) Slow virus replication: the role of macrophages in the persistence and expression of visna viruses of sheep and goats. J. Gen. Virol. 59, 345-356.

Narayan, O. and Clements, J.E. (1989) Biology and pathogenesis of lentiviruses. J. Gen. Virol. 70, 1617-1639.

Nisonoff, A. and Rivers, M.M. (1961) Recombination of a mixture of univalent antibody fragments of different specificity. Arch. Biochem. Biophys. 93, 460-462.

Oliver, R.E., Gorham, J.R., Parish, S.F., Hadlow, W.J. and Narayan, O. (1981) Ovine progressive pneumonia: pathologic and virologic studies on the naturally occurring disease. Am. J. Vet. Res. 42, 1554-1559.

Palsson, P.A. (1976). Maedi and Visna in sheep. In: R.H. Kimberlin (Ed.), Slow Virus Diseases of Animals and Man, pp.17-43. North-Holland Publishing, Amsterdam.

Parham, P., Androlewicz, M.J., Brodsky, F.M., Holmes, N.J., Ways, J.P. (1982) Monoclonal antibodies: purification, fragmentation and application to structural and functional studies of class I MHC antigens. J. Immunol. Methods 53, 133-173.

Payne, A., York, D.F., De Villiers, E-M., Verwoerd, D.W., Querat, G., Barban, V., Sauze, N. and Vigne, R. (1986) Isolation and identification of a South-African lentivirus from Jaagsiekte lungs. Onderstepoort J. Vet. Res. 53, 55-62.

Pederson, N.C., Ho, E., Brown, M.L. and Yamamoto, J.K. (1987) Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. Science 235, 790-793.

Peluso, R., Haase, A. T., Stowring, L., Edwards, M. and Ventura, P. (1985) A Trojan horse mechanism for the spread of visna virus in monocytes. Virology 147, 231-236.

Peterson, G.L. (1983) Determination of total protein. Methods Enzymol. 91, 95-119.

Philipson, L. (1967) Water-organic solvent phase systems. In: Maramorosch K., Koprowski, H., eds. Methods in Virology. New York: Academic Press, 235-244.

Porath, J. and Olin, B. (1983) Immobilized metal ion affinity adsorption and immobilized metal ion affinity chromatography of biomaterials. Serum protein affinities for gel-immobilized iron and nickel ions. Biochemistry 22, 1621-1630.

Porter, R.R. (1959) The hydrolysis of rabbit gamma-globulin and antibodies with crystalline papain. Biochem. J. 73, 119-126.

Pritchard, G.C. and Dawson, M. (1987) Maedi-Visna virus infection in commercial flocks of sheep in East Anglia. Vet. Rec. 120, 208-209.

Querat, G., Barban, V., Sauze, N., Filippi, P., Vigne, R., Russo, P. and Vitu, C. (1984) Highly lytic and persistent lentiviruses naturally present in sheep with progressive pneumonia are genetically distinct. J. Virol. 52, 672-679.

Querat, G., Audoly, G., Sonigo, P. and Vigne, R. (1990) Nucleotide sequence analysis of SA-OMVV, a Visna-related ovine lentivirus: Phylogenetic history of lentiviruses. Virology 175, 434-447.

Reyburn, H.T., Roy, D.J., Blacklaws, B.A., Sargan, D.R. and McConnel, I. (1992) Expression of Maedi-Visna virus major core protein, p25: development of a sensitive p25 antigen detection assay. J. Virol. Meth. 37, 305-320.

Ridder, R., Schmitz, R., Legay, F. and Gram, H. (1995) Generation of rabbit monoclonal antibody fragments from a combinatorial phage display library and their production in the yeast *Pichia pastoris*. Bio/Technology 13, 255-260.

Riddles, P.W., Blakeley, R.L. and Zerner, B. (1983) Reassessment of Ellman's reagent. Methods Enzymol. 91, 49-60.

Rimstad, E., East, N.E., DeRock, E., Higgins, J. and Pedersen, N.C. (1994) Detection of antibodies to caprine arthritis-encephalitis virus using recombinant *gag* proteins. Arch. Virol. 134, 345-356.

Romanos, M.A., Clare, J.J., Beesley, K.M., Rayment, F.B., Ballantine, S.P., Makoff, A.J., Dougan, G., Fairweather, N.F. and Charles, I.G. (1991) Recombinant *Bordetella pertussis* Pertactin p69 from the yeast *Pichia pastoris* high level production and immunological properties. Vaccine 9, 901-906.

Rylatt, D.B., Kemp, B.E., Bundesen, P.G., John, M.A., O'Reilly, E.J., Cottis, L.E., Mile, S.J., Khan, J.M., Dinh, D.P., Stapleton, D. and Hillyard, C.J. (1990) A rapid whole-blood immunoassay system. Med. J. Aust. 152, 75-77.

Sakano, H., Rogers, J.H., Huppi, K., Brack, C., Traunecker, A., Maki, R., Wall, R., Tonegawa, S. (1979) Domains and the hinge region of an immunoglobulin heavy chain are encoded in separate DNA segments. Nature 277, 627-633.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1990) Molecular cloning: a laboratory manual. Cold Spring Harbour Laboratory, Cold Spring Harbor, N.Y.

Santos, J.I., Galvao-Castro, B., Mello, D.C., Pereira, H.G., Pereira, M.S. (1987) Dot enzyme immunoassay. A simple, cheap and stable test for antibody to human immunodeficiency virus (HIV). J. Immunol. Methods 99, 191-194.

Sargan, D.R., Bennet, I.D., Cousens, C., Roy, D.J., Blacklaws, B.A., Dalziel, R.G., Watt, N.J. and McConnell, I. (1991). Nucleotide sequence of EV1, a British isolate of meadi-visna virus. J. Gen. Virol. 72, 1893-1903.

Schiestl, R. and Gietz, D. (1989) High efficiency transformation of intact yeast cells using single-stranded nucleic acids as a carrier. Curr. Genet. 16, 339-346.

Schupbach, J., Popovic, M., Gilden, R.V., Gonda, M.A., Sarngadharan, M.G. and Gallo, R.C. (1984) Serological analysis of a subgroup of human T-lymphotrophic retroviruses (HTLV-III) associated with AIDS. Science 224 (4648), 503-505.

Scott, J.V., Stowring, L., Haase, A.T., Narayan, O. and Vigne, R. (1979) Antigenic variation in visna virus. Cell 18, 321-327.

Seimenis, A., Papadopoulos, C., Mastroyanni, M. and Mangana, O. (1985) Slow virus diseases of the sheep in Greece. In: Slow Viruses in Sheep, Goats and Cattle. pp.105-110. Commission of the European Communities.

Sherman, M.Y. and Goldberg, A.L. (1992) Involvement of the chaperonin dnaK in the rapid degradation of a mutant protein in *Escherichia coli*. EMBO J. 11(1), 71-77.

Siddle, K., Gard, T., Thomas, D., Cranage, M.P., Coombs, R.R.A. (1984) Red cell-labelled monoclonal antibodies for assay of human chorionic gonadotropin and luteinising hormone by reverse passive haemagglutination. J. Immunol. Methods 73, 169-176.

Sigurdardottir, B. and Thormar, H. (1964) Isolation of a viral agent from the lungs of sheep affected with maedi. J. Infect. Dis. 114, 55-60.

Sigurdsson, B. (1954) Maedi, a slow progressive pneumonia of sheep: an epizoological and a pathological study. Brit. Vet. J. 110, 255-270.

Sigurdsson, B., Palsson, P.A. and Grimsson, H. (1957) Visna, a demyelinating transmissable disease of sheep. J. Neuropath. Exp. Neurol. 16, 389-403.

Sigurdsson, B. and Palsson, P.A. (1958) Visna of sheep. A slow demyelinating infection. Brit. J. Exp. Path. 39, 519-528.

Sihvonen, L. (1980) Studies on transmission of maedi virus to lambs. Acta Vet. Scan. 21, 689-698.

Sihvonen, L. (1981) Early immune responses in experimental maedi. Res. Vet. Sci. 30, 217-222.

Simard, C.L. and Briscoe, M.R. (1990) An enzyme-linked immunosorbent assay for detection of antibodies to maedi-visna in sheep. A simple technique for production of antigen using sodium dodecyl sulfate treatment. Can. J. Vet. Res. 54, 446-450.

Sonigo, P., Alizon, M., Staskus, K., Klatzmann, D., Cole, S., Danos, D., Retzel, E., Tiollais, P., Haase, A. and Wain-Hobson, S. (1985) Nucleotide sequence of the visna lentivirus: relationship to the AIDS virus. Cell 42, 369-382.

Smith, D.B. and Johnson, K.S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene 67, 31-40.

