

**AN INVESTIGATION INTO THE SEROLOGICAL AND MOLECULAR
DIAGNOSIS OF
JAAGSIEKTE SHEEP RETROVIRUS (JSRV)**

by

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Submitted in partial fulfilment of the requirements for the degree of

MASTER OF MEDICAL SCIENCE

in the

Department of Virology

Molecular Biology Section

Faculty of Health Sciences

Nelson R. Mandela School of Medicine

University of KwaZulu-Natal

Durban

2005

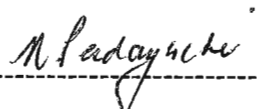
DECLARATION

The experimental work described in this thesis was carried out at the University of Kwa Zulu-Natal, Nelson R Mandela School of Medicine, Faculty of Health Sciences, Department of Virology under the supervision of Dr. Denis F York and University of KwaZulu-Natal, Department of Biochemistry, Pietermaritzburg. 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.



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29th November 2005

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DEDICATION

This thesis is dedicated to my late father,

Mariemuthu Cooposamy Naidoo

ACKNOWLEDGEMENTS

My heartfelt gratitude and appreciation go to the following individuals and departments for their help and assistance with this project:

My supervisor, Dr Denis F York, who has encouraged, supported and was instrumental in teaching me all the Molecular Biology that I have utilised during the course of this project. If it were not for his patience and understanding, I would not have continued with this project. I am extremely grateful for his advice during the most trying period of my life and for being there to listen to all my problems. Thank you.

Professor Alan Smith: Head of Department of Virology, for the support that he has given to me and for the use of the facilities in the Department of Virology in the undertaking of my project. I wish to also acknowledge the financial support provided for the purchase of reagents.

Professor Theresa Coetzer, Department of Biochemistry, University of KwaZulu-Natal, Pietermaritzburg, for the use of the laboratory facilities during the procedure for the immunisation of the chickens.

Jonathan Edwards, Department of Biochemistry, University of KwaZulu-Natal, Pietermaritzburg, for the assistance rendered during the procedure for the immunisation of the chickens at the Ukulinga Farm (Pietermaritzburg).

Dr Fanny Kiepiela, Head of the HPP unit, DDMRI, University of KwaZulu-Natal, for the support and words of encouragement whilst working on this project.

The University of KwaZulu-Natal, for the financial assistance in the form of fee remission towards the Master's degree.

The Medical Technologists in the Department of Histopathology for their help in the immunohistochemical aspect of the project, even though it was not included in the thesis.

Dr Anita Naicker, of the Department of Optics and Imaging, for allowing me the use of the microscopes and imaging equipment.

Mr. Claus Hallwirth for the provision of journal articles which I would have otherwise not been able to obtain. I thank him for his support and help during the course of my study.

Mrs. Coral Vinsen, for encouraging me to complete the dissertation.

Mr. Paul Chulule, Siva Danaviah, Michelle Gordon and Karen Bishop from the Africa Centre for their help and words of encouragement during the completion of this thesis.

Jeremy Norton and Leigh-Anne Edwards for their assistance in this project

I am extremely grateful to my husband Rajen, my mother, Mrs. P Naidoo and children Thanishiya, Shalendra and Yerdashin for their tolerance, patience and support during the

eight years that it took me to complete this project and the final dissertation. Please forgive me for my temperamental attitude during this period.

Last, but not least the 'Almighty God' whose help I constantly sought through prayer to help me to complete the thesis.

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ABBREVIATIONS

A ₂₆₀	Absorbance at 260 nanometre
Ab	Antibody
Ag	antigen
AEV	Avian Erythroblastosis virus
AIDS	Acquired immunodeficiency syndrome
AM	alveolar macrophages
APS	Ammonium persulfite
ASLV	Avian Leukosis-Sarcoma Virus
ATP	adenosine triphosphate
BAC	Bronchioloalveolar Carcinoma
B-Elisa	Blocking enzyme-linked immunosorbent assay
bp	base pair
β-gal	β-galactosidase
BSA	bovine serum albumen
CA	capsid
cDNA	RNA to DNA
cm	centimetre
CMV	cytomegalovirus
[Ag]	concentration of antigen



Da	daltons
°C	degrees centigrade
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
ddTTP	dideoxythymidine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytosine triphosphate
ddGTP	dideoxyguanosine triphosphate
d H ₂ O	distilled water
dNTP	deoxynucleoside triphosphate
ds DNA	double-stranded DNA
DMSO	dimethylsulfoxide
DTT	dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
EM	Electron Microscope
EDTA	ethylenediaminetetracetic acid disodium salt
ELISA	enzyme-linked immunoassay
ENTV	enzootic nasal tumour retrovirus
Et Br	ethidium bromide
ExJSRV	exogenous JSRV-like retrovirus

FPLC	Fast Pressure Liquid chromatography
g	gram
gag	group specific antigen
g/ml	grams per millilitre
gp	glycoprotein
GST	glutathione S-transferase
H ₂ O ₂	hydrogen peroxide
HBAC	human bronchiolo-alveolar carcinoma
HCl	hydrochloric acid
HERV	Human endogenous retrovirus-K
HIV	human immunodeficiency virus
hr	hour
HRPO	horseradish peroxidase
HTLV	human T-lymphotrophic/leukaemia virus
in	inch
IPTG	isopropyl β-thiogalactopyranoside
ICTV	International Committee on the Taxonomy of Viruses
Ig	immunoglobulin
IN	Integrase
JS	Jaagsiekte
JSRV	Jaagsiekte Sheep Retrovirus
JSRV-CA	JSRV-capsid antigen

kb	kilobase
kDa	kiloDalton
kg	kilograms
L	Litre
LB	Luria Bertani
LTR	Long terminal repeats
M	molar
MA	matrix
mabs	monoclonal antibodies
mg	milligram
mGcap	methylGuanosine cap
MgCl ₂	magnesium chloride
min	minute/s
ml	millilitre
mM	millimolar
mm	millimetre
MMTV	Mouse Mammary Tumour Virus
MPMV	Mason-Pfizer Monkey Virus
mRNA	messenger ribonucleic acid
MSV	Murine Sarcoma virus
MuLV	Murine (Mouse) leukaemia virus
m/v	mass by volume

NaOH	Sodium Hydroxide
NaCl	Sodium Chloride
N	normal
NC	nucleocapsid
Ng	nanograms
NL	normal lung
nm	nanometer
nM	nanomolar
OD	optical density
OPD	O Phenylenediamine 2 Hydrochloric acid
orf-X	open reading frame-X
OPA	ovine pulmonary adenocarcinoma
p	polypeptide
pmoles/ μ l	picomoles per microlitre
PAC	Peripheral adenocarcinoma
PAC	peripheral airway cells
PAS	Periodic Acid Schiff
PBMC	peripheral blood monocytes
PB+	primer binding site for positive strand DNA synthesis
PB-	primer binding site for minus strand DNA synthesis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethyleneglycol

pJSRV ₂₁	plasmid JSRV 21
pol	polymerase
PR	Protease
RE	restriction endonuclease
rpm	revolutions per minute
RNA	ribonucleic acid
RSV	Rous Sarcoma virus
RT	reverse transcriptase
3SR	Self-Sustained Sequence Replication
Sag	superantigen
SAIDS	simian 'AIDS' viruses
SBAC	Sheep bronchioloalveolar carcinoma
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	second/s
SMRV	Squirrel Monkey Retrovirus
SIV	simian immunodeficiency virus
SPA	Sheep pulmonary adenomatosis
SU	surface glycoprotein
tRNA	transfer ribonucleic acid
TAE	Tris-acetate ethylenediaminetetraacetate
TFB	Transformation buffer

TSP	Tropical Spastic Paraparesis
TBE	Tris–Borate-Ethylenediaminetetraacetate
Temed	N,N,N'N'-tetramethyl-ethylenediamine
T _m	temperature
TM	transmembrane
μg	microgram
μl	microlitre
μM	micromolar
uv	ultraviolet
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

ABSTRACT

The Jaagsiekte Sheep Retrovirus (JSRV), an exogenous type B/D-retrovirus with about 10-15 endogenous counterparts in all normal sheep genomes, causes Jaagsiekte (JS) or ovine pulmonary adenocarcinoma (OPA), a contagious lung cancer of sheep. This sheep lung cancer has been identified as the best natural out-bred model that can be used to study human epithelial tumours. The close similarity between the pathology of the sheep disease and Human Bronchiolo-alveolar carcinoma are highly suggestive that the human disease could have a similar aetiology and mechanism to the sheep disease. However, in the case of sheep at the time of the study there was a need for an assay that could be used to screen for infected sheep.

The isolation, cloning and subsequent sequencing of the first full-length exogenous and endogenous forms of JSRV contributed greatly towards JSRV research. Until recently the diagnosis of OPA was based mostly on clinical presentation with confirmation by micro and macro examination of the affected lungs by expert pathologists. In the absence of a specific humoral response no serology-based tests were available to diagnose the disease early in live animals. Control and management of the disease was primarily by regular flock inspections and prompt culling of the suspected cases.

The objective of this research project was therefore to assess and investigate the serological and molecular diagnosis of JSRV. In an attempt to develop a serology based assay three proteins were identified as candidate diagnostic antigens, the group specific antigen JSRVp26, the transmembrane and the *orf-X* proteins. Genes coding for all three proteins were isolated, cloned and expressed. The JSRV p26 was sufficiently purified and

its potential as a diagnostic antigen was evaluated in both a Western blot and ELISA. Our studies confirmed that there were no circulating antibodies to the JSRV capsid protein. Evidence suggested that the immune response was localised to the lungs. Lung lavage samples were therefore collected from infected and normal sheep and analysed for the presence of JSRV p26 antibodies using an in-house JSp26 peroxidase conjugate in an antigen capture assay. This assay lacked sensitivity but the results indicated that there was a specific localised immune response to JSRV in the lungs of OPA affected sheep. This was confirmed with an in-house antigen capture assay that we developed. JS antigen was detected in the lung and nasal fluid of affected sheep, but not in equivalent samples from normal sheep.

Three molecular assays were investigated for their sensitivity and specificity, the LTR-*gag* PCR, U3/LTR hemi-nested PCR and the PCR that covered the V1/V2 region. The U3/LTR hemi-nested assay was 2 logs more sensitive than the LTR-*gag* PCR. However, it detected the endogenous JSRV5.9A1 loci at higher concentrations. This was overcome by designing a more specific primer P3M for the first step of the U3/LTR hemi-nested PCR and the use of the AmpliTaq Gold DNA polymerase. This assay proved to be both sensitive and specific enough to screen for the infectious exogenous JSRV in peripheral blood samples from individual sheep.

It is now possible to use this assay to selectively eradicate the disease from a flock through a selective culling programme. Furthermore, the assay could be made quantitative by the inclusion of concentration standards.

CHAPTER 1

CHAPTER ONE

REVIEW OF THE LITERATURE

1 INTRODUCTION

Jaagsiekte Sheep Retrovirus (JSRV) is the causative agent of a contagious lung cancer in sheep that is known as Ovine Pulmonary Adenocarcinoma (OPA). The disease has been called by many other names including Ovine Pulmonary Carcinoma (OPC), Sheep Pulmonary Adenomatosis (SPA) and Bronchioloalveolar carcinoma (BAC) (Done, 1990; Hecht *et al*, 1996; De Martini *et al*, 1997; Palmarini *et al*, 1997 and Palmarini *et al*, 2001). In South Africa the disease is often referred to as jaagsiekte, a Dutch term used to describe the accelerated respiration that is so pronounced towards the latter stages of the disease that the animal appears to have been rapidly chased (*jagt*: to drive or chase; *ziekte*: a sickness), hence the name jagtziekte (Mitchell, 1915). However, OPA is now the official name to be used when referring to the Jaagsiekte sheep retrovirus induced disease. This was decided upon at an international workshop on JSRV/OPA that was held in Missillac, France in June of 2001 when all the researchers in this field were brought together (York *et al*, 2003a). OPA has been one of the most intensively studied lung tumours of domestic animals (de Kock, 1929; Dungal, 1938; Tustin, 1969; Hod *et al*, 1977; Nobel and Perk, 1978). Liebow 1960, defined Bronchioloalveolar (peripheral) carcinoma as “one whose cells tend to line the walls of distal airspaces without destroying them and spread via aerogenous and lymphatic routes” (Liebow, 1960). OPA is normally associated with different breeds of sheep and more rarely in goats and wild moufflon (Sharp *et al*, 1986 and De Las Heras *et al*, 1991). OPA shares pathological characteristics with human bronchiolar-alveolar cell carcinoma, an epithelial tumour that is not significantly associated with smoking and represents a quarter of human lung cancer cases (Wu *et al*, 1988 and Barsky *et al*, 1994). Recently the World Health Organisation (WHO) conveyed a more precise definition of human lung tumours. With regard to this new classification OPA would be defined as a ‘mixed adenocarcinoma showing acinar, papillary and bronchioloalveolar growth patterns’ (Palmarini *et al*, 2001).

JSRV is closely related to a retrovirus, Enzootic Nasal Tumour Virus (ENTV), the agent responsible for enzootic nasal tumours (nasal adenocarcinomas) of both sheep and goats (Cousens *et al*, 1999). The disease pathologies though distinctly different, are caused by two very similar viruses that would allow any control strategies that are designed for the one, including vaccination control, to be used for the other (De Las Heras *et al*, 2003b). The same would apply for both serology and molecular based diagnostic assay.

OPA (jaagsiekte) is important as a natural animal model of retroviral carcinogenesis of the secretory epithelium of the respiratory system, as epithelial neoplasms occur less frequently in animals as compared to humans (De Martini and York, 1997). OPA is characterised by a long incubation period and once the typical symptoms of the disease manifests, recoveries do not occur (Hecht *et al*, 1996). Much research has been undertaken in understanding the pathogenesis of OPA and the biology of its causative agent. This has resulted in OPA being selected as a model system to study a lung cancer at the molecular level (Palmarini *et al*, 2001). Therefore, the purpose of this study is to utilize the information available to establish a possible serological as well as a molecular technique to detect the aetiological agent as early as possible.

1.1 HISTORY

The earliest reports of OPA appeared in a letter written in 1825 by Veldkornet P. Aucampi in the Rhenoster district of the Cape of Good Hope where 800 sheep were reported to have died of OPA (Tustin, 1969). Hutcheon, a veterinarian employed by the Cape Government, first described symptoms and macroscopic pathology in 1891 (Hutcheon, 1891 cited by Tustin 1969). The disease was known to be contagious and Hutcheon advised farmers that the slaughter of affected animal was essential to prevent spread of the disease. The incidence of OPA as reported by these farmers at that time (late 1890's to early 1900's) was about two percent. The disease was probably brought into South Africa when merino sheep were imported from Spain in the early 1800's and the sheep developed the disease at about four years of age. In March 1912, Sir Arnold Theiler, Director of Veterinary Research at Onderstepoort carried out a series of experiments that were later continued by Mitchell to deduce the possible mode of transmission of the disease. After a series of experimental transmission attempts using blood, bronchial exudates, and suspensions of affected lung tissue as inoculum and by placing healthy sheep in contact with the affected

sheep, Mitchell was confident that the disease was capable of being transmitted by physical contact (Mitchell, 1915). Various aspects of Mitchell's experimental procedure, especially the short incubation period, led De Kock in 1929 to disclaim that Mitchell had transmitted the disease by physical contact of diseased sheep with normal sheep (Tustin, 1969). De Kock described a form of ovine interstitial pneumonia characterised by an excessive accumulation of round cells of the lymphoid series, and an atypical proliferation of bronchiolar epithelium referred to as Graaf Reinet Disease. What De Kock described was different to the proliferation seen in Jaagsiekte sheep and Chronic Catarrhal Pneumonia with the presence of giant cell formations in the alveolar exudates. He stated that there might be two lung diseases affecting the sheep, namely, a form of pneumonia called "Graaf Reinet Disease" and OPA "Graaf Reinet" disease and Montana Progressive Pneumonia were histopathologically similar (Tustin, 1969). De Kock in 1929 concluded that the neoplasms in OPA were probably of the nature of multiple papilliform cyst-adenomata. He also tried to determine the infectiousness of OPA by exposing healthy sheep to affected sheep. He was the first to transmit the disease successfully in this way, that is, by co-habitation (De Kock, 1929).