Sporeno, E., Paonessa, G., Salvati, A.L., Graziani, R., Delmastro, P., Ciliberto, G. and Toniatti, C. (1994) Oncostatin M binds directly to gp130 and behaves as interleukin-6 antagonist on a cell line expressing gp130 but lacking functional oncostatin M receptors. J. Biol. Chem. 269, 10991-10995.

Sreekrishna, K., Nelles, L., Potenz, R., Cruze, J., Mazzaferro, P., Fish, W., Fuke, M., Holden, K., Phelps, D., Wood, P. and Parker, K. (1989) High level expression purification and characterization of recombinant human tumor necrosis factor synthesized in the methylotrophic yeast *Pichia pastoris*. Biochem 28(9), 4117-4125.

Stavitsky, A.B., Arquilla, E.R. (1958) Studies of proteins and antibodies by specific hemagglutination and hemolysis of protein-conjugated erythrocytes. Int. Arch. Allergy 13, 1-38.

Strebel, K., Daugherty, D., Clouse, K., Cohen, D., Folks, T. and Martin, M.A. (1987) The HIV "A" (sor) gene product is essential for virus infectivity. Nature 328, 728-730.

Suttajit, M. and Winzler, R.J. (1971) Effect of modification of N-Acetylneuraminic acid on the binding of glycoproteins to influenza virus and on susceptibility to cleavage by neuraminidase. J. Biol. Chem. 246(10), 3398-3404.

Suzuki, T., Ueda, S. and Samijima, T. (1982) Enzyme-linked immunosorbent assay for diagnosis of equine infectious anaemia. Vet. Microbiol. 7, 307-316.

Thomas, L.M., Huntington, P.J., Mead, L.J., Wingate, D.L., Rogerson, B.A. and Lew, A.M. (1992) A soluble recombinant fusion protein of the transmembrane envelope protein of equine infectious anemia virus for ELISA. Vet. Microbiol. 31, 127-137.

Tiley, L.S., Brown, P.H., Le, S-Y., Maizel, J.V., Clements, J.E. and Cullen, B.R. (1990) Visna virus encodes a post-transcriptional regulator of viral structural gene expression. Proc. Natl. Acad. Sci., U.S.A. 87, 7497-7501.

Tillack, T.W., Scott, E.R. and Marchesi, V.T. (1972) The structure of erythrocyte membranes studied by freeze-etching. Localization of receptors for phytohemagglutination and influenza virus to the intramembraneous particles. J. Exp. Med. 135, 1209-1227.

Tomita, M., Furthmayr, H. and Marchesi, V.T. (1978) Primary structure of human erythrocyte glycophorin A. Isolation and characterization of peptides and complete amino acid sequence. Biochemistry 17, 4756-4770.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U.S.A. 76, 4530-4535.

Tschopp, J.F., Brust, P.F., Cregg, J.M., Stillman, C.A. and Gingeras, T.R. (1987a) Expression of the *lacZ* gene from two methanol-regulated promotors in *Pichia pastoris*. Nucl. Acids Res. 15, 3859-3876.

Tschopp, J.F., Sverlow, G., Kosson, R., Craig, W. and Grinna, L. (1987b) High-level secretion of glycosylated invertase in the methylotrophic yeast, *Pichia pastoris*. Bio/Technology 5, 1305-1308.

Vallee, H. and Carre, H. (1904) Sur la nature infectieuse de l'anemie de cheval. C R Acad. Sci. 139, 331-333.

Van der Molen, E.J., Vecht, U. and Houwers, D.J. (1985) A chronic indurative mastitis in sheep, associated with maedi/visna virus infection. Vet. Quart. 7, 112-119.

Vasudevachari, M.B., Uffelman, K.W., Mast, T.C., Dewar, R.L., Natarajan, V., Lane, H.C., Salzman, N.P. (1989) Passive hemagglutination test for the detection of antibodies to human immunodeficiency virus type 1 and comparison of the test with enzyme-linked immunosorbent assay and western blot (immunoblot) analysis. J. Clin. Microbiol. 27, 179-181.

Vedvick, T., Buckholz, R.G., Engel, M., Urcan, M., Kinney, J., Provow, S., Siegel, R.S. and Thill, G.P. (1991) High-level secretion of biological active aprotonin from the yeast *Pichia pastoris*. J. Ind. Microbiol. 7, 197-201.

Veenhuis, M., van Dijken, J.P. and Harder, W. (1983) The significance of peroxisomes in the metabolism of one-carbon compounds in yeasts. Adv. Microb. Physiol. 24, 1-82.

Vigne, R., Filippi, P., Querat, G., Sauze, N., Vitu, C., Russo, P. and Delori, P. (1982) Precursor polypeptides to structural proteins of visna virus. J. Virol. 42, 1046-1056.

Vigne, R., Filippi, P., Querat, G., Jouanny, C. and Sauze, N. (1985). Molecular biology of maedi/visna viruses. In: Slow Viruses in Sheep, Goats and Cattle. pp. 27-44. Commission of the European Communities.

Vigne, R., Gourdou, I., Mazarin, V., Querat, G., Sauze, N., Audoly, G., Filippi, P., Rousselot, C., Vitu, C. and Russo, P. (1990) Regulatory genes of visna virus. Development Biol. Standards 72, 213-222.

Vitu, C., Russo, P., Filippi, P., Vigne, R., Querat, G. and Giauffret, A. (1982) Une technique ELISA pour la détection des anticorps anti-virus maedi-visna. Étude comparative evec l'immunodiffusion en gélose et la fixation du complément. Comp. Immunol. Microbiol. Infect. Dis. 5, 469-481.

Vizard, A.L., Anderson, G.A. and Gasser, R.B. (1990) Determination of the optimum cut-off value of a diagnostic test. Prev. Vet. Med. 10, 137-143.

Voller, A., Bidwell, D.E. and Bartlett, A. (1982) ELISA techniques in virology. New Develop. Pract. Virol. 5, 59-81.

Wade-Evans, A.M., Woolhouse, T., O'Hara, R. and Hamblin, C. (1993) The use of African horse sickness virus VP7 antigen, synthesised in bacteria, and anti-VP7 monoclonal antibodies in a competitive ELISA. J. Virol. Meth. 45, 179-188.

Waeber, U., Buhr, A., Schunk, T. and Erni, B. (1993) The glucose transporter of *Escherichia coli*. Purification and characterization by Ni^{+} chelate affinity chromatography of the IIBCGlc subunit. FEBS Letters 342(1), 109-112.

Wang, I.I.G., Steel, S., Wisniewolski, R. and C.Y. Wang (1986) Detection of antibodies to human T-lymphotrophic virus type III by using a synthetic peptide of 21 amino-acid residues corresponding to a highly antigenic segment of gp41 envelope protein. Proc. Natl. Acad. Sci. USA 83, 6159-6163.

Wasniowska, K., Duk, M., Czerwinski, M., Steuden, I., Dus, D., Radzikowski, C., Bartosz-Bechowski, H., Konopinska, D. and Lisowska, E. (1992) Analysis of peptidic epitopes recognized by the three monoclonal antibodies specific for the same region of glycophorin A but showing different properties. Mol. Immunol. 29(6), 783-791.

Wasniowska, K., Czerwinski, M., Halasa, J. and Lisowska, E. (1993) An immunoblotting procedure for screening glycophorins and band 3 protein in the same blots. Identification of glycophorin and band 3 variant forms. J. Immunol. Methods 160(2), 253-260.

Watt, N.J., Roy, D.J., Mc Connel, I. and King, T.J. (1990) A case of visna in the United Kingdom. Vet. Record 126, 600-601.

Wegner, G.H. (1990) Emerging applications of the methylotrophic yeasts. FEMS Microbiol. Rev. 87, 279-283.

Welsh, C.J.R., Steinitz, M., Clark, M.R., Coombs, R.R.A. (1987) The detection of heat-aggregated IgG (as a model for immune complexes) by reverse passive haemagglutination using a human monoclonal rheumatoid factor coupled to erythrocytes. J. Immunol. Methods 104, 271-274.

Wilson, K.M., Gerometta, M., Rylatt, D.B., Bundesen, P.G., McPhee, D.A., Hillyard, C.J., Kemp, B.E. (1991) Rapid whole blood assay for HIV-1 seropositivity using an Fab-peptide conjugate. J. Immunol. Methods 138, 111-119.

Wilson, K.M., Catimel, B., Mitchelhill, K.I. and Kemp, B.E. (1994) Simplified conjugation chemistry for coupling peptides to F(ab') fragments: autologous red cell agglutination assay for HIV-1 antibodies. J. Immunol. Methods 175, 267-273.

Winter, G. and Milstein, C. (1991) Man-made antibodies. Nature 349, 293-299.