1.2 DISTRIBUTION

Ovine pulmonary adenocarcinoma (OPA) and progressive interstitial pneumonia (*maedi*) caused great confusion because both diseases existed in sheep and native languages or place names were used to describe the diseases (Shirlaw, 1959; Wandera, 1971). Despite the variation in the description of adenomatous lesions of Jaagsiekte, it is a specific disease and is found worldwide (Wandera, 1971). Jaagsiekte was first reported in England in 1888 by both McFadyean and Dykes, when they thought the lesions were caused by lungworms, and in Germany by Eber A. in 1899 (cited from Tustin, 1969). During the 1930's large-scale outbreaks were reported in Iceland where Dungal and associates were the first to transmit the disease by means of parenteral inoculation of suspensions containing the infectious agent. OPA is the most common pulmonary tumour of sheep and occurs in many countries. It is unexplainable why countries like Australia and New Zealand have escaped. Both these countries have imported sheep (merino) directly and via Britain and South Africa from Spain (De Kock, 1929). A possible explanation for this occurrence is that the sheep which were imported into both these countries served as flock founders that may have created a bottleneck such that one of the parent viruses needed to generate the

infectious viruses was not present in one of the flocks (Bai *et al*, 1999). Based on circumstantial evidence the original home of OPA could be Spain, France or Germany.

1.3 INCIDENCE AND ECONOMIC IMPORTANCE

The incidence of OPA in sheep farming communities will vary depending on the management of the flock (Sharp, 1981), susceptibility to the environment, that is, confinement in close winter quarters; and breed or strain of sheep. OPA was found to be most serious in flocks not previously exposed and lowest in those exposed longest (Hutt, 1964). A typical example is the epidemic suffered by Iceland during the 1930's and 1940's when there were widespread outbreaks due to OPA, which caused enormous losses to the farmers (Dungal, 1946).

In 1933, 20 Karakul sheep from Haile, Germany were imported into Iceland. These sheep were kept in quarantine for two months before distribution throughout 14 sheep farms in Iceland. Epidemiological studies show that two of the rams were carriers of a chronic pulmonary infection of the lungs, *maedi*, and one of them a carrier of OPA. In the winter of 1934, OPA started to appear on many of the farms and losses greater than 50% were reported over the next two or three years. The Gottorp strain of sheep was particularly susceptible while the Adolbol stock was conspicuously resistant but not widely distributed when the disease struck (Perk, 1982). The epizootic was at its peak from 1936–1945. OPA was eradicated from Iceland by destroying all sheep in the infected areas in the period 1944 to 1951. This eradication campaign was successful and no cases of OPA have been seen in Iceland since 1952 (Sigurdsson, 1958).

The actual mortality or number of animals involved in OPA infected flocks has varied in different countries and can actually be as much as up to 50%. It tends to drop to 1% to 5% in flocks where it is endemic (Wandera, 1971). In Scotland, 20% of all mature sheep necropsied at the Veterinary Investigation centres had OPA (cited from York, 1987). The prevalence rate in Scotland is much higher than in England and this suggests that there are breed differences in susceptibility. Lately it has become a significant problem in the UK, especially in East Anglia and on the east coast and Scottish Borders (Kayne, 2004). In South Africa, Merino and Karakul and their crossbreeds are possibly more susceptible than English breeds. The sex of the animal does not seem to play a role in its susceptibility

(Verwoerd *et al*, 1994). Losses of 30% or more in certain flocks were not unusual when the disease was first introduced into South Africa in the 1800's (Tustin, 1969).

Early reports on the natural occurrence of OPA in goats were controversial because of the unconvincing data and inadequate differentiation between alveolar epithelialisation and true adenocarcinomatous lesions (Tustin, 1969). OPA does occur naturally in goats in some countries where the prevalence is low, but it has not been diagnosed naturally in the Southern African species (Verwoerd *et al*, 1994). However, it has been experimentally transmitted to kids in South Africa. It appears that goats are less susceptible to JSRV than sheep, because in one experiment a viral inoculum of OPA that produced extensive lesions in more than 90% of newborn lambs after two to three months produced only small-circumscribed lesions in two out of five kids after nine to thirteen months (Tustin *et al*, 1988). Transmission studies in Europe provided similar results (Verwoerd *et al*, 1994). Further support for the natural low prevalence rate amongst goats is the epidemic of OPA in Iceland in the 1930's and 1940's. OPA was not seen in goats even though the goats were in close contact with the JS affected sheep (Tustin, 1969).

1.4 TRANSMISSION

De Koch, 1929 and Dungal, 1946 showed that the disease could be transmitted by co-habitation of healthy sheep with affected ones. Dungal experimentally proved in two ways that the exhaled air of an affected sheep contained the infectious agent. According to Tustin, 1969, Dungal and Markson as well as the others successfully transmitted the disease by exposing sheep to droplet infection by means of an aerosol spray. Tustin in his review of OPA found that transmission by means of parenteral inoculations of suspensions of diseased lung tissue and filtrates was also successful. This was accomplished by intrapulmonary, intrapleural, intratracheal or subcutaneous routes or by various combinations of these routes together with others such as intranasal. In an experiment carried out by Tustin at Onderstepoort, OPA was transmitted using neoplastic cells grown in tissue culture. Of the two day-old lambs that were inoculated one died of OPA after 249 days and the other was sacrificed after 253 days. Advanced lesions were present in the lungs. On repetition of this experiment using cells from another sheep, the transmission experiment failed (Tustin, 1969).

Experimental data therefore indicate that the main natural route of transmission is aerogenous and the spread of infection is facilitated by close contact. An attempt was made to determine whether intra-uterine infection took place. Results based on experimental evidence of OPA indicated that prenatal infection was not of significance (Tustin, 1969)

1.5 AETIOLOGY

Years of research in many countries failed to reveal the agent responsible for the disease, though many theories were put forward regarding the infectious, neoplastic nature and causal agent of OPA (Wandera, 1971). De Kock was the first to report on the successful experimental transmission of the disease by contact of healthy sheep with sick sheep in 1929 (Verwoerd *et al*, 1994). These results were later confirmed by Dungal in 1946. His experiments were designed so as to exclude all other possibilities of infection except through inhalation (Dungal, 1946).

The first ever report of a possible retroviral involvement in the aetiology of OPA was made in Israel when typical retrovirus particles were observed in adenomatous lesions (Perk *et al*, 1971). Retroviruses were found in cell cultures established from OPA lungs (Malmquist *et al*, 1972) and reverse transcriptase activity in purified extracts of lung tumour tissue. Transmission studies were not carried out and the probable presence of *maedi-visna* lentivirus in the material studied posed a problem as far as interpretation of results was concerned (Perk *et al*, 1974). Martin Scott and Sharp were the first to report the transmission of Jaagsiekte with lung extracts containing reverse transcriptase (Martin *et al*, 1976). Their findings were confirmed later (Verwoerd *et al*, 1980).

Several factors have emerged that clearly implicate retroviral involvement in OPA. A viral aetiology was suggested when lung tumours were induced with cell free filtrates of the lung discharge, lung lavage fluid or tumour homogenate (Martin *et al*, 1976). Secondly, reverse transcriptase activities with magnesium preference, associated with material of the density of retroviral particles of 1.175–1.18 g/ml, were found in the tumour material (Perk *et al*, 1974; Verwoerd *et al*, 1980; Herring *et al*, 1983). Thirdly, the same tumour derived materials contain proteins that cross-react with antisera to the capsid (Sharp *et al*, 1983; Kajikawa *et al*, 1990) and nucleocapsid proteins (He *et al*, 1992) of Mason–Pfizer Monkey Virus (MPMV) and Mouse Mammary Tumour Virus (MMTV), a Type D and Type B

oncovirus, respectively. Fourthly, retrovirus particles can be seen by electron microscopy in lung tumours and lung fluid (Payne *et al*, 1983; De Martini, 1988). Fifthly, the incubation period in the experimental studies is inversely proportional to the reverse transcriptase level in the inoculum, and may be as short as a few days to weeks (Verwoerd *et al*, 1980; Herring *et al*, 1983). Reverse transcriptase and viral proteins have been found only in tumour and lung fluid, never in other tissues, strengthening the physical linkage between the virus and the tumour. Further, the complete genome of the Jaagsiekte Sheep Retrovirus (JSRV) has been cloned from viral particles secreted in lung exudates of affected animals and sequenced. The genome is 7,462 nucleotides long and exhibits a genetic organization characteristic of the Type B and D oncoviruses (York *et al*, 1991; York *et al*, 1992). The exogenous form of JSRV has been consistently and exclusively found to be present in tumours and lung fluids and also on occasions used as inocula to induce OPA (Palmarini *et al*, 1996). These factors clearly implicate a retrovirus (exJSRV) of probably Type B or D classification in the pathogenesis of OPA (York *et al*, 1991; York *et al*, 1992).

The final confirmation that JSRV is exclusively responsible for the disease and transformation was provided by Palmarini *et al* (1999). They constructed an infectious and pathogenic full length JSRV proviral clone pJSRV₂₁ from a tumour genomic DNA library derived from a natural case of OPA. The U3 region of the upstream LTR in pJSRV₂₁ was replaced with the human cytomegalovirus (CMV), which is the immediate early promoter that is highly active in these cells. The CMV promoter was positioned so that the resulting RNA transcript would be very similar to wild-type JSRV RNA. When the resulting plasmid (pCMV2JS₂₁) was transfected into 293T cells substantial amounts of JSRV₂₁ virus were released into the supernatant. Four newborn blackface lambs were inoculated intratracheally with concentrated JSRV₂₁ stocks obtained from transfected 293T cells. The resulting tumours were histologically identical to spontaneous and experimentally induced OPA, and the tumour cells expressed JSRV CA antigen. Infection with OPA was also confirmed molecularly. This proved conclusively that JSRV is the aetiological agent of OPA and that it is necessary and sufficient to induce OPA (Palmarini *et al*, 1999). Recently another infectious molecular clone JSRV_{JS7} was obtained from a sheep lung cell line JS 7. (De Martini *et al*, 2001). His group constructed an infectious clone and managed to infect sheep and recover the virus from the infected sheep. Thus fulfilling Kochs postulates (Palmarini *et al* 2000; De Martini *et al*, 2001).

Other tumours that are associated with retroviruses and have similarities to OPA are the Caprine and Ovine nasal adenocarcinomas (De Las Heras *et al*, 1991; De Las Heras *et al*, 1991). Similarities such as experimental transmission using cell-free filtrate (De Las Heras *et al*, 1995), retroviral particles, particle-associated reverse transcriptase activity and antigenic cross-reactivity of ENTV capsid proteins with that of MPMV capsid proteins p27 and recombinant JSRV capsid proteins exist, indicating that ENTV has a Type D retroviral capsid (De Las Heras *et al*, 1991; De Las Heras *et al*, 1993). These studies have found that the goat viral products appear to be different from the OPA virus with a slightly different antigenic cross-reactivity. A type D-related virus has also been isolated from bovine cells, but its classification is uncertain and its role in disease, if any, is unknown (York *et al*, 1989). The sheep and goat nasal adenocarcinoma and OPA models emphasise the need for research into the mechanisms of transformation of secretory cells of the respiratory tract caused by a family of ungulate retroviruses. These ENTV's have been shown to be antigenically and genomically related to JSRV (Cousens *et al*, 1999).

1.6 CLINICAL SIGNS AND PATHOLOGICAL FEATURES

For natural infections the incubation period varies from about nine months to two to three years (Dungal, 1938). Experimentally, because of the large number of lesions induced, the incubation period in lambs can be reduced to as little as three weeks by inoculation of concentrated lung rinse material containing the aetiological agent in high concentration (Verwoerd *et al*, 1994).

The onset of clinical signs is insidious in natural cases. Although the physical condition of the animal may appear to be good, the respiratory rate is more rapid than normal after an animal has been driven. As the disease progresses, affected animals have a reduced appetite, body weight decreases and the animal tends to lag behind the flock when it is driven. Marked respiratory distress is evident on exercise, the respiratory movements being short and jerky. Tachypnoea and dyspnoea eventually become evident even at rest. Spasmodic bouts of coughing occur and there is a great increase in the amount of secretion from the lungs, resulting in the production of a watery nasal fluid. This is regarded as almost pathognomic for OPA (Mitchell, 1915; Dungal, 1938; Verwoerd *et al*, 1994). In countries where OPA is endemic, a clinical diagnosis of OPA is based on inducing flow of this fluid (up to 300ml can be collected) by raising the rear legs and lowering the head of

animals suspected to have the disease (referred to as the wheelbarrow test), upon thoracic auscultation, moist rales may be heard (Tustin, 1969).

There is generally no fever, but pyrexia may occur as a result of pneumonia following secondary bacterial infection (Verwoerd *et al*, 1994). Because no convenient test for detection of infected sheep existed, estimating the incubation period accurately has been difficult, although it is estimated at about five to six months. Lambs as young as three months have been diagnosed with OPA, but two to four year old sheep are most commonly affected (Tustin, 1969). In clinically affected animals a peripheral lymphopenia is marked by a decrease in CD4 T lymphocytes and a corresponding neutrophilia can assist clinician's diagnosis but the changes are not pathognomic (Sharp, 2000). Anatomic diagnosis of OPA is based on gross lesions and histopathology, but differentiation from other causes of chronic pneumonopathies of sheep is not always easy (De Martini and York, 1997). In advanced cases of Jaagsiekte the lungs are three or more times their normal weight (up to 2kg) and fluid almost fills the thoracic cavity. Interestingly the lungs do not collapse when the thorax is opened (Verwoerd *et al*, 1994). Lungs affected by OPA contain large, firm, gray masses in the anteroventral regions of one or both lungs, occasionally with similar, smaller (1-2 centimetres) nodules in posterodorsal parts of the lung. The intermediate lobe is frequently involved (De Martini and York, 1997). The lungs commonly exude clear fluid from the cut surface of the bronchioles of the affected areas and the trachea may be filled with foamy fluid. This is seen in the classical pathological form of OPA (De Las Heras *et al*, 2003b). Some cases are complicated by areas of necrosis in the tumour masses and by chronic or acute bacterial pneumonia that may hide or overshadow the tumour lesion pattern (De Las Heras *et al*, 2003b).

Another pathological form of OPA is more nodular and is referred to as being atypical. Nodules and areas of diseased tissue of various sizes (smallest being less than 1mm in diameter) are sometimes scattered throughout the normal lung tissue. Large lesions consist of very dense tumorous tissue with a relatively firm consistency, and may protrude slightly above the affected lung tissue. The distribution of lesions suggests that the primary lesion or lesions grow by expansion. Intrapulmonary spread of the infection with the development of new foci probably occurs both aerogenously and via the lymph and blood streams. Each new focus is seen grossly as a small greyish-white, semi-transparent nodule which at first is barely visible to the naked eye. These new foci expand and coalesce with neighbouring

nodules to develop into larger lesions. Lesions of experimentally induced cases tend to be more multicentric than those caused by natural infection (Verwoerd *et al*, 1994).

Intra- and extra-thoracic metastasis of the tumour may occur (Tustin, 1969; Verwoerd *et al*, 1994). In South Africa extrapulmonary metastasis is rare, whereas it frequently occurs in Awassi or fat-tailed sheep in Israel, where metastasis both within and outside the thorax may be found in 30% to 50% of cases (Herring *et al*, 1983; De Martini *et al*, 1988). The ability of OPA tumours to metastasize has led to them being transplanted in mice, thereby deriving cell lines from nude mice. This supports the theory that OPA tumours are neoplastic rather than proliferative in nature (Palmarini *et al*, 2001). About 10% of OPA tumours metastasise to regional pulmonary lymph nodes and metastasis to heart and skeletal muscle have been found less frequently. Neoplastic masses of OPA are composed of cuboidal to columnar epithelial cells in single layers or proliferating to form projections (or acinar-like structures) into the alveoli. Surrounding normal alveoli are usually compressed and may contain infiltrates of large alveolar macrophages with foamy cytoplasm (De Martini and York, 1997).

Ultra structural studies of OPA lesions indicate that tumour cells of Type II cell origin are usually well differentiated maintaining the appearance of the cells from which they originated (Verwoerd *et al*, 1994; Palmarini *et al*, 2001), with microvilli, desmosomes, and intracellular lamellar bodies. Tumour cells of Clara-cell origin contain cytoplasmic dense bodies instead of lamellar bodies (De Martini and York, 1997).

Scanning electron microscopy reveals that the surfaces of the neoplastic cells are usually covered by abundant microvilli, in stark contrast to the smooth surface of normal alveolar epithelial cells. A single transformed cell proliferates to form a single layer lining the alveolar lumen, which then develops into a grape-like cluster with papilliform projections eventually filling the alveolar space and spreading to adjacent alveoli. The most common non-malignant cells, found mainly in advanced lesions, are macrophages. They have an "angry" appearance, being large and possessing a ruffled surface indicating a state of activity (Verwoerd *et al*, 1994).

1.7 PATHOGENESIS

The natural route of infection is the respiratory route. The primary lesion in OPA is neoplastic transformation of Type II secretory epithelial cells (pneumocytes) and possibly also of the non-ciliated terminal bronchiolar epithelial cells (Verwoerd *et al*, 1994). Cells that have been transformed and where viral replication has occurred (Payne *et al*, 1983) proliferate and form clusters that invade and eventually obliterate the alveolar lumen, resulting in death due to hypoxia. Type II pneumocytes produce large amounts of surfactant containing clear and viscous fluid, which is stored in the air passages and aggravates respiratory distress. This surfactant leads to coughing and the formation of aerosol droplets containing the virus or tumour cells. Clara cells on the other hand are considered to synthesise, store and secrete protein elements of the extracellular lining layer of the bronchioles (Verwoerd *et al*, 1994; Palmarini *et al*, 2001)

OPA transcripts and to a lesser extent proviral DNA have been demonstrated in the draining lymph nodes and also in several anatomically dispersed lymphoid tissues such as the spleen, thymus, bone marrow and PBMC's (peripheral blood monocytes) indicating that JSRV probably establishes a disseminated infection. It has been found that the virus is exposed at much higher levels in OPA tumours than in lymphoid tissues particularly those at the non-local sites. The significance of JSRV in the overall pathogenesis of OPA is unknown. Presently sheep with clinical signs of OPA have been examined and therefore it is not known whether lymphoid infection precedes or is a result of JSRV replication in the pulmonary epithelium. Further studies will be required to define which cell types are involved, at which stages during the period between infection and the appearance of clinical illness, and the effect of JSRV on effector functions of the infected lymphoid cells (Sharp, 1997). A characteristic feature of OPA is a presence of large numbers of alveolar macrophages (AMs) usually in areas adjacent to the adenomatous lesions. The exact role of the AMs in the pathogenesis of OPA is not known, but one of its roles according to Eckert, 1983, is to take up the excess surfactant in the lungs (Eckert *et al*, 1983). The normal pathway leading to the proliferation of AMs is via the pulmonary interstitium. Blood monocytes enter the alveolus via the pulmonary interstitium and become macrophage precursors. These macrophage precursors differentiate into AMs under the influence of a growth factor supplied by mature AMs (Myer *et al*, 1987). The activated macrophages produce substances that stimulate the proliferation of Type II cells.

In the OPA lungs, the increase in AMs is due to the tumour cells secreting a chemotactic factor, which stimulates the proliferation of AMs. The chemotactic factor secreted by OPA belongs to the category of polypeptide lymphokines that includes the growth and tumour cell necrosis factors. After chemotactic stimulation, macrophages have been shown to produce both growth-stimulatory and inhibitory factors, depending on their interaction with other lymphoid cells (Verwoerd *et al*, 1994).

OPA is often closely associated with the lentivirus infection (Payne *et al*, 1986; De Martini *et al*, 1988). This lentivirus remains in a latent form in the monocytes, which are activated during their maturation. The presence of large numbers of activated macrophages in OPA lungs creates an ideal environment for lentivirus replication. Further, there is a possibility that JSRV has an immunosuppressive effect, similar to that of MPMV to which it is related. This effect makes adenomatous lungs more susceptible to lentivirus infection than normal lungs (Payne *et al*, 1986). It has been shown that the South African lentivirus isolate causes mild immunosuppression in sheep. The degree to which this happens coincides with the latent period before the appearance of signs of OPA in co-infected animals. Lentiviruses, in addition to causing mild interstitial pneumonia may lead to secondary infections in animal (Myer *et al*, 1988).

1.8 OVINE PULMONARY ADENOCARCINOMA (OPA) AND HUMAN BRONCHIOLO-ALVEOLAR CARCINOMA (HBAC)

Human Bronchiolo-Alveolar Cell Carcinoma (HBAC) is a rare tumour, which arises from both bronchiolar and alveolar epithelium. No apparent or yet detectable cause for lung damage has been proved, though an infective basis for these tumours has been suggested (Della *et al*, 1976). The 1999 World Health Organisation (WHO) imparts a more defined classification upon human lung cancer and classifies it into five major subtypes-acinar (gland forming), papillary, bronchiolo-alveolar, solid with mucous formation and mixed adenocarcinoma (Travis *et al*, 2000). Human Bronchiolo-alveolar carcinoma (HBAC) is defined as 'a type of adenocarcinoma with a pure bronchiolar-alveolar carcinoma growth pattern and no evidence of stromal, vascular or pleural invasion' (Palmarini *et al*, 2001). HBAC being one of the subtypes of adenocarcinoma represents 2% to 6% of all lung cancers.

HBAC consists of uniform cuboidal cells that arise distal to the terminal bronchioles and spreads along the alveolar septae without causing significant amounts of lung destruction. This is known as a lepidic growth pattern (Busick *et al*; 2000). There are three major subtypes of HBAC, namely, non-mucinous, mucinous and sclerotic HBAC (Fujimoto *et al*, 1999). Approximately 41% to 60% are mucin producing, 21% to 45% are non-mucin producing, 12% to 14% are a mixture and up to 7% of the cases of the tumours are difficult to classify. The mucin- producing tumours show gross and microscopic mucin production and tend to be multicentric. These tumours consist of tall columnar cells with abundant apical cytoplasmic mucin that is diastase-digested, PAS (Periodic Acid Schiff) negative and mucicarmine negative and small basally oriented nuclei. The tumour cells grow along thin alveolar septae. The surrounding airspaces are often filled with mucin, creating a colloid-like appearance (Travis *et al*, 2000).

Non-mucinous HBAC is more solitary. These tumours consist of cuboidal cells proliferating along alveolar septae giving rise to a hobnail or saw-toothed appearance. Nuclear inclusions are present in non-mucinous tumour cells and show certain properties. They are PAS positive, stain with immunohistochemistry for surfactant apoprotein, and ultrastructurally appear to consist of 40nm branching microtubules that are thought to arise from the inner nuclear membrane. This type of HBAC may consist of Clara cells or Type 11 pneumocytes (Travis *et al*, 2000).

The third type of HBAC is termed sclerotic. This type of HBAC has the tumour cells radiating from a central region of scarring (Fujimoto *et al*, 1999). The alveolar septae are thickened by fibrosis without loss of alveolar architecture (Travis *et al*, 2000). HBAC normally progress to a multifocal pattern that results from intrapulmonary metastases. In some cases the different foci are multiclonal (Barsky *et al*, 1994b).

The incidence of HBAC is increasing in the USA and Japan and represents up to 25% of all primary lung carcinomas (Palmarini *et al*, 2001). Overall primary lung cancer has increased drastically between 1955 and 1990 and appears to be more prevalent in men (74%) than women (26%). Over this 35-year period, there has been a dramatic increase of HBAC from less than 5% to 24% of total human lung cancers, with a male to female ratio of around unity. Patients with HBAC appear to be younger at diagnosis and more likely to

be female (Barsky *et al*, 1994a) and less likely to be cigarette smokers as compared to those with non-HBAC. The cause of HBAC is unknown.

OPA is histologically similar to HBAC and is found to exhibit a diffuse and multifocal character. No cases of sheep to human infection with the retrovirus have been reported. There have been reports of sheep farmers developing the diffuse form of HBAC (Barsky *et al*, 1994b) that histologically bear resemblance to OPA and consequently a viral cause for the disease cannot be ignored and its possible link to JSRV cannot be excluded (Cremer *et al*, 1992; Barsky *et al*, 1994a; Palmarini *et al*, 1997; De Las Heras *et al*, 2000).

Malassez was the first researcher in 1876 to describe HBAC (Malassez, 1876). Duran-Reynals 1958, had reported that many scientists have observed this similarity of HBAC to OPA (Duran-Reynals *et al*, 1958). Both OPA and HBAC are well-differentiated, multifocal tumours of alveolar type II (pneumocytes) or non-ciliated bronchiolar (Clara) cells. The neoplastic tissues are preferentially localised at the periphery of the lungs and tend to grow within the lung for a prolonged period terminating in a late metastatic process. Both share the same histological and ultrastructural features as well as having a peak occurrence in adults. In the human cell tumour, as in the sheep carcinoma, apical mucin granules, tonofibrils, microvilli and neoplastic cells with cytosomes are found. This tumour development is similar to bronchiolar alveolar embryogenesis. The term benign and malignant pulmonary adenomatosis has been applied to HBAC. In the so called “benign” form the constituent tumour cells lining the alveolar walls are peg-shaped and form a single investing layer of cells. Spread to both the hilar lymph glands and elsewhere is of rare and late occurrence. In the more malignant varieties the neoplastic cells form focal mural clumps of cells, which project, into the alveolar lumen. The neoplastic cells are several layers in thickness and display abnormal mitosis and other evidence of malignant change. The more malignant tumours are more liable to secrete mucus giving the lung a macroscopic appearance similar to Type Three-pneumococcal pneumonia. Intranuclear eosinophilic inclusion bodies may be found occasionally in some of the tumour cells, but upon Electron microscopic (E.M.) examination no virus aggregates were seen (Della Porta *et al*, 1976). Lung cancer is one of the most common of human malignancies and the prevalence of carcinomas in human beings exceeds haematopoietic neoplasms. In domestic animals, most importantly sheep and goats, bronchiolar and mainly peripheral adenocarcinomas are the most prevalent lung neoplasms, and also in which experimentally

induced carcinomas occur (Cremer *et al*, 1992). Since OPA exhibits all the criteria included in the definition of Bronchiolar-alveolarcarcinoma, this spontaneous, natural animal model can therefore provide basic information on the cell origin of HBAC. The disease pathogenesis from initial infection through to the development of preneoplastic lesions and eventual development of neoplastic events, natural history, epidemiology, host susceptibility of the disease that could lead to a selection of agents and modalities for chemotherapy in experimental animals and humans (Hod *et al*, 1977; Perk *et al*, 1974; Perk *et al*, 1982; Cremer *et al*, 1992).

1.8.1 Importance of HBAC AND JSRV

Human Bronchioloalveolar Carcinoma (HBAC) closely resembles OPA clinically, histologically and ultrastructurally. This similarity has raised questions about the role that JSRV could be playing in the development of human lung cancer and it was thought that the cause of BAC might have retroviral aetiology as well. It was O'Connell *et al* in an abstract that first suggested that a retroviral connection might exist owing to the presence of JSRV-related sequences and major capsid proteins in human BAC/PAC (peripheral adenocarcinoma) (O'Connell *et al*, 1998). Work by De las Heras *et al* (2000), revealed that 23% of 249 human lung tumours that have been tested by immunohistochemistry employing a rabbit antiserum towards the major capsid protein of JSRV proved to be immunoreactive (De Las Heras *et al*, 1997; De Las Heras *et al*, 2000). These results support the assumption that some pulmonary tumours may be associated with a JSRV-related virus. No virologic or molecular data was available to support this view. Subsequently, Yousem *et al*, 2001, reported that by using both PCR and RT-PCR, that the association of BAC with JSRV was very weak, if present at all. A follow up study published by Hiatt and Highsmith support the study by Yousem *et al*, 2001. The former reported that in the 24 human lung tumours that were investigated for integrated JSRV, no amplifiable DNA was present in all the samples (Hiatt *et al*, 2002).

Nevertheless, OPA remains a unique animal model that can be used as a natural model to uncover the mechanisms of human lung adenocarcinoma.

1.9 OVERVIEW OF THE FAMILY *RETROVIRIDAE*

Retroviruses are among the first viruses that were studied and because of HIV this family of viruses currently dominates research efforts worldwide. They are also the best models for studying carcinogenesis in the whole organism, since they induce tumours with extremely high efficiency. Evidence that viruses could cause cancer first came from a series of studies on chicken sarcoma by Peyton Rous beginning in 1911. Retroviruses are a large family of enveloped RNA viruses, which share the same structure, composition and replicative properties (Coffin, 1992a). Most of the retroviruses we currently know infect vertebrates, but as a group, they have been identified in virtually all organisms including invertebrates. Some retroviruses can induce only a benign viremia with no outward adverse effects and can become established in the germ line as “endogenous viruses” from generation to generation (Coffin, 1992a).

1.9.1 Classification of Retrovirus

Retroviruses are unified into a family ‘The *Retroviridae*’ by important features of virion structure, their replication cycle and pathogenicity (Mathews, 1982). The taxonomy of the *Retroviridae* has been divided into seven genera, and then into subgenera and species and two more groups have been added (the Fish retroviruses and the Gypsy group of endogenous viruses of insects) both await final classification. The seven genera are: alpharetroviruses, betaretroviruses, gammaretroviruses, deltaretroviruses, epsilonretroviruses, lentiviruses and spumaviruses (Hunter *et al*, 2000). A listing of the retrovirus by genera, subgenera and species is presented in Figure 1.1 (Burmeister *et al*, 2001; Coffin *et al* 2000). Classification is based on sequence similarity within the pol (polymerase) gene, but other correlated features, including the presence or absence of additional genes are sometimes used. Table 1.1 provides a taxonomic organization of the Family *Retroviridae* and examples of some known retrovirus groups (Porterfield, 1989; Fields *et al*, 1996; Coffin *et al*, 1997a).

Historically retroviruses have also been classified by appearance, morphology or by cellular location of the viral particles. Virions can be further described according to the virion structure (types A to D), utilization of particular cell receptors, lifestyle whether endogenous (passed from parent to offspring as a provirus integrated into the germline) or

exogenous by the presence or absence of oncogenes or other pathogenic properties (Fields *et al*, 1991). Distinguishable types A to D contain A, B, C and D particles. (Paul *et al*, 1992).