Woodward, M.P., Young, W.W. and Bloodgood, R.A. (1985) Detection of monoclonal antibodies specific for carbohydrate epitopes using periodate oxidation. J. Immunol. Methods 78, 143-153.

Woodward, T.M., Carlson, J., McClelland, C. and DeMartini, J.C. (1994) Analysis of lentiviral genomic variation by denaturing gradient gel electrophoresis. Biotechniques 17(2), 366-371.

Woodward, T.M., Carlson, J.O., de la Concha-Bermejillo, A. and DeMartini, J.C. (1995) Biological and genetic changes in ovine lentivirus strains following passage in isogenic twin lambs. J. Acq. Imm. Def. Syn. Hum. Ret. 8, 124-133.

Zanoni, R., Krieg, A. and Peterhans, E. (1989) Detection of antibodies to caprine arthritis-encephalitis virus by protein G enzyme-linked immunosorbent assay and immunoblotting. J. Clin. Microbiol. 27, 580-582.

Zanoni, R.G., Nauta, I.M., Pauli, U. and Peterhans, E. (1991) Expression in *Escherichia coli* and sequencing of the coding region for the capsid protein of Dutch maedi-visna virus ZZV 1050: Application of recombinant protein in enzyme-linked immunosorbent assay for the detection of caprine and ovine lentiviruses. J. Clin. Microbiol. 29(7), 1290-1294.

Zink, M.C. and Johnson, L.K. (1994) Pathobiology of lentivirus infections of sheep and goats. Virus Res. 32, 139-154.

Zweig, M.H. and Campbell, G. (1993) Receiver-operating characteristic (ROC) plots: A fundamental evaluation tool in clinical medicine. Clin. Chem. 39(4), 561-577.

APPENDIX 1

10X One-Phor-All Buffer *PLUS*:
100 mM Tris acetate, 100 mM magnesium acetate, 500 mM potassium acetate (pH 7.5)

Reagents for transformation of *E. coli*
SOB medium

Reagent	Final concentration
Tryptone	2%
Yeast Extract	0.5%
NaCl	10 mM
KCl	2.5 mM
$MgCl_2$	10 mM
$MgSO_4$	10 mM

To prepare SOB medium, combine tryptone, yeast extract, NaCl and KCl in water. Autoclave. Prepare a 2 M stock of Mg^{2+}, comprised of 1 M $MgCl_2$ and 1 M $MgSO_4$. Filter sterilise through a 0.22 µm membrane. Prior to use, combine the media with the magnesium solution. Add 100 µg/ml ampicillin. Final pH is 6.8-7.0. Store at 4°C, and use within two weeks. To prepare as a solid medium, add 1.2-1.5% agar.

TFB (Standard Transformation Buffer)
Use double-distilled water for preparation of all solutions.

Reagent	Amount/liter	Final concentration
KCl (ultrapure)	7.4 g	100 mM
$MnCl_2 \cdot 4\ H_2O$	8.9 g	45 mM
$CaCl_2 \cdot 2\ H_2O$	1.5 g	10 mM
$HACoCl_3$ *	0.8 g	3 mM
K-MES **	20 ml of 0.5 M stock (pH6.3)	10 mM

* Hexamine cobalt (III) trichloride
** Equilibrate a 0.5 M solution of MES (2-[N-morpholino]-ethone sulfonic acid) to pH 6.3 using concentrated KOH. Filter sterilise the solution through a 0.22 µm membrane. Store in aliquots at -20°C.

To prepare the TFB, add the salts as solids, then filter the solution through a 0.22 µm membrane. Final pH should be 6.2 ± 0.10. Pour aliquots into sterile flasks and store at 4°C. TFB is stable for about one year.

SOC medium: Identical to SOB, but contains 20 mM glucose in addition to listed reagents. To prepare SOC medium, combine tryptone, yeast extract, NaCl and KCl in water. Autoclave. Prepare a 2 M stock of glucose. Filter sterilise through a 0.22 µm membrane. Cool medium then add glucose to a final concentration of 20 mM. Prepare a 2 M stock of Mg^{2+}, comprised of 1 M $MgCl_2$ and 1 M $MgSO_4$. Filter sterilise through a 0.22µm membrane. Prior to use, combine the media with the magnesium

solution. Add 100 µg/ml ampicillin. Final pH is 6.8-7.0. Store at 4°C, and use within two weeks. To prepare as a solid media, add 1.2-1.5% agar.

2X YT Medium

Reagent	Final Concentration
Tryptone	16 g/l
Yeast extract	10 g/l
NaCl	5 g/l

Dissolve above ingredients in 900 ml of distilled H_2O. Adjust the pH to 7.0 with NaOH. Adjust the volume to 1 liter. Sterilise by autoclaving for 20 minutes. To prepare as a solid medium, add 1.2-1.5% agar.

2X YT-G Medium

Reagent	Final Concentration
Tryptone	16 g/l
Yeast extract	10 g/l
NaCl	5 g/l

Dissolve above ingredients in 800 ml of distilled H_2O. Adjust the pH to 7.0 with NaOH. Adjust the volume to 900 ml. Sterilise by autoclaving for 20 minutes. Once the medium has cooled, aseptically add 100 ml of a sterile 20% glucose solution (final concentration 2% glucose). To prepare as a solid medium, add 1.2-1.5% agar.

2X YT-G Medium + 100 µg/ml ampicillin
See protocol for 2X YT-G medium, above. At the same time as the glucose is added, also add 1 ml of a 100 mg/ml ampicillin stock solution (final concentration µg/ml).

Ampicillin stock solution
Dissolve 100 mg of the sodium salt of ampicillin in 4 ml of water. Sterilise by filtration and store in small aliquots at -20°C.

Reagents for small-scale isolation of plasmid DNA (Alkaline Lysis Method)
Solution I: 100 mM Tris-HCl (pH 7.5), 10 mM EDTA, 400 µg of heat-treated RNAse I/ml.
Solution II: 0.2 M NaOH, 1% (w/v) SDS.
Solution III: 3 M potassium, 5 M acetate. To prepare 100 ml, mix 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of distilled water.

pGEX Sequencing Primers

Primer	Sequence (5'- 3')	Nucleotide location
5' pGEX	GGGCTGGCAAGCCACGTTTGGTG	869-891
3' pGEX	CCGGGAGCTGCATGTGTCAGAGG	1020-998

AOX1 Sequencing Primers

Primer	Sequence (5'- 3')	Nucleotide location
5' AOX1	GACTGGTTCCAATTGACAAGC	855-875
3' AOX1	GCAAATGGCATTCTGACATCC	1347-1327

Media recipes for the growth and expression of recombinant proteins in *P. pastoris*

Stock Solutions

10X YNB (Yeast Nitrogen Base)

Dissolve 134 g of yeast nitrogen base (YNB) without amino acids in 1000 ml of water and filter sterilise. Store at 4°C. Stable for one year.

500X B (Biotin)

Dissolve 20 mg biotin in 100 ml of water and filter sterilise. Store at 4°C. Stable for one year.

100X H (Histidine)

Dissolve 400 mg of L-histidine in 100 ml of water and filter sterilise. Store at 4°C. Stable for one year.

10X D (Dextrose)

Dissolve 200 g of D-glucose in 1000 ml of water. Autoclave for 15 minutes. Stable for one year.

10X M (Methanol)

Mix 5 ml of methanol with 95 ml of water. Filter sterilise and store at 4°C. Stable for two months.

10X GY (Glycerol)
Mix 100 ml of glycerol with 900 ml of water. Sterilise by autoclaving. Store at room temperature. Stable for one year.

100X AA (Amino Acids)
Dissolve 500 mg each of L-glutamic acid, L-methionine, L-lysine, L-leucine and L-Isoleucine in 100 ml of water. Filter sterilise and store at 4°C. Stable for one year.

1 M potassium phosphate buffer, pH 6.0
Combine 132 ml of 1 M K_2HPO_4, 868 ml of 1 M KH_2PO_4 and confirm that the pH = 6.0 ±0.1 (if the pH needs to be altered, use phosphoric acid). Sterilise by autoclaving and store at room temperature. Stable for one year.

Growth
YPD
Dissolve 10 g of bacto yeast extract and 20 g of peptone in 900 ml of water (include 20 g of bacto agar if making YPD plates) and autoclave for 20 minutes. Add 100 ml of 10X D. Makes 1 liter. The liquid medium is stored at room temperature. YPD plates are stored at 4°C. Stable for several months.

Transformation
RDB (Regeneration Dextrose Base)
Dissolve 186 g of sorbitol and 20 g of agar in 700 ml of water and autoclave. Cool and maintain in a 45°C water bath. Add a prewarmed (45°C) mixture of stock solutions: 100 ml of 10X D, 100 ml of 10X YNB, 2 ml of 500X B, 10 ml of 100X AA and 88 ml of sterile water. Makes 1 liter. After mixing, pour the plates immediately. The plates are stored at 4°C and should last for several months.

RD (Regeneration Dextrose-top agar)
Composition is the same as RDB, except use only 10 g of agar. Maintain the autoclaved solution in a 60°C water bath prior to addition of prewarmed mixture of stock solutions. During transformation, use as a molten solution at 45°C.

Screening
MD (Minimal Dextrose)
Combine 800 ml of autoclaved water with 15 g agar and autoclave. Cool autoclaved mix to about 60°C and then add: 100 ml of 10X YNB, 2 ml of 500X B, and 100 ml of 10X D. Makes 1 liter. After mixing, pour the plates immediately. MD stores well for several months at 4°C.

MM (Minimal Methanol)
Combine 800 ml of autoclaved water with 15 g of agar and autoclave. Cool the autoclaved mix to 60°C and then add: 100 ml of 10X YNB, 2 ml of 500X B, and 100 ml of 10X M. Makes 1 liter. After mixing, pour the plates immediately. MM stores well for several months at 4°C.

Protein Expression

BMGY (Buffered Minimal Glycerol-complex Medium)

Combine 10 g of yeast extract, 20 g peptone and 700 ml water and autoclave. Let it cool to room temperature then add the following and mix well: 100 ml 1 M potassium phosphate buffer, pH 6.0, 100 ml 10X YNB, 2 ml 500X B, 100 ml 10X GY. Store at 4°C. The shelf life of this solution is approximately two months.

BMMY (Buffered Minimal Methanol-complex Medium)

Combine 10 g of yeast extract, 20 g of peptone and 700 ml of water and autoclave. Let it cool to room temperature then add the following and mix well: 100 ml 1 M potassium phosphate buffer, pH 6.0, 100 ml 10X YNB, 2 ml 500X B, 100 ml 10X M. Store at 4°C. The shelf life of this solution is approximately two months.

APPENDIX 2
DNA SEQUENCE OF GST-TM-p25 (COM2) AND ITS COMPARISON WITH THE SA-OMVV SEQUENCE
TM PORTION

```
                                                                  40         50         60
MVVCOM2.DNA                                                       AAGCTATAGTCGATAGAATGATGCT
                                                                  :::::::::::::::::::::::::
OLVSAOMV.DNA                                                      AAGCTATAGTCGATAGAATGATGCT
                          8180       8190       8200       8210       8220
                           70         80         90        100        110        120
MVVCOM2.DNA    CTATCATGAATTGGATTGTTGGCATTATCAACACTATTGTGTAACCTCTACTAGAACAGA
               ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
OLVSAOMV.DNA   CTATCATGAATTGGATTGTTGGCATTATCAACACTATTGTGTAACCTCTACTAGAACAGA
            8230       8240       8250       8260       8270       8280
                       130        140        150        160        170        180
MVVCOM2.DNA    AGTAGCACAATATGTAAATTGGACAAGATATAAGGATAATTGCACATGGCAGCAGTGGGA
               ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
OLVSAOMV.DNA   AGTAGCACAATATGTAAATTGGACAAGATATAAGGATAATTGCACATGGCAGCAGTGGGA
            8290       8300       8310       8320       8330       8340
                       190        200
MVVCOM2.DNA    AGAAGAGATAGAGCAACACGAGGCA
               :::::::::::::::::::::::::
OLVSAOMV.DNA   AGAAGAGATAGAGCAACACGAGGCA
            8350       8360       8370
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APPENDIX 2 (CONTINUED)
DNA SEQUENCE OF GST-TM-p25 (COM2) AND ITS COMPARISON WITH THE SA-OMVV SEQUENCE
p25 PORTION

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                                                                         230
IVVCOM2.DNA                                                         GTATGAAAGAAGGGC
                                                                    :::::::::::::::
LVSAOMV.DNA                                                         GTATGAAAGAAGGGC
                 820        830        840        850        860        870
             240        250        260        270        280        290
IVVCOM2.DNA  TACACGAAAAACAGGAGGATAAAGAAAAGAAGGTAGAACAACTCTACCCAAACTTGGAAA
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
LVSAOMV.DNA  TACACGAAAAACAGGAGGATAAAGAAAAGAAGGTAGAACAACTCTACCCAAACTTGGAAA
                 880        890        900        910        920        930
             300        310        320        330        340        350
IVVCOM2.DNA  AACACAGAGAAGTGTATCCTATTGTAAATTTGCAGGCTGGAGGGAGAAGTTGGAAAGCGG
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
LVSAOMV.DNA  AACACAGAGAAGTGTATCCTATTGTAAATTTGCAGGCTGGAGGGAGAAGTTGGAAAGCGG
                 940        950        960        970        980        990
             360        370        380        390        400        410
IVVCOM2.DNA  TAGAGTCAGTGACATTCCAGCAGCTGCAAACAGTAGCAATGCAGCATGGACTTGTGTCCG
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
LVSAOMV.DNA  TAGAGTCAGTGACATTCCAGCAGCTGCAAACAGTAGCAATGCAGCATGGACTTGTGTCCG
                1000       1010       1020       1030       1040       1050
             420        430        440        450        460        470
IVVCOM2.DNA  AGGATTTTGAAAGACAATTAGCATATTATGCCACTACATGGACAAGCAAAGATATATTAG
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
LVSAOMV.DNA  AGGATTTTGAAAGACAATTAGCATATTATGCCACTACATGGACAAGCAAAGATATATTAG
                1060       1070       1080       1090       1100       1110
             480        490        500        510        520        530
IVVCOM2.DNA  AAGTACTAGCCATGATGCCTGGAAATAGGGCGCAAAAAGAGTTAATACAGGGGAAATTAA
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
LVSAOMV.DNA  AAGTACTAGCCATGATGCCTGGAAATAGGGCGCAAAAAGAGTTAATACAGGGGAAATTAA
                1120       1130       1140       1150       1160       1170
             540        550        560        570        580        590
IVVCOM2.DNA  ATGAGGAAGCAGAAAGGTGGGTAAGGCAGAATCCCCCAGGGCCAAATGTCCTTACTGTGG
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
LVSAOMV.DNA  ATGAGGAAGCAGAAAGGTGGGTAAGGCAGAATCCCCCAGGGCCAAATGTCCTTACTGTGG
                1180       1190       1200       1210       1220       1230
             600        610        620        630        640        650
IVVCOM2.DNA  ATCAGATTATGGGAGTCGGACAAACAAATCAACAGGCATCGCAAGCTAATATGGATCAGG
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
LVSAOMV.DNA  ATCAGATTATGGGAGTCGGACAAACAAATCAACAGGCATCGCAAGCTAATATGGATCAGG
                1240       1250       1260       1270       1280       1290
             660        670        680        690        700        710
VVCOM2.DNA   CAAGACAGCTTTGCTTGCAGTGGGTAATAACAGCCTTGAGATCAGTAAGACATATGTCAC
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
LVSAOMV.DNA  CAAGACAGCTTTGCTTGCAGTGGGTAATAACAGCCTTGAGATCAGTAAGACATATGTCAC
                1300       1310       1320       1330       1340       1350
             720        730        740        750        760        770
VVCOM2.DNA   ACAGACCAGGAAATCCTATGCTGATAAAACAAAAGAATAGTGAAAGTTATGAAGATTTTA
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
LVSAOMV.DNA  ACAGACCAGGAAATCCTATGCTGATAAAACAAAAGAATAGTGAAAGTTATGAAGATTTTA
                1360       1370       1380       1390       1400       1410
             780        790        800        810        820        830
VCOM2.DNA    TAGCAAGATTGCTAGAAGCAATTGATACAGAACCCGTCACGGATCCTATAAAAACATATT
             ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
VSAOMV.DNA   TAGCAAGATTGCTAGAAGCAATTGATACAGAACCCGTCACGGATCCTATAAAAACATATT
                1420       1430       1440       1450       1460       1470
```

p25 PORTION (CONTINUED)

```
                 840        850        860        870        880        890
MVVCOM2.DNA      TAAAAGTAACTCTGTCGTTCACAAATGCTAGCACAGATTGTCAAAAACAAATGGACAGAG
                 ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
OLVSAOMV.DNA     TAAAAGTAACTCTGTCGTTCACAAATGCTAGCACAGATTGTCAAAAACAAATGGACAGAG
                      1480       1490       1500       1510       1520       1530
                  900        910        920        930        940        950
MVVCOM2.DNA      TGTTAGGGACAAGAGTCCAACAGGCATCAGTAGAAGAAAAAATGCAAGCATGTCGGGACG
                 ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
OLVSAOMV.DNA     TGTTAGGGACAAGAGTCCAACAGGCATCAGTAGAAGAAAAAATGCAAGCATGTCGGGACG
                      1540       1550       1560       1570       1580       1590
                  960        970
MVVCOM2.DNA      TAGGATCAGAAGG
                 :::::::::::::
OLVSAOMV.DNA     TAGGATCAGAAGG
                      1600
```