GENUS	SUBGROUP	SPECIES
1. Alpharetroviruses		ALV (avian leucosis virus)
2. Betaretroviruses	Type B viruses	MMTV (mouse mammary tumor virus)
	Type D viruses	MPMV (Mason-Pfizer monkey virus)
		SRV (simian retrovirus)
		JSRV (Jaagsiekte retrovirus)
		ENTV (sheep enzootic nasal tumor virus)
3. Gammaretroviruses	Mammalian Type C	MuLV (murine leukaemia virus)
		GaLV (gibbon ape leukaemia virus)
		FeLV (feline leukaemia virus)
		REV (Reticuloendotheliosis virus)
4. Deltaretroviruses	Primate T-cell lympho- tropic viruses	HTLV (human T-cell lymphotropic virus)
		STLV (simian T-cell lymphotropic virus)
		BLV (bovine leukaemia virus)
5. Epsilonretroviruses		Wally dermal sarcoma virus
6. Lentiviruses	Immunodeficiency viruses	HIV (human immunodeficiency virus)
		SIV (simian immunodeficiency virus)
		FIV (feline immunodeficiency virus)
		BIV (bovine immunodeficiency virus)
	Ungulate Lentiviruses	MVV (Maedi-Visna virus)
		CAEV (caprine arthritis encephalitis virus)
		ELAV (equine infectious anemia virus)
7. Spumaretroviruses		SFV (simian foamy virus)
		FFV (feline foamy virus)
		BFV (bovine foamy virus)
		EFV (equine foamy virus)

(Burmeister et al, 2001)

Figure 1.1 A Listing of Retroviruses by genera, subgenera and species

Type A particles, about 60 to 90 nm in diameter, are only found intracellularly and are not as infectious as the extracellular forms (Lu *et al*, 1978). These particles lack the lipid containing outer envelope, as they do not bud from the membrane. Type A particles are composed of two forms: intracisternal and intracytoplasmic. The intracytoplasmic virions are intermediates in assembly of Type B and D virions, subsequently associating with the

cell membrane to initiate budding. Intracisternal A type particles are derived from endogenous retrovirus-like genetic elements non-enveloped and believed to be non-infectious (Coffin, 1992a).

B type particles have a diameter of approximately 125-130 nm. They contain a double membrane that consists of phospholipids and glycoproteins. The representative form is the mature virions of the Mouse Mammary Tumour Virus. B type particles are assembled via budding of A particles into an immature form resembling an A particle with an envelope and mature into a virion with a condensed, eccentric core and prominent spines on its surface (Coffin, 1992a). The major polypeptides have a molecular weight of 28 kilo Daltons (kDa) (p28), 14 kDa (p14) and 10 kDa (p10) respectively. Two major polypeptides are the glycoproteins with molecular weights of 52 kDa (gp52) and 36 kDa (gp36) that are probably located in the viral envelope (Tzramoto *et al*, 1974). This group includes both endogenous and exogenous viruses. Exogenous virions are transmitted vertically via milk. MMTV is an unusual simple retrovirus in that it has an extra gene that has a novel and important role in mediating host infections and tumorigenesis. This gene is referred to as *sag* (superantigen) and is located in the 3' *orf* region of the LTR that encodes the mls superantigen. It is a cell surface protein that interacts with specific $v\beta$ chains of the T-cell receptor to induce them to send an activation signal to the infected cell. When expressed from an endogenous MMTV provirus, Sag proteins lead to depletion of specific T-cell subsets (Coffin *et al*, 1997a).

Type C particles are about 80-120 nm in diameter and include two retroviral groups, the mammalian and avian C-type viruses. These particles are assembled entirely at the plasma membrane without a precursor core being formed. An electron-dense crescent on the membrane accumulates spikes on the outer surface and extrudes from the membrane until the whole particle is formed and budded off. There are two types of extracellular particles. "Immature" forms have a central electron-dense core about 45 nm in diameter surrounded by an envelope with short spikes. "Mature" forms have a central condensed, electron-dense core within an envelope with even shorter spikes (Porterfield, 1989).

D type particles are characteristic of most of the retroviruses which have been found in primates, the prototype being the Mason-Pfizer Monkey virus (MPMV) and the Simian "AIDS" (SAIDS) virus (Coffin, 1992a). A more recent inclusion is the Jaagsiekte Sheep

Retrovirus (JSRV). They resemble B particles in assembly, maturation and morphology. The viral cores assemble in the cytoplasm before migrating to and budding from the plasma membrane. D particles are produced by budding type A cytoplasmic particles. Type D particles have a complete intracellular nucleocapsid and an eccentric location of the core in mature particles, but have less prominent surface projections and a characteristic cylindrical core. Type D retroviruses comprise both endogenous and exogenous viruses of New and Old World Primates and sheep. No oncogene-containing viruses are known and insertional activation of cellular oncogenes has not been reported (Coffin *et al*, 1997a).

1.9.2 The Genome Organisation

All retrovirus genomes consist of two molecules of RNA, which are single stranded positive sense and have 5' methylated cap structure and 3' poly (A) tail (essentially resembling mRNA). Retrovirus genomes have four unique features. They are the only viruses that are truly diploid and the only RNA virus whose genome is copied via cellular transcriptional machinery (without any participation from a virus-encoded polymerase). Retroviral genomes require a specific cellular RNA (tRNA) for replication. They are the only positive sense RNA virus whose genome does not serve directly as mRNA immediately after infection. These two molecules are physically linked as a dimer by hydrogen bonds. In addition, there is a third type of nucleic acid present in all particles, a specific type of tRNA [tryptophan (trp); proline (pro); lysine (lys); or pro-glutamine (glu)] required especially by mammalian type C for replication. It is the primer on which reverse transcriptase initiates DNA synthesis (Coffin, 1992a).

1.9.3 Virion RNA Genome

The various components of the viral genome are arranged in the 5' to 3' order. Figure 1.2 is a schematic representation of a prototype viral RNA genome. The R Region is a short sequence of 18 to 250 nucleotides, which forms a direct repeat at the both ends of the genome, which is therefore “terminally redundant”. At the 5' end it is adjacent to the capping group and at the 3' end found immediately preceding the poly (A) tract. It plays a role during reverse transcription in permitting the transfer of nascent DNA from one end of the genome to the other.

Table 1.1 : RETROVIRUS GROUPS					
Subfamily	Genus	Example isolates	Genome	FAMILY: RETROVIRIDAE Virion Morphology	Type/Pathogenesis
α-retroviruses	Avian leucosis-sarcoma	Rous sarcoma virus (RSV)	Simple ¹	Central spherical core "C-particles"	Exogenous; oncogene-containing (<i>src</i>)
		Avian myeloblastosis virus (AMV)			Exogenous; oncogene-containing (<i>myb</i>)
γ-retroviruses	Mammalian C-type	Avian erythroblastosis virus (AEV)	Simple	Central spherical core "C-particles"	Exogenous; oncogene-containing (<i>erb-A</i> and <i>erb-B</i>)
		Avian leucosis virus (ALV)			Exogenous; endogenous; causes leukemia of various sorts, osteopetrosis
β-retroviruses	Mammalian B-type viruses	Rous-associated virus (RAV) 1-50	Simple	Eccentric, spherical core "B-particles"	Exogenous; cause B-lymphoma, osteopetrosis, and other diseases
		RAV-0			Endogenous; benign
δ-retroviruses	Mammalian D-type viruses	Moloney murine leukaemia virus (MoMLV)	Simple	Eccentric, cylindrical core, complete intracellular nucleocapsid	Exogenous, endogenous; causes T-cell lymphoma, immunodeficiency, neurological and many other diseases
		Harvey murine sarcoma virus (Ha-MSV)			Exogenous; oncogene-containing (<i>H-ras</i>)
Lentivirinae	Lentiviruses	Abelson murine leukemia virus (A-MuLV)	Complex ²	Central spherical core	Exogenous; oncogene-containing (<i>abl</i>)
		Feline leukemia virus (FeLV)			Exogenous, endogenous; causes T-cell lymphoma, immunodeficiency, and many other diseases; oncogene-containing (<i>myc</i>)
Spumavirinae	"Foamy" viruses	Simian sarcoma virus	Complex	Central, spherical core	Exogenous; oncogene-containing (<i>sis</i>) (SSV)
		Numerous endogenous and exogenous viruses, mostly in mammals			
HTLV-BLV group	HTLV-BLV group	Reticuloendotheliosis virus (REV); spleen necrosis virus (SNV)	Complex	Central spherical core	Exogenous viruses of birds; causes malignancies, immunodeficiency and neurological diseases
		Mouse mammary tumour virus (MMTV)			Endogenous and exogenous; transmission vertically via milk; causes mostly mammary carcinoma, some T-lymphoma; no oncogene-containing members
Lentivirinae	Lentiviruses	Mason-Pfizer monkey virus (MPMV)	Simple	"D-particles"	Exogenous and endogenous; immunodeficiencies in monkeys
		"SAIDS" viruses			Exogenous and endogenous; immunodeficiencies in monkeys
Spumavirinae	"Foamy" viruses	Squirrel monkey retrovirus (SMRV)	Complex	Eccentric, cylindrical core, complete intracellular nucleocapsid	Endogenous; benign
		Jaagsiekte Sheep retrovirus (JSRV)			Exogenous and endogenous; causes carcinoma
Lentivirinae	Lentiviruses	Human T-cell leukaemia (or lymphotropic) virus (HTLV)	Complex	Cone-shaped core	Exogenous; causes T-cell lymphoma; associated with neurological disorders
		Bovine leukaemia virus (BLV)			Causes B-cell lymphoma
Spumavirinae	"Foamy" viruses	Human immunodeficiency virus (HIV-1/2)	Complex	Central, spherical core	Exogenous; cause of AIDS
		Simian immunodeficiency virus (SIV)			Exogenous; causes AIDS-like disease in certain monkeys
Lentivirinae	Lentiviruses	Feline immunodeficiency virus (FIV)	Complex	Central, spherical core	Exogenous; immunodeficiency
		Visna/maedi virus (MVV)			Exogenous; causes neurological and lung disease in sheep
Spumavirinae	"Foamy" viruses	Equine infectious anaemia virus (EIAV)	Complex	Central, spherical core	Exogenous; causes anaemia
		Caprine arthritis-encephalitis virus (CAEV)			Exogenous; arthritis; neurological disorder and pneumonia
Spumavirinae	"Foamy" viruses	Bovine syncytial virus (BSV)	Complex	Central, spherical core	Exogenous, apparently benign
		Simian foamy virus (SFV)			
Spumavirinae	"Foamy" viruses	Feline syncytium foaming virus (FSFV)	Complex	Central, spherical core	Exogenous, apparently benign
		Human foamy virus (HFV)			

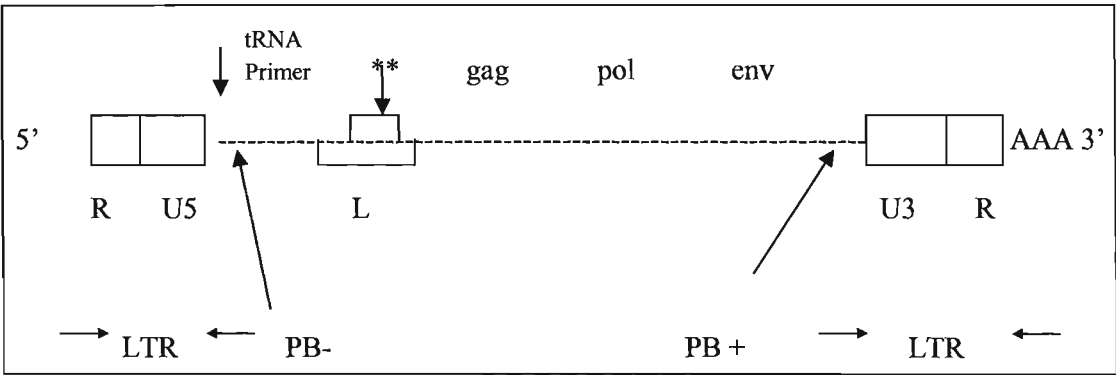
¹ The retroviral genome is made up of 4 major coding regions: *gag*, *pro*, *pol* and *env*.

² In addition to major coding regions have accessory regulatory coding genes e.g HTLV/BLV have *tax* and *rex*

(Porterfield, 1989; Coffin et al, 1997a and Fields et al, 1996)

The U 5 sequence is a unique, non-coding region of 75 to 250 nucleotides that is the first part of the genome to be reverse transcribed, forming the 3' end of the provirus genome or LTR (Long Terminal Repeats). The R and the primer-binding site flank U5 region. The primer-binding site is 18 nucleotides in length, is found perfectly complimentary to the 3'-terminal nucleotides of the tRNA, and is used by the virus to begin reverse transcription.

The Leader (L) sequence is a relatively long (90 to 500 nucleotides) non-translated region downstream of the transcription start site and therefore present at the 5' ends of all virus m RNA's. The leader region is found between the Primer binding site and the beginning of gag.



(Coffin *et al*, 2000)

Figure 1.2 Schematic representation of a prototype Viral RNA Genome

The above diagram shows a number of elements that participate in reverse transcription, viral gene expression and virion assembly. The 5' end of the viral genome has a cap structure 7Mg (7-methylguanosine). The 3' end has a poly –A tail. R represents a short repeat at either end of the genome. U5 is a unique sequence found after the 5'R region. PB- is the primer site for the minus strand DNA synthesis (tRNA binding site). L-leader region. PB+ is the primer site for positive strand DNA synthesis and U3 is the unique sequence at the 3' end. Genes encoding virion polypeptides: gag, pol and env are also shown.

This sequence has two functional roles. It contains the donor site for the generation of all spliced sub-genomic mRNA's encoded by the virus. The second function encoded in the L region is to specify incorporation of genome RNA into virions. The Polypurine Tract is a short run of approximately 10 adenine and guanine residues responsible for initiating positive strand synthesis during reverse transcription and is located downstream from the env.

A unique non-coding region the U3 of 200 to 1200 nucleotides forms the 5' end of the provirus. It extends from PB to R at the 3' end of the viral genome. At the end of the

genome, between U3 and the poly (A) sequence, is the other copy of the R region, which sometimes contains the poly (A) additional signal. During the retrovirus replication cycle, the U5, U3 and R elements form the large, directly repeated structure found at either end of the retroviral genome, the long terminal repeats (LTR's). They are important for viral replication and essential for stable integration into the host cell genome (Coffin *et al*, 2000).

1.9.4 Virion Structure

The virion contains several enzymes necessary for the early events of replication that include reverse transcriptase (RT), ribonuclease H, integrase (IN) as well as protease (PR) used in processing the virion proteins. All replication competent retroviruses to date contain four coding domains *gag*, *pro*, *pol* and *env*, which occupy the body of the DNA. *Gag* encodes the internal structural protein of the virus (gag protein from the original name “group specific antigen”) reflecting the so-called antigenic properties of this protein. Gag is proteolytically processed into the mature proteins of the matrix (MA), capsid (CA), and nucleocapsid (NC).

Pol encodes the enzyme reverse transcriptase (RT), which contains both DNA polymerase and associated Rnase H activities, and integrase, which mediates replication of the genome. Pro encodes the viral protease which acts late in assembly of the viral particles to process proteolytically the proteins encoded by *gag*, *pro* and *pol*. *Env* encodes the surface (SU) glycoprotein and transmembrane (TM) protein of the virion. (Coffin *et al*, 2000).

Replication-competent retroviruses have all the functions for completing the replication cycle and yield infectious progeny virions. Replication-deficient retroviruses have one or more mutations in essential viral functions and depend on “helper” virus to provide the missing functions. ASLV and MuLV provide replication-functions for avian erythroblastosis virus (AEV) and murine sarcoma virus (MSV) respectively (Paul *et al*, 1992). Figure 1.3 shows a schematic representation of a mature (prototype) virion.