APPENDIX 2 (CONTINUED)
DNA TRANSLATION OF SEQUENCED GST-TM-p25 (COM2)

```
  1        Ser Ser Lys Ile Gly Ser Gly Ser Ala Trp Ile Gln Ala Ile Val
  1      A TCC TCC AAA ATC GGA TCT GGT TCC GCG TGG ATC CAA GCT ATA GTC

 16   Asp Arg Met Met Leu Tyr His Glu Leu Asp Cys Trp His Tyr Gln His
 47   GAT AGA ATG ATG CTC TAT CAT GAA TTG GAT TGT TGG CAT TAT CAA CAC

 32   Tyr Cys Val Thr Ser Thr Arg Thr Glu Val Ala Gln Tyr Val Asn Trp
 95   TAT TGT GTA ACC TCT ACT AGA ACA GAA GTA GCA CAA TAT GTA AAT TGG

 48   Thr Arg Tyr Lys Asp Asn Cys Thr Trp Gln Gln Trp Glu Glu Glu Ile
143   ACA AGA TAT AAG GAT AAT TGC ACA TGG CAG CAG TGG GAA GAA GAG ATA

 64   Glu Gln His Glu Ala Gly Ser Pro Gly Ile Arg Met Lys Glu Gly Leu
191   GAG CAA CAC GAG GCA GGA TCC CCG GGA ATT CGT ATG AAA GAA GGG CTA

 80   His Glu Lys Gln Glu Asp Lys Glu Lys Lys Val Glu Gln Leu Tyr Pro
239   CAC GAA AAA CAG GAG GAT AAA GAA AAG AAG GTA GAA CAA CTC TAC CCA

 96   Asn Leu Glu Lys His Arg Glu Val Tyr Pro Ile Val Asn Leu Gln Ala
287   AAC TTG GAA AAA CAC AGA GAA GTG TAT CCT ATT GTA AAT TTG CAG GCT

112   Gly Gly Arg Ser Trp Lys Ala Val Glu Ser Val Thr Phe Gln Gln Leu
335   GGA GGG AGA AGT TGG AAA GCG GTA GAG TCA GTG ACA TTC CAG CAG CTG

128   Gln Thr Val Ala Met Gln His Gly Leu Val Ser Glu Asp Phe Glu Arg
383   CAA ACA GTA GCA ATG CAG CAT GGA CTT GTG TCC GAG GAT TTT GAA AGA

144   Gln Leu Ala Tyr Tyr Ala Thr Thr Trp Thr Ser Lys Asp Ile Leu Glu
431   CAA TTA GCA TAT TAT GCC ACT ACA TGG ACA AGC AAA GAT ATA TTA GAA

160   Val Leu Ala Met Met Pro Gly Asn Arg Ala Gln Lys Glu Leu Ile Gln
479   GTA CTA GCC ATG ATG CCT GGA AAT AGG GCG CAA AAA GAG TTA ATA CAG

176   Gly Lys Leu Asn Glu Glu Ala Glu Arg Trp Val Arg Gln Asn Pro Pro
527   GGG AAA TTA AAT GAG GAA GCA GAA AGG TGG GTA AGG CAG AAT CCC CCA

192   Gly Pro Asn Val Leu Thr Val Asp Gln Ile Met Gly Val Gly Gln Thr
575   GGG CCA AAT GTC CTT ACT GTG GAT CAG ATT ATG GGA GTC GGA CAA ACA

208   Asn Gln Gln Ala Ser Gln Ala Asn Met Asp Gln Ala Arg Gln Leu Cys
623   AAT CAA CAG GCA TCG CAA GCT AAT ATG GAT CAG GCA AGA CAG CTT TGC
224   Leu Gln Trp Val Ile Thr Ala Leu Arg Ser Val Arg His Met Ser His
671   TTG CAG TGG GTA ATA ACA GCC TTG AGA TCA GTA AGA CAT ATG TCA CAC

240   Arg Pro Gly Asn Pro Met Leu Ile Lys Gln Lys Asn Ser Glu Ser Tyr
719   AGA CCA GGA AAT CCT ATG CTG ATA AAA CAA AAG AAT AGT GAA AGT TAT

256   Glu Asp Phe Ile Ala Arg Leu Leu Glu Ala Ile Asp Thr Glu Pro Val
767   GAA GAT TTT ATA GCA AGA TTG CTA GAA GCA ATT GAT ACA GAA CCC GTC

272   Thr Asp Pro Ile Lys Thr Tyr Leu Lys Val Thr Leu Ser Phe Thr Asn
815   ACG GAT CCT ATA AAA ACA TAT TTA AAA GTA ACT CTG TCG TTC ACA AAT

288   Ala Ser Thr Asp Cys Gln Lys Gln Met Asp Arg Val Leu Gly Thr Arg
863   GCT AGC ACA GAT TGT CAA AAA CAA ATG GAC AGA GTG TTA GGG ACA AGA

304   Val Gln Gln Ala Ser Val Glu Glu Lys Met Gln Ala Cys Arg Asp Val
911   GTC CAA CAG GCA TCA GTA GAA GAA AAA ATG CAA GCA TGT CGG GAC GTA

320   Gly Ser Glu Gly Glu Phe Ile Val Thr Asp *** Arg
```

APPENDIX 3
DNA HOMOLOGY SEARCH FOR MVV TM FRAGMENT CLONED IN pPIC9 EXPRESSION VECTOR

```
                    8214       8224       8234       8244       8254       826
OLVSAOMV.DNA        AAGCTATAGTCGATAGAATGATGCTCTATCATGAATTGGATTGTTGGCATTATCAACAC
                    X:::::::::::::::::::::::::::::::::::::::::::::::::::::::::
PMVVTM.SEQ          AAGCTATAGTCGATAGAATGATGCTCTATCATGAATTGGATTGTTGGCATTATCAACAC
                           40         50         60         70         80        90
                    8274       8284       8294       8304       8314
OLVSAOMV.DNA        ATTGTGTAACCTCTACTAGAACAGAAGTAGCACAATATGTAAATTGGACAAGATAT
                    ::X          :::::::::::::::::::::::::::::::::::::::::::
PMVVTM.SEQ          ATT---------CTACTAGAACAGAAGTAGCACAATATGTAAATTGGACAAGATAT
                                100        110        120        130
```

APPENDIX 3 (CONTINUED)
DNA TRANSLATION OF SEQUENCED MVV TM FRAGMENT CLONED IN pPIC9 EXPRESSION VECTOR

```
  1       Tyr Val Glu Phe His His His His His His Glu Ala Ile Val Asp
  1       TAC GTA GAA TTC CAT CAT CAT CAT CAT CAT GAA GCT ATA GTC GAT

 16  Arg Met Met Leu Tyr His Glu Leu Asp Cys Trp His Tyr Gln His Tyr
 47  AGA ATG ATG CTC TAT CAT GAA TTG GAT TGT TGG CAT TAT CAA CAC TAT

 32  Ser Thr Arg Thr Glu Val Ala Gln Tyr Val Asn Trp Thr Arg Tyr Ala
 95  TCT ACT AGA ACA GAA GTA GCA CAA TAT GTA AAT TGG ACA AGA TAT GCG

 48  Ala Ala Asn *** Phe
143  GCC GCG AAT TAA TTC
```

PATENTS

TITLES

1) SHEEP ERYTHROCYTE BINDING AGENT AND IMMUNOASSAYS EMPLOYING SAME

2) DETECTION OF ANTIBODY TO MAEDI-VISNA VIRUS

D.M. KISCH INC. , Johannesburg

INKOMSTE 1. 9. 94 REVENUE

REPUBLIC VAN SUID-AFRIKA
Form P.1
R ≈ 45.00
NR REPUBLIC OF SOUTH AFRICA 445

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

APPLICATION FOR A PATENT AND ACKNOWLEDGEMENT OF RECEIPT
(Section 30 (1) - Regulation 22)
The grant of a patent is hereby requested by the undermentioned applicant on the basis of the present application filed in duplicate.