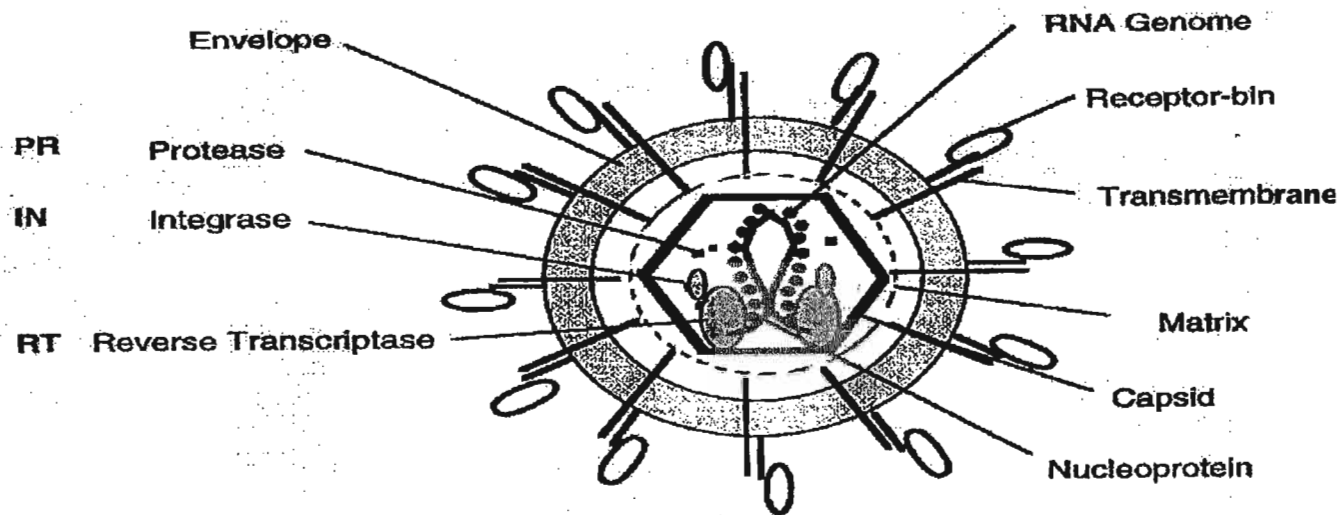


FIGURE 1.3 Schematic Representation of a Mature (prototype) Virion (Coffin, 1992a)

1 9.5 Overview of retroviral replication

The retroviral replication cycle is divided into early and late phases. The early phase starts with the binding of a virus particle (virion) to a cell receptor and continues to the formation of an integrated provirus. The SU envelope glycoprotein binds to a specific receptor on the surface of the host target cell. TM causes fusion of viral and cellular membranes and the virion core (capsid) is released in the cell cytoplasm. When the capsid enters the cytoplasm the core is unmasked [inside the core is RT (reverse transcriptase), IN (integrase), +RNA and tRNA] and free nucleotides (from host cell) in the cytoplasm enter the core and reverse transcription of genomic viral RNA into double stranded linear DNA takes place. All reverse transcription occurs in the cytoplasm. Long terminal repeats (LTR's) found on either side of the viral genome, are produced during reverse transcription and remain in a nucleoprotein complex. Viral DNA within the nucleoprotein complex migrates to the nucleus, where termini of viral DNA are covalently linked to host- cell DNA in a reaction to produce the provirus. This step is mediated by viral integrase.

The late phase begins with synthesis of viral transcripts from the provirus in the nucleus and continues through to release of progeny virions. LTR's in the provirus provide signals for cellular function that control synthesis and processing of viral RNA. All viral transcripts serve as genomic RNA and as mRNA for viral polyproteins. Spliced as well as

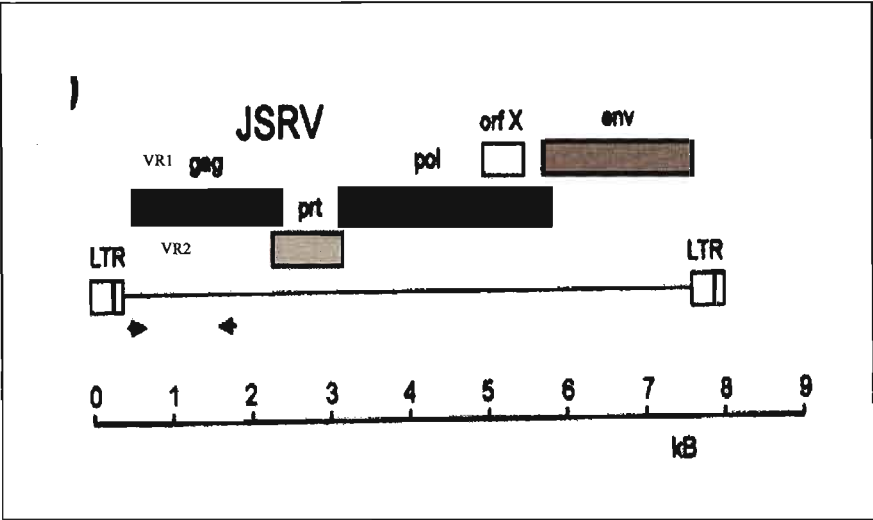
full-length viral RNA that are transported to the cytoplasm are translated on host-cell polysomes into virion polyproteins (precursor polypeptides). Full-length transcripts also interact with virion polyproteins and are assembled into immature virus particles. Through the budding process, immature cores are engulfed by the cell plasma membrane and thus acquire a lipid bilayer membrane that contains env glycoproteins. Proteolytic processing of gag, protease (prt) and pol polyproteins takes place during the budding step and continues in newly released particles. Finally, there is assembly into mature fully infectious virus particle (virion). The replication process proceeds without any significant effect to the infected cell. The cell continues dividing and is unaltered in any way except that it is now continually producing new virions (Paul *et al*, 1992; Coffin *et al*, 1997b).

1.9.6 Genomic Organisation of JSRV

The complete genomic sequence of the Jaagsiekte Sheep Retrovirus is derived from viral particles secreted in lung exudates of OPA affected animals. JSRV is a replication competent simple betaretrovirus with a genome 7,462 nucleotides long (Hunter *et al*, 2000; Palmarini *et al*, 2002). It exhibits a genomic organization which is a unique chimeric structure consisting of a type B envelope gene such as MMTV or HERV-K, type D gag and pol genes with *pro* in a different open reading frame (*orf*) to pol (York *et al*, 1991; York *et al*, 1992; Palmarini *et al*, 1999; Palmarini *et al*, 2001), and an additional open reading frame *orf-X* that overlaps pol and is not present in any other retrovirus that overlaps pol (York *et al*, 1991; York *et al*, 1992; Bai *et al*, 1996; Rosati *et al*, 2000) (see Figure 1.4). This *orf-X* region has a different codon usage to other genes within JSRV and a very hydrophobic predicted amino acid sequence that shows no homologies with any other known protein. The role of *orf-X* is unknown. Betaretroviruses have prominent surface spikes and an eccentric condensed core or less dense surface spikes and a cylindrical core. Capsid assembly for this group occurs in the cytoplasm in structures previously described as A-type particles (Palmarini and Fan, 2003).

A capsid type D retroviral sequence was isolated from an affected Peruvian sheep by a different approach (Hecht *et al*, 1994). Comparison of the MMTV and MPMV capsid and nucleocapsid amino acid sequence revealed a number of conserved regions that could be responsible for the observed shared epitopes with the OPA agent. These were used to amplify by PCR; viral sequences from cDNA reverse transcribed from putative viral RNA

in lung fluids of an OPA affected sheep from Peru. The capsid sequence obtained this way from a Peruvian case was identical with JSRV (which originated in the Republic of South Africa). Furthermore, on the amino terminal end, twenty amino acids of p25 capsid protein purified from OPA lung fluid in Scotland were identical to JSRV (Hecht *et al*, 1996). The identification of the infectious form of JSRV has been complicated by the presence of 15-20 copies of endogenous sequences fixed in the genomes of all sheep and goats when tumour and non-tumour DNA were probed with JSRV capsid or envelope regions (Hecht *et al*, 1994)



(Burmeister et al, 2001; Palmarini et al, 2004)

Figure 1.4 **Genomic structure of JSRV**

The numbered bar at the bottom indicates distances in kb. JSRV shows the canonical retroviral *gag*, *prt*, *pol* and *env* with *prt* in a different open reading frame from *pol*, the same for all betaretroviruses. An additional open reading frame (*orf-X*) overlaps *pol*.

Phylogenetic analysis of JSRV suggests that it evolved from the A-B-D lineage, diverging before MPMV but after MMTV and human endogenous retrovirus K (HERV-K). The transmembrane (TM) domain does not align with other type D virus but has a structural homology with MMTV and HERV-K 57(Palmarini *et al*, 1997).

1.10 DIAGNOSIS

1.10.1 Virus Host Interaction-Serological based diagnosis

The long pre-clinical incubation period of OPA and the short life span of sheep under modern management systems allows the disease to disseminate widely in a flock before it is recognised. Further the absence of an in vitro culture system makes diagnosis of this disease solely dependent on the end stage clinical signs and characteristic lung pathology. At the time this study commenced there was no specific pre-clinical detection system. Consequently the sub-clinical infection rate within the individual flocks and countries remained unknown and the options to enforce some sort of control were limited. Knowledge about the immune response in sheep is limited and circulating antibodies to the virus have not been found (Sharp *et al*, 1983; De Martini *et al*, 1988; Verwoerd, 1990; De Las Heras *et al*, 1995). A 25-28kda polypeptide (OPA p27) obtained from OPA lung fluid and tumour homogenates was shown to cross-react on Western immunoblots with antisera to the major capsid protein of Mason Pfizer monkey virus (MPMV) and mouse mammary tumour virus (MMTV), the prototype type D and type B retroviruses, respectively, as well as other type D retroviruses (Sharp *et al*, 1983; Perk *et al*, 1985; De Martini *et al*, 1987; He *et al*, 1992). The MPMV capsid-related antigen p27 is internationally accepted as a marker for JSRV and OPA. An immunoblotting and an enzyme linked immunoassay technique (ELISA) based on the recombinant capsid protein MPMV p27 of Mason Pfizer monkey virus (as a glutathione S-transferase (GST) fusion protein) was used as an antigen to detect antibodies against the homologous viral protein both in Italian sheep flocks with a prevalence of OPA and in north American sheep flocks with a high prevalence of chronic pneumonia (Kwang *et al*, 1995). Positive reactions were detected in sera from animals suffering from OPA.

Based on the results of previous experiments (Sharp *et al*, 1983; Kajikawa *et al*, 1990; He *et al*, 1992; Kwang *et al*, 1995) the usefulness of a recombinant JSRV-CA protein was evaluated as a potential diagnostic antigen in an immunoblotting assay. The study was designed to assess the antibody response in animals naturally affected with OPA and in animals free of the disease. The results of our study concurred with that of Kwang and associates (Kwang *et al*, 1995) where positive reactions were detected in sheep suffering from OPA. This study however also found positive reactions with sera from animals with

no detectable tumours at necropsy that originated from flocks with no clinical history of OPA. This lack of correlation between disease status and reactivity with the recombinant fusion proteins raised doubts concerning the specificity of the reaction and supported a view that this reaction was not specific to JSRV. Confirmation of this view was realised when the sera that was absorbed with lysates of clone pGEX–NM522 that contained GST but no proteins related to JSRV resulted in the complete abolition of all positive reactions. Sera that were absorbed with recombinant JSRV-CA, had reactivity abolished in only a few animals. This proved the non-specificity of the reaction (Ortin *et al*, 1998).

In further analysis of the JSRV viral proteins, a JSRV specific capsid antibody (CA) was generated. Palmarini and co-workers in 1995 developed a blocking enzyme linked immunosorbent assay (β -ELISA) and an immunohistochemical assay for the detection of JSRV major capsid proteins at the tissue and cellular level. Briefly, β -gal CA and GST-CA fusion proteins expressed in *E coli* (*Escherichia coli*) were used to immunize rabbits to generate a JSRV specific capsid (CA) antibody. β -gal-CA and the specific rabbit antiserum were used in the assay to detect JSRV-CA. Rabbit antiserum to JSRV-CA was used for the immunohistochemical technique. JSRV was detected only in the respiratory tract of sheep affected by OPA and specifically in the transformed epithelial cells of the alveoli of OPA-affected sheep (Palmarini *et al*, 1995).

1.10.2 Molecular Diagnosis

The ability to distinguish between exogenous JSRV from the endogenous background has allowed the introduction of more sensitive molecular techniques for the detection of the virus. The use of the Polymerase Chain Reaction (PCR) has extended previous observations based on immunological detection methods and such advanced techniques that can be used to detect JSRV RNA or DNA in OPA affected sheep or carrier sheep.

A Self-Sustained Sequence Replication (3SR) assay has been used to detect exogenous JSRV based on its single stranded RNA genome (York *et al*, 1993; De Martini *et al*, 1997). It is an isothermal reaction that uses three enzymes and a virus specific primer to synthesise many copies of cDNA of the single stranded target, but not double stranded endogenous viral DNA (Fahy *et al*, 1991). The reaction is highly efficient and within one hour can amplify the target more than a million-fold. The target is then detected in a slot-

blot using an internal probe. This technique has been used to detect the exogenous JSRV in lung lavage fluid and nasal exudates of OPA affected sheep, but not in samples from unaffected sheep. The specificity of this assay is limited by the presence of 15 to 20 copies of endogenous sheep retrovirus loci (ESRV) in normal sheep that may hybridise to JSRV DNA probes (York *et al*, 1993). As the gene targeted in the above assay was to be the JSRV p26 capsid protein this assay would also detect endogenous RNA transcripts if and when they were expressed, as the p26 gene is present in some of the endogenous JSRV loci. Evidence is available that suggests that the transcripts of the endogenous viruses are expressed.

The presence of these endogenous sequences has hampered studies at the molecular level. So an investigation was carried out to determine if the exogenous form of JSRV was associated with neoplasia in OPA affected animals. Initially, ESRV loci were found transcribed in a wide variety of normal sheep tissues. Molecular studies have demonstrated that JSRV is an exogenous virus distinct from the transcriptionally active endogenous retroviral sequences present in the ovine genome (York *et al*, 1992; Bai *et al*, 1996; Palmarini *et al*, 1996). To clarify the aetiological roles of ESRV (endogenous virus) and an exogenous JSRV-like retrovirus (ex JSRV) in OPA, ESRV proviral sequences were characterised and compared to ex JSRV. Molecular characterization of six ESRV loci revealed restriction sites specific for JSRV. Nucleotide sequences of ESRV's from sheep of different breeds were similar to those of JSRV in structural genes, but divergent in the U3 region. The most remarkable difference between the two types of sequences was that the endogenous U3 region was 47 base pairs longer than that of JSRV (York *et al*, 1992). Molecular markers that could be used to distinguish JSRV from ESRV were identified as *Kpn*1, *Nde*1, *Hind*111, *Sca*1, *Bam*HI and *Xho*1 sites in gag, a *Cla*1 site in the gag-pol junction and an *Eco*R1 site in the envelope transmembrane region. The ex-JSRV of the South African, Kenyan, Wyoming and Scotland had two common *Sca*1 sites whilst ESRV only had the first *Sca*1 site (Bai *et al*, 1996). These restriction sites were identified using endogenous JSRV proviral sequences. With the isolation of three endogenous JSRV retroviruses JS5.6A1, JS5F16 and JS5.9A1 and comparison to the South African strain, these restriction sites are no longer used to differentiate the exogenous JSRV from that of the endogenous virus. The only restriction site that is still valid is the *Sca*1 restriction site.

Sequencing part of the *gag* gene of endogenous (ESRV) and JSRV revealed a *ScaI* restriction site in the *gag* gene of JSRV (nucleotide position 1729) that was absent in ESRV (York *et al*, 1992; Palmarini *et al*, 1996). The *gag* gene of the ESRV is a conserved 612 amino-acid polypeptide corresponding to the boundaries of the capsid proteins (CA) of MPMV, Squirrel Monkey Retrovirus (SMRV) and MMTV. RT PCR of this region amplified a 229 base pair product in both affected and unaffected tissues. *ScaI* restriction of the products revealed that the exogenous form JSRV was exclusively and consistently present in tumour tissues and lung secretions of the affected animals. JSRV was not detected outside the respiratory tissues of OPA affected sheep nor in any tissue of the control sheep. Amplification of both endogenous and exogenous sequences can mask the detection of low copies of exogenous JSRV due to the relatively high background formed by ESRV. To address this question an exogenous virus specific hemi-nested PCR was developed utilizing primers in the U3 region of the LTR of JSRV where, as mentioned previously, major differences between endogenous and exogenous sequences exist. This technique is more sensitive than the previous *gag* PCR and *ScaI* digestion method. This assay has been used to analyse the tissue distribution of JSRV in sheep with natural and experimentally induced OPA (Palmarini *et al*, 1996).