PATENT APPLICATION NO.	AGENT'S REFERENCE
21 01	P/94/71806

FULL NAME(S) OF APPLICANT(S)

71 1. NATAL BLOOD TRANSFUSION SERVICE
2. THE AGRICULTURAL RESEARCH COUNCIL

ADDRESS(ES) OF APPLICANT(S)

1. 10 Robin Road, Pinetown
Durban, South Africa
2. Celtis Plaza
1085 Schoeman Street, Hatfield
Pretoria, South Africa

TITLE OF INVENTION

54 SHEEP ERYTHROCYTE BINDING AGENT AND IMMUNOASSAYS EMPLOYING SAME

THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2.
The earliest priority claimed is

THIS APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO.	21	01
THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND BASED ON APPLICATION NO.	21	01

THIS APPLICATION IS ACCOMPANIED BY :

X	1	A single copy of a provisional ~~xxxxxxxxxxxxxxxxxxxxx~~ complete specification of 9 pages.
	2	Drawings of sheets.
	3	Publication particulars and abstract (Form P.8. in duplicate).
	4	A copy of Figure of the drawings for the abstract.
	5	An assignment of invention.
	6	Certified priority document(s) (State number).
	7	Translation of priority document(s).
	8	An assignment of priority rights.
	9	A copy of Form P.2 and specification of S.A. Patent Application No. 21 01
	10	A declaration and power of attorney on Form P.3.
	11	Request for ante-dating on Form P.4.
	12	Request for classification on Form P.9.
	13	

DATED THIS 1 st DAY OF September 19 94

J. S. KISCH

Patent Attorney for the Applicant(s)

ADDRESS FOR SERVICE

74 D.M. KISCH INC.
Syfrets Park
23 Girton Road

RECEIVED
OFFICIAL DATE STAMP

D.M. KISCH INC., Johannesburg

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

INKOMSTE REVENUE 9.11.94

REPUBLIEK VAN SUID-AFRIKA Form P.8
R 30 00
REPUBLIC OF SOUTH AFRICA

DECLARATION AND POWER OF ATTORNEY
(Section 30 - Regulations 8, 22(i)(c) and 33)

PATENT APPLICATION NO.	LODGING DATE.	D.M.KISCH'S REFERENCE
21 01 94/6718	22 01-09-1994	P/94/71806

FULL NAME(S) OF APPLICANT(S)

71 1. NATAL BLOOD TRANSFUSION SERVICE
2. THE AGRICULTURAL RESEARCH COUNCIL

FULL NAME(S) OF INVENTOR(S)

72 1. CHRISTOFFEL HENDRIK BOSHOFF
2. JAN DIRK CONRADIE
3. JOHANNA MARIA ALETTA CONRADIE
4. R. Williams
5. D.F. York

EARLIEST PRIORITY CLAIMED	COUNTRY	NUMBER	DATE
NOTE : The country must be indicated by its International Abbreviation - see schedule 4 of the Regulations.	33 -	31 -	32 -

TITLE OF INVENTION

54 SHEEP ERYTHROCYTE BINDING AGENT AND IMMUNOASSAYS EMPLOYING SAME

* I / We

1. HARRY LAWRENCE DWYER
2. DANIEL WYNAND VERWOERD

hereby declare that --

1. ~~I/we am/are the applicant(s) mentioned above~~;

** 2. I/we have been authorised by the applicant(s) to make this declaration and have knowledge of the facts herein stated in the capacity of 1. FINANCIAL DIRECTOR 2. DIRECTOR of the applicant(s);

*** 3. the inventor(s) of the abovementioned invention is/are the person(s) named above and the applicant(s) has/have acquired the right to apply by virtue of *an assignment from the inventor(s);*

4. to the best of my/our knowledge and belief, if a patent is granted on the application, there will be no lawful ground for the revocation of the patent;

**** 5. ~~this is a convention application and the earliest application from which priority is claimed as set out above is the first application is a convention country in respect of the invention claimed in any of the claims~~; and

6. the directors and qualified staff of the firm of **D. M. KISCH INC**, patent attorneys, are authorised, jointly and severally, with powers of substitution and revocation, to represent the applicant(s) in this application and to be the address for service of the applicant(s) while the application is pending and after a patent has been granted on the application.

SIGNED AT PINETOWN, THIS 6 DAY OF OCTOBER, 1994

1. .....
NATAL BLOOD TRANSFUSION SERVICE

2. .....
SIGNATURE(s) - no legalization necessary
THE AGRICULTURAL RESEARCH COUNCIL

* In the case of applications in the name of a Company, Partnership or Firm, give full names of signatory/signatories, delete paragraph 1, and enter capacity of each signatory in paragraph
** If the applicant is a natural person, delete paragraph 2.
*** If the right to apply is not by virtue of an assignment from the inventor(s), delete "an assignment from the

D.M. KISCH INC.
Patent Attorneys &
Trade Mark Agents

South Africa
Local

ASSIGNMENT OF INVENTION

WHEREAS we

1. **CHRISTOFFEL HENDRIK BOSHOFF**
2. **JAN DIRK CONRADIE**
3. **JOHANNA MARIA ALETTA CONRADIE**
4. **ROY WILLIAMS**
5. **DENIS FRANCIS YORK**

of

1. 29 University Gardens, 308 Queen Mary Avenue, GLENMORE, DURBAN 4001
2. 2A Palmiet Drive, Westville, DURBAN 3630
3. 2A Palmiet Drive, Westville, DURBAN 3630
4. Plot 144, Mooiplaats, PRETORIA 0001, and
5. 8 Spring Grange Road, WESTVILLE 3630 respectively

are the inventors of an invention entitled: **"SHEEP ERYTHROCYTE BINDING AGENT AND IMMUNOASSAYS EMPLOYING SAME"**

AND WHEREAS **NATAL BLOOD TRANSFUSION SERVICE** of 10 Robin Road, Pinetown, and **THE AGRICULTURAL RESEARCH COUNCIL** of Celtis Plaza, 1085 Schoeman Street, Hatfield, Pretoria, have for good and sufficient consideration acquired the said invention from us for all countries

NOW THEREFORE this Deed witnesses that we have assigned the said invention to **NATAL BLOOD TRANSFUSION SERVICE** and **THE AGRICULTURAL RESEARCH COUNCIL** its successors, assigns or legal representatives, for all countries, together with the right to apply for Letters Patent in respect thereof in its own name, the assignment taking effect on or before **1 SEPTEMBER 1994.**

We agree that when requested, we will without charge to the assignee but at its expense sign all papers, take all rightful oaths, and do all acts which may be necessary, desirable or convenient for securing and maintaining patents for said invention in any and all countries and for vesting title therefore in said assignee, its successors, assigns or legal representatives.

	DATE	SIGNATURE
1.	17/10/94	C.H. Boshoff
2.	20/9/94	J.D. Conradie
3.	20/9/94	J.M.A. Conradie
4.	24/10/94	R. Williams
5.	17/10/94	D.F. York

No legalisation required

FIELD OF THE INVENTION

THIS invention relates to reagents suitable for use in performing immunoassays for a variety of analytes in sheep blood.

BACKGROUND OF THE INVENTION

South African Patent 88/6937 in the name of Agen Limited describes various immunoassay techniques based on erythrocyte agglutination. It does so with reference to earlier publications and also discloses as the subject matter of that invention an agglutination assay for analytes in human blood which may be performed without the need for sophisticated laboratory equipment. Although that patent specification makes reference to the use of the technique disclosed therein for conducting veterinary immunoassays, it does not provide details of any erythrocyte binding molecule which is specific for but non-auto-agglutinating for non-human erythrocytes. It has been found by the present applicants that mono-clonal anti-human glycophorin-A does not react with sheep erythrocytes.

The ease with which the Agen technique may be performed under field conditions and the potential affordability of such an assay

renders the Agen technique a candidate for disease diagnosis and particularly for the diagnosis of viral infections such as the Maedi visna virus. However, the implementation of that technique in sheep immunoassays is clearly dependent on the development of a conjugate which presents on the one hand a sheep erythrocyte binding molecule or functionality and, on the other, the appropriate analyte binding molecule or functionality. Such conjugate would in addition be required not to cause sheep erythrocytes to agglutinate except in the presence of the analyte for which the analyte binding molecule or functionality is specific. It has hitherto been unknown whether such sheep erythrocyte binder exists or could be developed and furthermore, if it could be raised, whether it would have sufficiently general functionality to enable it to be used for assaying or binding to the erythrocytes in the blood of different breeds of sheep.

OBJECT OF THE INVENTION

The development of such a general sheep erythrocyte binding molecule and conjugates thereof with various analyte binding molecules was an object of this invention and has now been

achieved, thus rendering the Agen technique [and others which rely on erythrocyte agglutination in the presence of an analyte] useful in sheep immunoassays.

DESCRIPTION OF THE INVENTION

According to the present invention there is provided a sheep erythrocyte binding agent [hereinafter referred to as **SEBA**] which is characterised in that it is capable of binding to sheep erythrocytes without causing agglutination of such erythrocytes in the absence of other binding agents but causing agglutination of the erythrocytes in the presence of albumin.

Further according to the invention there is provided an immunoassay reagent comprising a conjugate of SEBA and an analyte binding molecule characterised in that the reagent, when added to whole sheep blood, fails to cause agglutination of the erythrocytes in the absence of any other binding agents but causes agglutination in the presence of the analyte for which the analyte binding molecule is specific.

Yet further according to the invention there is provided an immunoassay reagent useful in diagnosing sheep for Maedi visna infection comprising a conjugate of SEBA and Maedi visna antigen [p.24 capsid protein].

Another aspect of the invention comprises a method for the *in vitro* diagnosis of infections in sheep comprising the step of using a conjugate as described above which contains SEBA according to the invention.

EXAMPLES OF THE INVENTION

EXAMPLE 1

Preparation of SEBA

The SEBA of the present invention was produced by generally following, with modifications, the procedure described by Kemp, B.E. *et al* (1988) **Science** 241:1352 for producing a human erythrocyte binding antibody. Sheep red blood cells obtained from a Dorper breed of sheep were prepared by washing the cells 3 times in 0,9% saline w/v and resuspended in saline to 10% final

concentration. Balb/c mice were injected intra-peritoneally with 100 μl of the 10% red blood cell suspension. The mice received at least three subsequent inoculations at three weekly intervals. Those mice with the strongest sheep erythrocyte agglutinating activity in their sera as measured by the Coombs assay [Coombs RRA *et al* (1987) J Immun Methods 101:1] received a booster immunization before they were sacrificed and their splenocytes fused with NSO/1 myeloma cells following standard hybridoma cloning procedures [Galfre and Milstein, C. Methods in Enzymology Academic Press Vol. II (1981) p.285]. Hybridoma cell supernatants were tested for their ability to agglutinate sheep red cells using the Coombs assay.

Isolation of SEBA

An hybridoma clone was identified that produces monoclonal antibodies specific for sheep erythrocytes. This monoclonal antibody does not auto-agglutinate sheep erythrocytes. In the presence of 30% albumin the monoclonal antibody will agglutinate the sheep erythrocytes. The monoclonal antibody was tested against the blood of more than a hundred sheep and found to recognize an epitope on erythrocytes of all the sheep tested. The sheep tested

were of different breeds including Dohne, Finn, Dorper, Merino and several different cross-breeds.

Using a Western immunoblot technique the monoclonal antibody was further shown to bind to a glycoprotein of the same molecular weight as that recognized by Jacalin, a lectin known to bind to glycophorin. Using an ELISA technique the monoclonal antibody will bind to erythrocyte ghosts immobilized on microtitre plates. Poly-L-lysine activated plates were used and intact red blood cells were absorbed onto the plates, whereafter they were lysed and the ghosts were washed *in situ* and used as solid phase. The binding of SEBA to sheep erythrocytes ghosts insolubilised in this manner was detected with rabbit anti-mouse IgG antibodies conjugated to a reporter enzyme.

The above properties of the monoclonal antibody produced by the method described strongly suggests that the SEBA of the present invention binds to the glycophorin of the sheep erythrocytes but this mechanism of binding has not yet been positively proved. For the purposes of practical utility, however, it is unimportant whether the

SEBA of the invention in fact binds to the glycophorin of the sheep erythrocyte or to any other erythrocyte membrane protein which occurs commonly across different breeds of sheep.

EXAMPLE 2

Preparation of SEBA-MVVAg Complex

Generally following the principle outlined by Wilson *et al* (1991) Journal of Immunological Methods 138:119 the Fab fragment of SEBA may be coupled to an immunodominant MVV antigen [p.24 capsid protein] [Querat *et al* (1990) Virology 175 434-447. The gene coding for this protein may be cloned and expressed in a prokaryotic expression system. This complex when added to sheep erythrocytes containing MVV antibodies should result in the agglutination of the erythrocytes. In the absence of the MVV specific antibodies no agglutination should take place.

GENERAL

It will be appreciated that the invention of the generally applicable sheep erythrocyte binding agent [SEBA] of the present invention

opens the field for numerous immunological applications of the invention. It also has application for confirmation of a blood sample as being sheep blood as it has been shown that SEBA does not bind human, bovine or goat erythrocytes. It is expected that further investigations will not detract from applicants' conclusion that the SEBA of the present invention is a species specific, universal sheep erythrocyte binding agent which meets the objectives of the invention set out above.

The invention is illustrated with regard to the ability of the SEBA-MVVAg conjugate to agglutinate sheep erythrocytes in the presence of Maedi visna antibodies. It is, however, not intended to be limited to such application and can be extended to conjugates with antigens to any other circulating antibodies or conjugates with antibodies to any circulating antigens. The invention thus also finds application in pregnancy tests for sheep and to confirm immunity following a dosing program.

Dated this 1st day of September 1994

TRUTER

Parent Attorney/Agent for the Applicant

D.M. KISCH INC., Johannesburg

INKOMSTE 17. 5. 95 REVENUE

REPUBLIC VAN SUID-AFRIKA
Form P.1
R 30,00
REPUBLIC OF SOUTH AFRICA

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978

APPLICATION FOR A PATENT AND ACKNOWLEDGEMENT OF RECEIPT
(Section 30 (1) - Regulation 22)
The grant of a patent is hereby requested by the undermentioned applicant on the basis of the present application filed in duplicate.

PATENT APPLICATION NO.		
21	01	

AGENT'S REFERENCE
P/95/72674

FULL NAME(S) OF APPLICANT(S)

71 THE AGRICULTURAL RESEARCH COUNCIL

ADDRESS(ES) OF APPLICANT(S)

Celtis Plaza
1085 Schoeman Street
Hatfield
Pretoria
Republic of South Africa

TITLE OF INVENTION

54 DETECTION OF ANTIBODY TO MAEDI-VISNA VIRUS

THE APPLICANT CLAIMS PRIORITY AS SET OUT ON THE ACCOMPANYING FORM P.2.
The earliest priority claimed is

	THIS APPLICATION IS FOR A PATENT OF ADDITION TO PATENT APPLICATION NO.	21	01
	THIS APPLICATION IS A FRESH APPLICATION IN TERMS OF SECTION 37 AND BASED ON APPLICATION NO.	21	01

THIS APPLICATION IS ACCOMPANIED BY :

X	1	A single copy of a provisional ~~xxxxxxxxxxxxxxxxxxxxxxxxx~~ specification of 7 pages.
	2	Drawings of sheets.
	3	Publication particulars and abstract (Form P.8. in duplicate).
	4	A copy of Figure of the drawings for the abstract.
	5	An assignment of invention.
	6	Certified priority document(s) (State number).
	7	Translation of priority document(s).
	8	An assignment of priority rights.
	9	A copy of Form P.2 and specification of S.A. Patent Application No. 21 01
	10	A declaration and power of attorney on Form P.3.
	11	Request for ante-dating on Form P.4.
	12	Request for classification on Form P.9.
	13	

DATED THIS 17 th DAY OF May 19 95

C.P. VAN BILJON
Patent Attorney for the Applicant(s)

RECEIVED
OFFICIAL DATE STAMP

ADDRESS FOR SERVICE

74 D.M. KISCH INC.
Syfrets Park
23 Girton Road

10. 7. 95
REVENUE

FORM P. 3.

D.M. KISCH INC., Johannesburg

REPUBLIC OF SOUTH AFRICA
PATENTS ACT, 1978
DECLARATION AND POWER OF ATTORNEY
(Section 30 – Regulations 8, 22(i)(c) and 33)

PATENT APPLICATION NO.	D.M.K. Ref: P/95/72674	LODGING DATE
21 || 01 | 95/3999		22/23 | 17-05-1995

FULL NAME(S) OF APPLICANT(S)

71 THE AGRICULTURAL RESEARCH COUNCIL

FULL NAME(S) OF INVENTOR(S)

72 CHRISTOFFEL HENDRIK BOSHOFF
DENIS FRANCIS YORK

EARLIEST PRIORITY CLAIMED	COUNTRY	NUMBER	DATE
NOTE: The country must be indicated by its International Abbreviation – see schedule 4 of the Regulations	33 —	31 —	32 —

TITLE OF INVENTION

54 "DETECTION OF ANTIBODY TO MAEDI-VISNA VIRUS"

• I/We. DANIEL WYNAND VERWOERD

hereby declare that–

1. ~~I/we am/are the applicant(s) mentioned above;~~

•• 2. I/we have been authorised by the applicant(s) to make this declaration and have knowledge of the facts herein stated in the capacity of Director of the Veterinary Institute of the ARC at Onderstepoort of the applicant(s);

••• 3. the inventor(s) of the abovementioned invention is/are the person(s) named above and the applicant(s) has/have acquired the right to apply by virtue of *an assignment from the inventor(s);*

4. to the best of my/our knowledge and belief, if a patent is granted on the application, there will be no lawful ground for the revocation of the patent;

•••• 5. ~~this is a convention application and the earliest application from which priority is claimed as set out above is the first application in a convention country in respect of the invention claimed in any of the claims; and~~

6. the directors and qualified staff of the firm of D.M. KISCH INC patent attorneys, are authorised, jointly and severally, with powers of substitution and revocation, to represent the applicant(s) in this application and to be the address for service of the applicant(s) while the application is pending and after a patent has been granted on the application.