1.11 AIMS OF THE STUDY

OPA is widespread in various parts of the world, except Australia and New Zealand. In the countries where it is known to occur, farmers have incurred considerable sheep losses. Since the mode of transmission appears to be aerogenous and the spread of disease facilitated by close contact, it is a particular hazard of intensified sheep husbandry (Mackay, 1966). Currently, there is no vaccine to protect sheep against Jaagsiekte.

Eradication of the disease requires the development of fast, sensitive and efficient serological and/or molecular assays which can confirm the early presence of the disease in living animals. The early diagnosis could ensure that all infected animals are removed immediately to eradicate the disease from the infected flock. The goal is a reliable assay that can diagnose OPA early. The Jaagsiekte Sheep Retrovirus does not produce detectable circulating antibodies; hence the inability to detect a JSRV-specific humoral response (Summers *et al*, 2002). Furthermore, the presence of 15 to 20 copies of sheep genome of the JSRV-related endogenous sequences compared with possibly only 1 exogenous copy

per neoplastic cell has caused great difficulty in detecting integrated exogenous JSRV (Sharp, 1997). Moreover, neoplastic cells are approximately only 40% to 50% of the total number of cells present in the neoplastic tissue, therefore increasing the ratio of endogenous to exogenous sequences (Palmarini *et al*, 1996). Thus the need to develop and test new, effective and sensitive assays for the identification of JSRV in sheep would be of enormous benefit to commercial breeding flock owners and emergent farmers in the underdeveloped areas in South Africa and elsewhere in the world. Farmers would be able to selectively identify and eliminate positive sheep from the affected flocks. This would reduce the prevalence of the disease and perhaps permit eradication of OPA from the flock. Further before new sheep are introduced into a flock, pretesting of all sheep could ensure that the flock remains free of the disease (De Martini *et al*, 1997).

In the light of what has been discussed above, the purpose of this study was to investigate the potential to develop both a serological and molecular diagnostic assay to help in the early diagnosis and removal of carriers of the OPA disease. To achieve these objectives the following approach was undertaken.

Specific primers based on the JSRV published sequence will be designed to target the conserved region of the *gag* (group-specific antigen) polyprotein and the transmembrane region of the *env* encoded proteins. The amplified products will be cloned and expressed. The expressed protein will then be investigated for their diagnostic potential in various assay formats.

The second objective will focus on a molecular diagnostic approach. Various molecular assays will be optimised and assessed for their ability to detect the exogenous infectious virus and distinguish it from the endogenous loci that are present in all sheep genomes.

CHAPTER 2

CHAPTER TWO

A SEROLOGICAL APPROACH TO THE DIAGNOSIS OF JSRV: ISOLATION, CLONING AND EXPRESSION OF THE JSRV GENES (JSP26 CAPSID GENE, JSTM AND JSORF-X)

2.1 INTRODUCTION

A major obstacle facing JSRV research was the inability to cultivate the etiological agent of JSRV *in vitro*. This made diagnosis of the disease entirely dependent on clinical signs that only appeared at the terminal stages of the disease. Sheep died very shortly after they showed symptoms of the disease. Confirmation of the disease was dependant upon micro- and macroscopic pathology, which required that the sheep be slaughtered. Without any specific type of testing procedure, the subclinical infection rate within individual flocks and flocks in different countries remained unknown and the means of managing the infection were limited and non-scientific (Kwang *et al*, 1995).

2.1.1 Production of Antibodies against semi-purified JS

The absence of an *in vitro* culture system to produce the virus meant that initially the only source of virus was that obtained from lambs that were experimentally infected with lung and nasal extracts from JSRV affected sheep lung material. Virus could be recovered from experimental and or natural cases of JS; however the virus was in low concentrations and was either associated with or attached to non-viral lung debris, which were difficult to remove from the virus. One of the common methods used to purify the virus involved rate zonal and isopycnic centrifugation of JSRV lung rinse material through sucrose gradients (Perk, 1982; York, 1984b). This procedure provided sufficient purification of the virus to identify the viral proteins. However, even the best purification procedures resulted in the virus co-purifying with significant amounts of immunoglobulins (Ig) and other non-viral material. Eight to ten polypeptides were constantly observed when JSRV lung rinse material was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and reverse transcriptase assay. Those proteins consistently present on SDS-

PAGE that were associated with high reverse transcriptase activity were considered as likely JSRV polypeptides.

The Western immunoblot technique, although useful in identifying specific antigens, is limited by the specificity and purity of the serum used. As there was no pure and specific serum available against all JSRV polypeptides at that time, a rabbit serum was made against semi-purified JSRV proteins and after extensive adsorption against normal sheep lung proteins and *Mycoplasma* species, only three possible JSRV antigens were identified as p26, p32 and p38 polypeptides. The higher molecular weight polypeptides were difficult to identify because of the non-specific staining in sheep lung extracts. This was probably due to the serum components being against lipopolysaccharides and proteins of surfactant origin as well as serum proteins. Further polypeptides p35, p50, p61, p75, p77, p81 and p84 were also identified because of their constant appearance in stained polypeptide patterns of OPA lung rinse material, following purification using different methods ((Verwoerd *et al*, 1983; York, 1984c)

2.1.2 Expression of Viral Proteins and their Use in ELISA Assays

Possibly one of the most useful breakthroughs in JSRV research was the observation by Sharp and Herring; 1983, who showed that JS lung fluid and tumour homogenates contained a 25 to 28 kDa) polypeptide that cross-reacted on Western Blots when they used antisera to the major capsid protein (p27) of Mason Pfizer monkey virus (MPMV), a type D oncovirus. A similar polypeptide with a molecular weight of 25 to 26 kDa was detected at the Onderstepoort Veterinary Institute in Pretoria using the rabbit antiserum to JSRV (Sharp and Herring, 1983; York, 1984c). The MPMV capsid related antigen p27 became an internationally accepted OPA marker protein as it was present in the lung and nasal fluid of all JS affected sheep. The capsid protein (CA) JSp26 is antigenically similar to the major capsid protein p27 of MPMV. In the case of MPMV, p27 is the most abundant protein (0.33% of total weight of virus particle) in the virus particle (Turner and Summers, 1999). The group specific antigen (*gag*) precursor poly protein has a calculated molecular weight of 68 kDa (York *et al*, 1992).

In the early 90's the JSRV genome was isolated, cloned and sequenced (York *et al*, 1992). It thereafter became a major focus to develop a diagnostic assay to detect the virus.

Unfortunately, very similar endogenous counterparts to the exogenous infectious JSRV were present in all sheep genomes. This made the classical molecular approach more difficult. At the same time that the molecular approach to develop a diagnostic assay was taking place, so was the cloning and expression of various JSRV genes and the production of antibodies to the virus and its proteins (York *et al*, 1992).

This chapter focuses on the approaches that were taken to isolate, clone, express and purify some key JSRV proteins so as to investigate their potential as diagnostic antigens. The approach is divided into two parts:

1. The cloning, expression and purification of JSRVp26 from both endogenous and exogenous forms of JSRV and to investigate its use as a diagnostic antigen
2. To develop antibodies to JSp26 and to investigate their potential in an antigen assay.

2.2 MATERIALS AND METHODS

2.2.1 Sources of DNA

Dr. York provided the following JSRV cDNA clones, namely, JS 382 and JS 107. Other sources of DNA included JS 4844 (South African sheep with JSRV); NLDNA (DNA from normal sheep) and JS DNA7 (a cell line from Dr Mike Sharp, Scotland). Genomic DNA was isolated from three different Australian sheep designated MSCU, LPW and 230909. Additional normal sheep lung was obtained from the local abattoir.

2.2.1.1 DNA Extraction from Tissue

A number of protocols have been used to isolate nucleic acids. For the purposes of extracting DNA for PCR, genomic DNA was extracted from tissue using two methods, the BIO 101 Fast DNA Kit (BIO 101, Inc., La Jolla, CA, USA: Southern Cross Biotechnology) with the Fast PrepTM Instrument as well as the Salting Out Procedure by Millar *et al*, 1988.

2.2.1.1.1 Fast Prep™ DNA System

The principle behind the FastPrep DNA procedure is the ability to lyse cells with minimal shearing of the nucleic acid by shaking the tube up and down (and with a slight twisting motion) at very high speeds. The lysing matrix contains a chaotropic DNA stabilising solution that is a mixture of detergents and salts. These detergents contribute to nuclease inactivity and provide lubrication during the lysing step to prevent DNA from being sheared. The rise in temperature during the lysing step contributes towards the inactivation of the nucleases and does not harm the DNA.

The Fast Prep system disrupts whole tissue, lyses cells and stabilises nucleic acid from tissues by the use of highly energetic mechanical means and careful choice of reagents, thus eliminating the need for lysing enzymes or grinding and homogenising equipment).

The method outlined below was used following the manufacturer's instructions (BIO 101 Fast DNA Kit protocol). Briefly, 200 milligram (mg) of tissue was sandwiched between a small and large bead in a homogenisation tube. This formed the lysing matrix. One millilitre of cell lysis /DNA solubilising solution was added to the tube. The samples were placed in the Fast Prep 120 instrument and processed for 5-15 seconds (sec) at a speed of five. The tubes were removed and spun in a Sorvall MC 12V (Du Pont) centrifuge at 13,000 revolutions per minute (rpm) for 5 minutes (min) to pellet protein and any cell debris. The supernatant (600µl) was transferred to a clean 1.5ml Eppendorf tube. An equal volume of DNA Binding solution was added and incubated at room temperature for 5 min. The samples were spun for 1 min, the supernatant removed and discarded. The pellet was gently resuspended in 500 microlitre (µl) of SEWS-M solution, which is a salt/ethanol combination to wash the pellet. The pellet was washed for 1 min and the supernatant discarded. DNA elution buffer (100µl) was added to the pellet and allowed to incubate for 2-3 min at room temperature. After centrifugation for 1 min, the resuspended DNA was transferred to a clean tube using a large bore tip to avoid mechanical shearing of the DNA (BIO 101 Fast DNA Kit protocol).

2.2.1.1.2 Extraction of DNA from Tissue or Cells using a Salting out Procedure

A high salt concentration was used to dehydrate and precipitate the cellular proteins, while the DNA remains soluble. The yield of DNA obtained from this technique is comparable to that obtained using the phenol-chloroform method.

Tissue from sheep lung was weighed out (100mg) and sliced finely using a scalpel blade. This was added to a 50ml centrifuge tube containing 5ml of nuclei lysis buffer A (Appendix D1). About 600 micrograms (μg) of Proteinase K (Roche Diagnostics GmbH, Mannheim Germany) in lysis buffer and 4ml of 10% (w/v) SDS (Appendix D2) (800 μl /ml buffer A) was added to the tissue and incubated overnight at 37°C. RNase (20 μl of 10mg/ μl stock RNase (Roche Diagnostics, Germany) was added to the tissue. Once the tissue was digested, 1.5ml of a 6M sodium chloride (NaCl) solution (final concentration 1M NaCl) was then added. The tube was gently inverted for 10 min at room temperature. NaCl was added to the solution until it had turned milky and become very viscous. The solution was centrifuged at 2500 rpm for 15 minutes at room temperature using a Sorvall MC 12V (Du Pont) centrifuge to remove the proteins. The clear supernatant was removed to a 50ml centrifuge tube. To this an equal volume of chloroform/isoamylalcohol (24:1/Appendix D3) was added and mixed as before, but for 5 min. The tubes were centrifuged at 2500 rpm for 10 min at 8°C. The supernatant was transferred to a clean tube, and an equal quantity of chloroform was added. The solution was mixed as before by gentle inversion and then centrifuged at 3500g (gravity) for 10 min at 8°C. The supernatant was transferred to a clean McCartney bottle. The addition of equal quantities of isopropanol resulted in the precipitation of strands of DNA upon gentle inversion. These strands were hooked out with a sealed hooked Pasteur pipette and dipped into 70% ice-cold ethanol (Appendix D4) to wash the DNA. The DNA was resuspended in 400 μl Tris-EDTA buffer pH 8 (Appendix D5) (Millar *et al*, 1988).

2.2.1.2 Measurement of DNA Concentration

A popular method to determine the DNA concentration and its purity is the use of ultraviolet absorbance spectrophotometry. The amount of ultraviolet radiation absorbed by a solution of DNA or protein is directly proportional to the amount present in the sample. Nucleic acid solutions are measured at 260 nanometre (nm) and proteins at 280 nm. Pure

DNA in solution should have an A_{260}/A_{280} ratio of purity ranging from 1.8 to 2.0. A ratio less than 1.8 indicates the presence of contaminants such as proteins or phenols and these samples should be purified further. The concentrated DNA solution was diluted one in two hundred using water as the diluent and to zero the spectrophotometer. Absorbance measurements were determined at both 260 nm and 280 nm using a Beckman Du^R-5 spectrophotometer (Beckman Instruments Inc. Fullerton, California). DNA with an absorbance value at A_{260} of 1 has a concentration of 50µg/ml. This together with the dilution factor was used to determine the exact concentration of DNA in solution (Brown, 1986a; Sambrook *et al*, 1989a).

2.2.1.3 Designing of Primers to amplify and clone the genes into pGEX Expression Plasmids

A standard method that is used to isolate and clone genes is the Polymerase Chain Reaction (PCR) technique. This is an extremely useful technique since very small amounts of target material is required. Primers were synthesised by the Department of Biochemistry of the University of Cape Town using the Milligen Oligonucleotide Synthesiser. The primers were to amplify the JSRVp26 capsid gene, JSRVTM (transmembrane) and the JSRV*orf-X* (open reading frame unknown (X)). The DNA starting materials were from plasmids JS 382 and JS 107. Genomic DNA included JS 4844, JS sheep lung and JS 7 DNA (cell line from Scotland where each cell has one integrated exogenous JSRV genome).

The fragments amplified included the capsid region of the *gag* gene (653 bp), the transmembrane region of the *env* gene (710 bp) and the *orf-X* gene (497 bp). The capsid gene has a molecular weight of between 25-27 kDa.

The primers were designed so that the amplicons would have specific restriction sites at their 5' ends to facilitate cloning of the amplicons in frame with the expression vector. Table 2.1 outlines the nucleotide sequence and genomic locations of the PCR primer pairs used to amplify JSp26, TM (Transmembrane) and *orf-X* (open reading frame) genes. The PCR primers used were designed to flank the codons of the genes to be expressed. The *orf-X* primers were designed solely for the study (York *et al*, 1992).

Table 2.1 **Primer Sequences and Genomic Locations of PCR Primers for the amplification of JSp26, TM and *orf-X* Genes**

PRIMER	SEQUENCE 5'-3'	GENE	*LOCATION
JSp26 Bam HI forward	ttaaggatcctgtcttcgaaaataacaa	<i>Gag</i>	1035-1053
JSRTp26 Bam H1 rev.	ggatcctgcatagcaatgcctgca	<i>Gag</i>	1699-1679
JSp26 Bam H1	tataggatccccgtcttcgaaaataacaac	<i>Gag</i>	1034-1054
JSRTp26 EcoR1	gaattctgcatagcaatgcctgca	<i>Gag</i>	1699-1679
JSTM Bam H1	Tcccggatccggcttgagcttaattat	<i>Env</i>	6483-6500
JSRT env Bam H1	ttaaggatcccggtctgcccccgca	<i>Env</i>	7194-7176
JSorf X Bam H1	aattggatccatgcagcccgaaaatcccat	<i>orf-X</i>	4606-4625
JSorf X Sal 1	actagtcgacttaataattgatgtctaaggcgttg	<i>orf-X</i>	5106-5082

gaattc *Eco*R1 restriction site; ggatcc *Bam*HI restriction site; gtcgac *Sal*I restriction site

*Nucleotide locations refer to JSRV sequence published by York *et al*, 1992

2.2.1.4 The Standard PCR Reaction

2.2.1.4.1 Amplification of JSp26 *Gag* Gene

The capsid region of the *gag* gene was amplified from plasmid JS 382 using primers JSp26 (nucleotide position 1034-1054) and antisense primer JSRTp26 (nucleotide position 1699-1679). Both primers had restriction sites designed into their 5' ends. JS 382 was a cDNA clone of a segment of a South African exogenous JSRV (York *et al*, 1992).