SIGNED AT Pretoria, THIS 6th DAY OF June, 19 95

D.J. Verwoerd

SIGNATURE(s)
(no legalization necessary)

*In the case of applications in the name of a Company, Partnership or Firm, give full names of signatory/signatories, delete paragraph 1, and enter capacity of each signatory in paragraph 2.
**If the applicant is a natural person, delete paragraph 2.
***If the right to apply is not by virtue of an assignment from the inventor(s), delete 'an assignment from the inventor(s)' and give details of acquisition of right
****For non-convention applications delete paragraph 5

D.M. KISCH INC.
Patent Attorneys &
Trade Mark Agents

South Africa
Local

ASSIGNMENT OF INVENTION

WHEREAS we

CHRISTOFFEL HENDRIK BOSHOFF

and

DENIS FRANCIS YORK

of 29 University Gardens, 308 Queen Mary Avenue, GLENMORE, DURBAN 4001

and

8 Spring Grange Road, WESTVILLE 3630

are the inventors of an invention entitled: **"DETECTION OF ANTIBODY TO MAEDI-VISNA VIRUS"**

AND WHEREAS **THE AGRICULTURAL RESEARCH COUNCIL** of Celtis Plaza, 1085 Schoeman Street, Hatfield, Pretoria, have for good and sufficient consideration acquired the said invention from us for all countries

NOW THEREFORE this Deed witnesses that we have assigned the said invention to **THE AGRICULTURAL RESEARCH COUNCIL** its successors, assigns or legal representatives, for all countries, together with the right to apply for Letters Patent in respect thereof in its own name, the assignment taking effect on or before 17 May1995

We agree that when requested, we will without charge to the assignee but at its expense sign all papers, take all rightful oaths, and do all acts which may be necessary, desirable or convenient for securing and maintaining patents for said invention in any and all countries and for vesting title therefore in said assignee, its successors, assigns or legal representatives.

DATE: 20/6/95

C.H. BOSHOFF

DATE: 20/6/95

D.F. YORK

No legalisation required

D.M. KISCH INC. , Johannesburg

Patent Attorneys & Trademark Agents
Attorneys, Notaries & Conveyancers

Form P.6

REPUBLIC OF SOUTH AFRICA

PATENTS ACT, 1978.

PROVISIONAL SPECIFICATION

(Section 30 (1) - Regulation 27)

PATENT APPLICATION NO.			LODGING DATE.		AGENT'S REFERENCE
21	01		22	17-05-1995	P/95/72674

FULL NAME(S) OF APPLICANT(S)	
71	THE AGRICULTURAL RESEARCH COUNCIL

FULL NAME(S) OF INVENTOR(S)	
72	1. CHRISTOFFEL HENDRIK SCHOEMAN 2. DENIS FRANCIS YORK

TITLE OF INVENTION	
54	DETECTION OF ANTIBODY TO MAEDI-VISNA VIRUS

DETECTION OF ANTIBODY TO MAEDI-VISNA VIRUS

FIELD OF THE INVENTION

THIS invention relates to immunoassay for detecting infection of sheep by Maedi-Visna virus and to new compounds used in such assay.

BACKGROUND TO THE INVENTION

Maedi-Visna Virus [MVV] is a non-oncogenic, exogenous retrovirus of the *Lentiviridae* subfamily. The major route of transmission of MVV is by horizontal spread through colostrum and milk. Animals become chronically infected and are characterised by increasing respiratory distress, persistent weight loss and a susceptibility to secondary infections. Since ovine lentivirus poses a worldwide threat to commercial sheep farming, various techniques are used for its serologic diagnosis. These include agar gel immunodiffusion [AGID], indirect enzyme-linked immunosorbent assay [ELISA], ELISA based on a double antibody sandwich blocking procedure and Western blot analysis. Most of these tests use tissue culture produced viral antigen. This source is labour intensive, expensive and subject to batch variation which makes it unsuitable for routine use. Studies have also shown that both p25 and TM epitopes are necessary to produce a sensitive and specific screening assay. This is due to the fact that the immune response tends to shift

during the course of disease from initially being anti-p25 specific, to a predominantly TM specificity towards the end of the disease.

OBJECT OF THE INVENTION

It is an object of the present invention to provide a novel recombinant antigen suitable for use in serologic diagnosis of MVV infection in sheep by techniques such as enzyme-linked immunosorbent assay [ELISA] and modifications thereof.

STATEMENT OF THE INVENTION

According to the present invention there is provided a fusion protein presenting both the immunodominant core p25 and the transmembrane epitopes of MVV. The protein is expressed by the nucleotide bases 737 to 1485 for the core p25 and nucleotide basis 8084 to 8357 for the transmembrane epitope based on the South African isolate of Maedi-Visna Virus [Querat *et al*, Virology Vol. 175, 1990, p. 434].

The DNA sequence may obviously be modified at any position provided that such modification does not affect the functional integrity of the sequence to code for the immunogenic fusion protein of the invention.

According to a further aspect of the present invention there is provided a vector for use as a suitable expression vehicle comprising a plasmid including the DNA sequence as set out above.

The plasmid is preferably the commercially available plasmid marketed as **pGEX system** by **Pharmacia** of Sweden.

The two DNA fragments as indicated above may be inserted at any position relative to each other provided the correct reading frame is maintained to create an expression cassette comprising both the core p25 and the transmembrane epitopes of MVV.

Thus, the invention essentially relates to the cloning of selected fragments of the MVV p25 and transmembrane [TM] proteins in the same expression cassette, expression of these two distinct epitopes as a single fusion protein in *E. coli*, its purification and its use in antibody detection assays like ELISA.

The invention will now be described to illustrate the approach taken to develop an antigen to specifically and sensitively diagnose MVV infection serologically.

EXAMPLE

PCR Amplification of DNA fragments: The region coding for the TM and core p25 proteins of SA-OMVV were amplified as a 273-bp TM and 748-bp core fragment respectively by the polymerase chain reaction [PCR]. The primers were designed according to the published sequence of SA-OMVV. The primers synthesized to amplify the TM and core p25 region had BamH1 and EcoR1 sites at the 5' end respectively to allow cloning of the amplified products. The nucleotide sequences of PCR primer pairs used to amplify SA-OMVV DNA encoding MVV-TM and core p25 proteins are indicated below.

PRIMER	SEQUENCE [5' to 3']
MVV TM 5' primer	5'-TCC CCC ggA TCC gAA gCT ATA gAT AgA-3'
MVV TM 3' primer	5'-TCC CCC ggA TCC TgC CTC gTg TTg CTC TAT-3'
MVV core 5' primer	5'-TCC CCC gAA TTC gTA TgA AAg AAg ggC TAC AC-3'
MVV core 3' primer	5'-TCC CCC gAA TTC TCC TTC TgA TCC TAC gTC-3'

After PCR amplification the DNA fragments could be visualized by using agarose gel-electrophoresis to verify the correct size of each fragment. These PCR products were subsequently purified and digested with either BamH1 or EcoR1 restriction enzymes.

CLONING OF PCR AMPLIFIED DNA FRAGMENTS

The 273-bp TM and 748-bp core p25 fragments were then ligated separated and consecutively to BamH1 and EcoR1 digested pGex-2T vector, which has been treated with calf intestinal phosphatase prior to ligation, to prevent self ligation of vector DNA. The vector containing the TM and core p25 fragments was sequenced to ensure that both fragments retained their identity and is located correctly within the vector's reading frame in order to be expressed as a functional full length protein. All recombinant DNA techniques were performed by standard methods.

EXPRESSION AND PURIFICATION OF FUSION PROTEIN

The TM-p25 fragments are expressed in *Escherichia coli* as a single fusion protein of glutathione S-transferase, extracted and affinity purified as described by the manufacturers. The purity and relative molecular size of the recombinant protein is analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis [SDS-PAGE].

UTILIZATION OF ABOVE FUSION PROTEIN IN ELISA

The use of this antigen in an antibody detection ELISA has shown to specifically and sensitively diagnose MVV infected sheep at all stages of the disease.

Clearly many variations of the invention may be devised without departing from the spirit of the invention.

Dated this 17th day of May 1995

C.F. VAN BILJON

Patent Attorney/Agent for the Applicant