The PCR master mix solution was made up of 10µl of 10x PCR buffer containing 15mM Magnesium chloride (MgCl₂) [Roche Diagnostics], 6µl MgCl₂ (25mM-Sigma stock), 5µl each of JSp26 (20 ρmoles/ul) and JSRTp26, 8µl of 2.5mM (200µM) deoxynucleoside triphosphates (dNTPs) (Appendix D6) and 1µl (1u/µl) of Taq DNA polymerase (Roche Diagnostics). To avoid contamination, the PCR mix was added to the required number of 0.2ml PCR tubes in a sterile hood. Template DNA was not added to the solution at this stage. A known amount of DNA (see Table 2.2) was added to each tube.

2.2.1.4.2 Amplification of JS *orf-X* gene

The main emphasis of this thesis is the cloning and expression of the JSp26 gene. However, we also attempted to clone and express additional genes such as open reading frame x (*orf-X*) and a section of the transmembrane (TM). A lot of work was performed in cloning these

genes; unfortunately we had great difficulty expressing them due to the hydrophobic nature of the expressed proteins. For completion they have been included in this thesis.

The *orf-X* gene is a novel open reading frame that was identified while analysing the genomic sequence. It is found at nucleotide positions 4465 to 5103. It theoretically codes for a protein that does not share homology with any known oncogene or viral protein. *Orf-X* is very hydrophobic (York *et al*, 1992). The *orf-X* gene was amplified from 500 nanograms (ng) of both exogenous and endogenous sources of DNA. The source of exogenous DNA was JS 7 cell DNA and JS 4844 (JS affected sheep lung). The source for the endogenous virus was three normal Australian sheep muscle tissue (LPW, MSCU and 230909). Primers JSorf-X (position 4606-4625) and anti-sense primer JSRTorf-X located at nucleotide position 5106-5082. The *orf-X* primer set has a *Bam*H1 restriction site on the 5' end of the forward primer and *Sal*I on the 5' end of the reverse primer. The primer sequence and gene location can be found in Table 2.1. The PCR reaction conditions were as discussed in 2.2.1.4. The expected amplicon size was 497 base pairs.

2.2.1.4.3 Amplification of the JS transmembrane (JSTM) gene

Plasmid JS 107 is a cDNA clone that covers the exogenous JRSV envelope region. This cDNA fragment had *Eco*RI linkers added to facilitate (about 2000 bases in length) cloning in λ ZAP11 (York *et al*, 1992). Direct amplification of the plasmid did not result in any product. Upon analysis of the plasmid sequence, it was discovered that the cDNA fragment, which had *Eco*RI restriction sites at both the 5' ends, besides being ligated in a manner opposite to what had been expected, had a further *Eco*R1 restriction site identified at nucleotide position 6900. In order to amplify the correct TM fragment, the plasmid was digested with *Eco*RI and re-ligated hoping to obtain the correct alignment of the digested fragments. The ligated product was then used as template to amplify the *TM* gene.

2.2.1.5 Plasmid Digest.

Plasmid JS 107 was digested with restriction enzyme *Eco*R1 as follows: to 1 μ g of plasmid DNA, 1 μ l of 10% Buffer H (Roche Diagnostics), 8 μ l of dH₂O and 1 μ l of *Eco*R1 enzyme (10units/ μ l) was added. This sample was incubated at 37°C for 120 minutes. The digested

sample was denatured at 65°C to inactivate the enzyme. The denatured sample was made up to a volume of 1ml with deionised water.

2.2.1.6 Ligation

About 9µl (9ng) of the digested product was ligated using 1µl of 10% ligase buffer and 0.5µl of T4 DNA ligase enzyme (Pharmacia Biotech). This sample was incubated at room temperature for 120 minutes and then kept at 4°C. 1µl of the ligated product was used to amplify a 710 base pair fragment using primers JSTM (nucleotide position 6483 to 6500) and JSRTENV (nucleotide position 7194 to 7176) in a standard PCR reaction as described in section 2.2.1.4.

Table 2.2 DNA Templates and Concentrations

TEMPLATE DNA	CONCENTRATION OF DNA	TYPE OF DNA	JSP26 PCR	JSTM PCR	ORFX PCR
JS 382	1ng/µl (exogenous)	Plasmid (<i>gag</i>)	2µl		2µl
JS7 DNA	57ng/µl (exogenous)	Scottish-transformed sheep	10µl		10µl
JS 4844	200ng/µl (exogenous)	S.A. sheep plasmid	3µl		3µl
JS NL ¹	600ng/µl	Normal lung	1µl		1µl
LPW ²	455ng/µl	Genomic DNA	1µl		1µl
MSCU ²	95ng/µl	Genomic DNA	5µl		5µl
23909 ²	382.5ng/µl	Genomic DNA	1.3µl		1.3µl
JS 107	6µg/µl (exogenous)	Plasmid (envelope)		1ng/µl	

- 1 Salting out method used
- 2 Fastprep DNA extraction method

2.2.1.7 PCR Cycling Conditions

The PCR reaction mix was denatured by heating to 94°C for 5 min. in a Perkin Elmer 2400 thermocycler. The samples were amplified for 35 cycles. Each cycle consisted of a denaturation step at 94°C for 30 sec., followed by annealing at 55°C for 30 sec and an extension at 72°C for 30 sec. The final extension step at 72°C for 10 min was to allow complete extension of all DNA products. The PCR products were kept at 4°C until they were removed from the thermocycler.

Verification of the correct size of each amplified product was established by running each completed PCR reaction product through a 1% (w/v) agarose gel containing a 0.005%

Ethidium Bromide (Appendix D7) in Tris Borate EDTA (TBE) buffer (Appendix D8). These products were compared to a Standard DNA molecular weight marker VI or marker 11 (Roche Diagnostics, Germany) and visualized by using a UV Gel Documentation system (GDS 5000 Gel Documentation system, Ultraviolet Products Ltd) that is attached to a Sony Thermal Printer linked to a computer. Once the image has been captured, it could be improved using the Imagestore 5000 and ultimately printed.

2.2.1.8 Separation and Detection of DNA Molecules

Electrophoresis is a technique used to detect and separate DNA molecules of different sizes, either for isolation, cloning, identification or restriction analysis. Before the 1970's, it had been difficult to visualise DNA. The weight of DNA molecules was based solely on the viscosity of the solution. The larger the DNA molecule, the more viscous the solution compared to smaller molecules.

DNA molecules like proteins and many other biological compounds carry an electrical charge. At neutral pH DNA molecules carry a negative charge and when placed in an electrical field migrate from the cathode towards the positive pole or anode. The rate of migration of a molecule depends on its shape and its electrical charge. Since most of the DNA molecules are the same shape and have very similar electrical charges, fragments of different sizes cannot be separated by standard electrophoresis. Fragments do separate out, if the electrophoresis is performed in a gel that is usually made of agarose, polyacrylamide or a mixture of both. A gel comprises a complex network of pores through which the DNA molecules must travel to reach the positive electrode. The smaller the DNA molecule, the faster it can migrate through the gel (Brown, 1986b). Larger molecules migrate more slowly because of frictional drag and their ability to worm their way through pores of the gel less efficiently than smaller molecules. Gel electrophoresis separates DNA molecules according to their size. The size of DNA fragment determines the type of gel support that is needed and the resolution required is determined by the concentration of agarose or polyacrylamide used. Table 2.3 shows the range of separation in gels containing different amounts of agarose.

The ability of polyacrylamide gels in separating small fragments of DNA 5-500 base pairs (bp) is due to their high resolving power. Fragments of DNA that differ in size by as little

as 1 bp can be separated from one another. The only disadvantage of polyacrylamide over agarose gels is that they are more difficult to prepare and handle (Sambrook *et al*, 1989b).

Table 2.3 Range of Separation in Gels Containing Different Amounts of Agarose

Amount of agarose in gel % (w/v)	Efficient range of separation of linear DNA molecules in kilobases (Kb)
0.3	5-60
0.6	1-20
0.7	0.8-10
0.9	0.5-7
1.2	0.4-6
1.5	0.2-3
2.0	0.1-2

DNA has different conformations such as super helical, circular, nicked circular and linear forms that migrate at different rates through the gel even though their molecular weights are the same. Maximum resolution of DNA is obtained by running gels at no more than 5V per cm (centimetre). Before any sample of DNA is loaded into the wells of the agarose gel, the DNA is mixed with a gel-loading buffer (Appendix D9) containing Bromophenol blue. This gel-loading buffer serves three purposes. It increases the density of the sample, thereby ensuring even loading of sample into wells. Colour is added to the sample to simplify the loading process and dyes are present such that in an electric field, they move towards the anode at a known rate. Bromophenol blue migrates at 300 bp independent of agarose concentration between 0.5% and 1.4% in 0.5% TBE (Sambrook *et al*, 1989b).

DNA in both agarose and polyacrylamide gels can be visualised directly by the use of a fluorescent dye, Ethidium bromide. The dye intercalates between stacked base pairs, extending the length of linear and nicked circular DNA molecules and making them more rigid. The electrophoretic mobility of linear DNA is reduced by about 15%. Ethidium bromide is a carcinogen and care should be taken when being handled and disposed.

The composition and ionic strength of the electrophoresis buffer affects the electrophoretic mobility of DNA. The two most commonly used electrophoresis buffers are Tris-acetate-ethylenediaminetetraacetate (TAE) and Tris-borate-ethylenediaminetetraacetate (TBE). TBE is the buffer of choice because of its higher buffering capacity compared to TAE. TAE tends to become exhausted during extended electrophoresis (the anode becomes alkaline and the cathode acidic).

Double stranded linear DNA fragments migrate approximately 10% faster through TAE than TBE, although the resolving powers of both systems are almost identical. Resolution of supercoiled DNA is better in TAE than in TBE (Sambrook *et al*, 1989b).

2.2.1.8.1 Agarose Gel Electrophoresis

For a 0.8% agarose gel, 0.8g of agarose was weighed out and added to 100ml of 0.5% TBE buffer in an Erlenmeyer flask. The flask was weighed (weight noted) and covered with cling wrap. A hole was made in the centre of the cling to allow evaporation to occur. The Erlenmeyer flask was heated in a microwave oven for 1 min on high. The solution was not allowed to overheat, as it would have foamed over the sides of the flask when agitated. The flask was removed from the oven; contents swirled to dissolve agarose and then reheated for a further 1 min until all the agarose had dissolved. The flask was re-weighed and the original weight made up by adding hot water. The solution was cooled to 60°C before adding 5µl (final concentration 0.5µg/ml) of a 10mg/ml of ethidium bromide solution. The agarose was left to cool before being poured into a rectangular- shaped gel-casting apparatus containing a 12 well comb. The agarose was allowed to set, and then left at 4°C for 15 min before the comb could be removed. The gel electrophoresis system used was the Hybaid Electro-4 electrophoresis system (S.A. Scientific, S.A.).

The gel was placed in an electrophoresis tank and about 450ml of 0.5% TBE buffer was added, so that the buffer was 3-5mm over the gel. The samples (10µl) containing Bromophenol blue loading buffer (2µl) was loaded along with a DNA molecular weight marker (Boehringer Mannheim, Mannheim Germany). A Hoeffer power pack was used to run the gel. The voltage was set at 80Volts (V) for approximately an hour or until the blue front had reached about half the way of the casting tray. The gel is removed from the tank using gloves and placed on a Transilluminator so that the gel could be viewed using a UV Gel Documentation system (GDS 5000 Gel Documentation system Ultraviolet Products Ltd) that is attached to a Sony Thermal Printer linked to a computer. Once the image is captured, it could be improved using the Imagestore 5000 and ultimately printed.

2.2.1.9 Restriction Enzymes

2.2.1.9.1 Restriction Digest

A restriction digest is performed by adding to the DNA a buffer to ensure maximal activity of the restriction enzyme that is usually at pH 4, the correct ionic strength provided by Sodium Chloride (NaCl) and Magnesium ion (Mg^{2+}) concentration. Included is also a reducing agent like Dithiothreitol (DTT) that can stabilise the enzyme and prevent its inactivation. One unit of enzyme is sufficient to cut one microgram (μg) of DNA in 1 hour at 37 °C (temperature at which most restriction enzymes work best). After a restriction digest, incubating the solution at 65°C for 15 min inactivates the enzyme. This ensures complete inactivation of the enzyme and guards against accidental digest of other DNA molecules that may be added at a later stage (Brown, 1986b).

2.2.1.10 Purification of amplified fragments

The amplified PCR products were purified using the Wizard PCR DNA purification System (Promega Corporation, Madison, USA; Catalogue No.A7170) as well as the Qiagen PCR DNA Purification system (Qiagen, GmbH, Hilden, Germany). The methods followed were according to the manufacturer's instructions. PCR products were purified to remove the enzymes, primers, nucleotides, salts, ethidium-bromide detergents or any other impurities of PCR. For purposes of this study, the purification of one PCR product, JSp26 will be described using the Qiagen PCR purification kit. The PCR product was transferred to a clean 1.5ml microcentrifuge tube for direct purification. A purification buffer (PB) was added such that its volume was 5% that of the PCR product. The sample was vortexed briefly and then transferred to a 2ml micro-centrifuge fitted with a spin column. The column contains a silica-gel membrane for binding up to 10 μg of DNA in high-salt buffer and elution in low-salt buffer. The contents of the micro-centrifuge tube were spun for 30-60 sec. The supernatant is discarded and 750 μl of a wash buffer PE (ethanol wash) is added to the column. The tube with the column is centrifuged as before, and the supernatant discarded. The column is given a further spin to remove all traces of ethanol. The DNA is eluted with either Tris-EDTA (TE) buffer or sterile distilled water depending on the application that it is used for. For instance, TE would be suitable for cloning

purposes and water for sequencing. Since the product was being used for cloning, I used 50µl TE as the elution buffer. The product was stored at -20°C until it was required.

2.2.1.11 Cloning of PCR Products

The amplified purified products from Section 2.2.1.10 were cloned into Amersham's pMoss Blue T Vector (Amersham Life Sciences, UK; RPN 1719) according to the manufacturer's instructions. This vector was designed for simplified cloning of PCR products. Many thermostable DNA polymerases such as *Taq* DNA polymerase leave single 3'A (adenosine) nucleotide overhangs on their reaction products and so are able to be ligated directly to the vector containing compatible single T (thymidine) nucleotides overhangs. The pMoss Blue T vector has been specifically constructed by digestion with *EcoRV* followed by the addition of a single 3'dT residue at each end. There are many steps to the process of construction of a recombinant DNA molecule. The most important and critical being the ligation, which is the joining of pieces of DNA by an enzyme called ligase. No further manipulation of this vector is required for ligation with amplified DNA.

An advantage of the pMoss Blue T vector is that it allows for blue-white selection of recombinants through insertional inactivation of the *lac Z* gene that it carries. The bacterial and plasmid genes complement each other to produce functional β -galactosidase molecules. Successful transformation of plasmid into the bacterial host results in a blue colony (β -galactosidase is synthesised). If, however, should the expression of the *lac Z* gene fragment be altered by disruption of the reading frame by cloning a gene into the fragment, the colony would be white (β -galactosidase is not synthesised). In other words, when X-gal and an inducer of the enzyme such as IPTG is added to the agar, along with ampicillin, then non-recombinant colonies will be coloured blue and recombinants with a disrupted *lac Z* gene will be white (Brown, 1986c).

The amplified JMTM and JSorf-X amplicons were subjected to the same experimental manipulations as JSp26, the *gag* fragment, which is the example to be used from Section 2.2.1.10 for cloning into pMOSS Blue T vector and for cloning into the pGEX Expression system. Thereafter the experimental procedures differed.

The concentration of the PCR products was estimated by running the product on an agarose gel and comparing it to a DNA molecular marker VI. The vector to insert ratio used should be in the range of 1:5, 1:7.5 or 1:10 for optimal cloning. The amount of insert that was required for ligation into the standard amount of 50ng of vector was calculated by simply multiplying the size of the insert, which in this case was 653 bp, by 1.3×10^{-1} .

This amount of JSp26 DNA was added to a tube containing 1 μ l of 10% ligase buffer, 0.5 μ l of 100mM DTT, 0.5 μ l of 10mM ATP, 1 μ l of 50ng/ μ l vector and 0.5 μ l of T4 DNA ligase representing 2-3 Weiss units. The volume was made up to 10 μ l with nuclease free water. The ligation reactions were prepared on ice. The ligation reaction was mixed gently with a pipette tip and then incubated at 16°C overnight. Further to test the efficiency of the ligation reaction 2 μ l (4.5ng/ μ l) of the positive control insert provided was also used in the ligation reaction.

The ligation reactions were then transformed, using antibiotic sensitive *Escherichia coli* (*E.coli*) cells. All the cells in each antibiotic-resistant clone that remained after selection contains plasmids with the same inserted DNA fragment, but different clones carry different fragments (Lodish *et al*, 1999b). The ligated samples were then used to transform MOS BLUE competent cells. A sufficient number of 82mm petri dishes with Luria Bertani agar (LB-Appendix D10) containing 100 μ g/ml ampicillin (Appendix D11) and 15 μ l/ml tetracycline (Appendix D12) were prepared in advance and kept at 4°C away from the light. Before being used, the plates were kept at 37°C so that they were warm enough for plating of the transformation mix. A suitable number of micro-centrifuge tubes were pre-chilled on ice. A tube of frozen MOSS Blue competent cells was thawed on ice and mixed to suspend the cells evenly. 20 μ l of competent cells was pipetted into each of the pre-chilled tubes. To each tube of competent cells, 1 μ l of ligation mix and test plasmid (provided in the cloning kit) was added directly to the cells and mixed very gently. The tubes were left on ice for 30 min. The cells were heat shocked at 42°C for 40 sec in a water bath. The tubes were immediately placed on ice for 2 min. SOC medium (Appendix D15) (80 μ l) pre-warmed to 37°C was added to each tube. The tubes were shaken at 250 rpm at 37°C for 1 hour (hr) in a Brunswick Orbital Shaker. Meanwhile the LB agar plates containing ampicillin (Appendix D16) were prepared for blue-white screening of recombinants by spreading 35 μ l of 20mg/ml X-gal (Appendix D13) and 20 μ l (100mM) of IPTG (Appendix D14) per plate. The plates were left for at least half an hour to allow the

X-gal/IPTG to soak before plating the transformation mix. 100µl of each transformation mix was spread onto each plate. The plates were incubated overnight at 37°C. Recombinants were screened in two ways. Firstly, the colonies were screened by direct colony PCR. Secondly, the positive colonies from that screening process were then screened using the standard mini-prep method.

Ten white colonies were selected from the LB agar plate. Sterile water (50µl) was added to ten 1.5µl micro-centrifuge tubes. Using a sterile loop, colonies were picked by touching the loop tip to a colony then to an agar stock plate. For PCR analysis the tip of the loop was then transferred to a tube containing water. The agar plate containing ampicillin was incubated overnight at 37°C. The tubes were vortexed to disperse the bacteria and boiled at 100°C on a dry block (Techne Dri-block DB2A) for 5 min to lyse the cells and expose the DNA. The tubes were centrifuged at 12,000 rpm for 1 min to pellet the cell debris. To a 0.2ml thin-walled PCR tube containing 40µl of PCR master mix, 10µl of the supernatant was added and the tubes placed in a thermal cycler with the same cycling conditions as in 2.2.1.7 being followed. The amplified products were run on a 1% agarose gel and the size of the products determined as 653 bp. Once the clones on the plates were identified as that producing the correct product, these clones were then grown in 3mls of liquid LB medium (Appendix D24) with 100µg/ml of ampicillin and incubated overnight at 37°C.

The alkaline lysis mini-prep method was used 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). This 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. Cells from the overnight culture were harvested by centrifugation at 12,000g, for 2 min and resuspended in 100µl Solution I (Appendix D17) by vortexing vigorously. The function of this step is to lyse the bacteria by hyperlytic osmosis, releasing DNA and other contents. To this 200µl of Solution II (Appendix D18) was added and mixed by inverting the tube several times. This step denatures chromosomal DNA and proteins. The solution was incubated for 5 min at room temperature, neutralized with 150µl of Solution III (Appendix D19) and mixed by inverting the tube several times. The idea behind this step is to selectively precipitate proteins, polysaccharides and genomic DNA. The use of this buffer allows plasmid DNA to remain in the supernatant, without being precipitated. The solution was incubated on ice

for 5 min, centrifuged (12,000g x 5 min) and the DNA containing supernatant transferred to a clean tube. An equal volume of phenol-chloroform-isoamylalcohol pH 8 (24:1:1-Bisolve) was added to the supernatant and vortexed for 15 sec. The solution was centrifuged as before for 2 min and the aqueous phase transferred to another clean tube. 800µl of absolute ethanol (AR Merck) was added to the aqueous layer, vortexed and allowed to settle for 2 min. The solution was centrifuged as before, but for 5 min and the supernatant discarded. The pellet was washed with 1ml of 70% ethanol (ambient temperature) (Appendix D4) and centrifuged for 5 min. The washed pellet was allowed to air dry at room temperature for 10min and then resuspended in 25µl of TE (pH8) buffer containing 20µg/ml of RNase (Roche Diagnostics, Germany). This step ensures that the total RNA's is degraded from the sample. The tubes were incubated at 37°C for 15 min. To verify the presence of the correct size of the insert, 1µl (+/-160ng/µl) of DNA was digested with the appropriate restriction enzymes for 1 hr at 37°C. To 1µl of DNA extracted, 1µl of 1% restriction buffer B and 1µl of the enzyme *Bam*HI (Roche) was added in a total volume of 10µl that excluded the enzymes and analysed by agarose gel electrophoresis as described in 2.2.1.8.1.

2.2.1.11.1 Large Scale Isolation of pMOSS DNA

The plasmid containing the cloned fragment pMOSS JSp26 was grown overnight at 37°C in a large volume (100ml) of LB broth containing 100µg/ml of ampicillin. The plasmid was purified using the Qiagen® plasmid maxi kit (Southern Cross Biotechnology, Cape Town) according to the manufacturers' method. This extraction provided a large stock of DNA plasmid for further procedures such as cloning into a pGEX expression vector. About 10µg (34µl) of plasmid DNA was digested in a total volume of 50µl using the appropriate restriction enzyme (25 units) and 10% enzyme buffer (5µl) for 2 hrs at 37°C. The amount of restriction enzyme that was used was equivalent to less than 5% of the total volume of the digest. The restriction digest was run on a 1% agarose gel and the insert excised out of the gel using a clean sterile scalpel blade. The DNA was extracted from the low melting gel using the Wizard® PCR Preps DNA Purification System (Promega Corporation, Madison, USA).

Briefly, the excised DNA fragment was transferred to a 1.5ml micro-centrifuge tube, weighed and incubated at 70°C until the agarose had melted completely. One millilitre of

resin was added to the melted agarose slice and mixed thoroughly for 20 sec. The DNA was purified using Promega's Vac Man[®] Lab vacuum manifold (catalogue number A7231) as follows. A wizard mini-column was prepared by attaching the syringe barrel that was provided to the luer-lok extension of the mini-column. The top of the mini-column/ syringe barrel assembly was inserted into the vacuum manifold. The resin/ DNA mixture was pipetted into the syringe barrel and a vacuum applied to draw the resin DNA into the mini-column. The vacuum to the column was broken and the column washed by adding 2ml of 80% isopropanol (Merck) to the syringe barrel and reapplying the vacuum to draw the solution through the column. The resin was dried, by continuing to draw the vacuum for a further 30 sec. The syringe barrel was removed and the mini-column transferred to a clean 1.5ml that was centrifuged (10,000 x 2 min.) to remove any residual isopropanol. The mini-column was transferred to a fresh tube and 50µl of TE added. After a waiting period of 1 min the mini-column was centrifuged (10,000 x 20sec) to elute the DNA fragment. The DNA was stored at -20°C.

2.2.1.12 The pGEX Plasmid Expression Vector System

The expression of foreign genes from plasmids transformed into *E.coli* is a tool increasingly used in the investigation of the functions of proteins (Grieco *et al*, 1992). Plasmid expression vectors have therefore been constructed that direct the synthesis of foreign polypeptides in *E.coli* as fusions with the C-terminus of Sj26, a 26 kDa glutathione S-transferase (GST) encoded by the parasitic helminth *Schistosoma japonicum*. The plasmid *pSj5* directs the synthesis of Sj26 in *E.coli* under the control of the IPTG-inducible tac promoter. Through a series of manipulations, *pSj5* was modified into the expression vector pGEX-1. This vector contains a tacpromoter (for chemically inducible high level protein expression), followed by the complete coding sequence of Sj26 in which the normal termination codon is replaced by a polylinker containing unique recognition sites for *Bam*H1, *Sma*1 and *Eco*R1. The polylinker is followed by TGA translation termination codons in all three reading frames; the β -lactamase coding gene *Ap^R* (for ampicillin resistance); *ori* (origin of DNA replication); a fragment of the *lac* operon containing the over-expressed *lac I^q* allele of the *lac* repressor and part of the *lacZ* with very mild elution conditions for release of fusion proteins from the affinity matrix, thus minimising effects on antigenicity and functional activity. The resultant fusion protein gene is expressed as an intracellular product and is able to be purified under non-

denaturing conditions by glutathione affinity chromatography. The protein of interest can then be cleaved from the GST by using a specific protease if it has been included in the expression vector like factor Xa for pGEX 3X or thrombin (pGEX 2-T) and Pre-Scission fusion proteins that possess the complete amino-acid sequence of GST also demonstrate GST enzymatic activity and can undergo dimerisation similar to that observed in nature. Mammalian GST isozymes can be purified by affinity chromatography on immobilized glutathione followed by competitive-elution with excess reduced glutathione, a property shared both by the native Sj26 and Sj26 synthesised in *E.coli*. It is this observation that has led to the development of the pGEX expression system. The crystal structure of the recombinant *Schistosoma japonicum* GST from the pGEX vectors has been determined and matches that of the native protein (Smith and Johnson, 1988). In this study pGEX-1, its derivative pGEX 2-t and pGEX 4-t were used as expression vectors for JSp26, JSTM and orf-X viral proteins respectively.

2.2.1.12.1 Restriction Enzyme and Dephosphorylation of pGEX Vector DNA

The pGEX vector DNA (Pharmacia, Uppsala, Sweden) was linearised, by digesting maximally 5µg of DNA in a total volume of 50µl with 20 units of appropriate restriction enzyme for 5 hr at 37°C. The One-Phor-All buffer Plus™ (OPA-Appendix D20) from Amersham Pharmacia Biotech was used, since it is compatible with virtually all restriction enzymes and many modifying enzymes. A small aliquot of the digested mixture was examined by agarose gel electrophoresis as described in 2.2.1.8, 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 group had to be removed before ligation to prevent recircularisation of the vector. Calf-intestinal alkaline phosphatase (Amersham-Pharmacia Biotech) was diluted in OPA buffer such that a total of 0.1 units (1-2µl of diluted enzyme) were added to the digested pGEX DNA, which was then incubated for 30 min at 37°C. The alkaline phosphatase was heat inactivated at 85°C for 15 min. The de-phosphorylated product was purified by ethanol precipitation. An equal volume of phenol-chloroform-isoamyl alcohol (Sigma Capital, Durban. 24:1:1) was added to the product, vortexed and centrifuged (12,000g for 1 min) to separate the phases. The upper aqueous phase was transferred to a fresh tube and an equal amount of chloroform-isopropanol v/v (24:1) was added. The solution was mixed and centrifuged as before to separate the phases. The aqueous layer was transferred to a clean tube and one-tenth volume of the aqueous layer of

3M Sodium Acetate (pH 5.2) (Appendix D21) and 2.5 volumes of 95% ethanol added. The solution was mixed and placed at -20°C overnight or at -70°C for 30 min. The sample was centrifuged (12,000 x 15 min) and the supernatant removed. The pellet was washed with 1ml 70% ethanol and re-centrifuged for 2 min, and every drop of ethanol removed. The pellet was air-dried at 37°C and dissolved in 20µl of TE. The pGEX DNA was stored at -20°C for use later.

2.2.1.12.2 Ligation of Insert to pGEX-1 DNA

The linearised vector and insert DNA was used at a vector insert molar ratio of 1:5 (7.5). The molar number of ends of linear DNA was calculated according to the following formula where 1 optical density (OD) unit at 260nm is equivalent to 50µg/ml of linear double stranded pGEX DNA consisting of 4900 bp were moles of ends is equal to 2 times (g of DNA) divided by number of bp times 649daltons/bp.

The final ligation reaction mix consisted of not more than 200ng of DNA, 1mM ATP, 5 units of FPLC pure T4 DNA ligase, 2µl of 10 x One-Phor-All buffer Plus (OPA⁺) in a total volume of 20µl. The ligation mix was incubated overnight at 16°C and thereafter was terminated by heating at 65°C for 10 min. The ligation mix was used to transform competent cells.

2.2.1.12.3 Preparation of Competent Cells and Transformation with pGEX DNA

The protocol followed was based on the procedure described by (Hanahan, 1983). The compositions of buffer and media used are described in the Appendix D. Using sterile techniques, a loopful of *E.coli* strain JM105 from frozen glycerol stocks, was streaked onto a SOB agar plate and grown overnight at 37°C. The following day, polypropylene tubes were pre-cooled on ice. A streak of well-isolated colonies was transferred from the SOB (Appendix D22) plate using a sterile plastic loop and resuspended in TFB (Appendix D23) by vortexing at a moderate speed. The cells were incubated on ice for 30 min. DMSO was added to the resuspended culture and gently vortexed and incubated on ice for 5 min. DTT was added next and incubated as before, but for 10 min. Finally DMSO was added, and incubated for a further 5 min on ice. The cells (200µl) were aliquoted into pre-cooled polypropylene tubes. For transformation, the pGEX DNA to a maximum volume of 20µl

(10-50ng) was added to the competent cells and incubated on ice for 30 min. The cells were heat shocked for exactly 45 sec in a 42°C water bath. The tubes were immediately chilled on ice for 2 min and 800µl of SOC medium (Appendix D15) warmed to 37°C was added and the tubes incubated at 37°C for 60 min with moderate agitation using an Orbital shaker. The incubated cells were spread onto LB agar plates (Appendix D10) containing 100µg/ml ampicillin and incubated overnight (Hanahan, 1983).

2.2.1.12.4 Small-scale Isolation of pGEX DNA

Colonies appearing after incubation as described above were picked and screened as in section 2.2.1.11 using the two methods to determine which clones were recombinants. There was no blue white screening of recombinants with this plasmid as it lacked the F containing Lac ZσMI5 gene. To further verify the presence of the correct sized insert about 2µl of the recombinant pGEX DNA was digested as described in section 2.2.1.9.1 and analysed by agarose gel electrophoresis as described previously in section 2.2.1.8.1.

2.2.1.12.5 Large-scale Screening of pGEX DNA

Since the insert was cloned into one restriction enzyme site and therefore required digestion with one enzyme, the isolated recombinant clones were further investigated to ensure that the insert was in the correct reading frame. The plasmids containing the cloned fragments were purified using the Qiagen maxi preparation method (Qiagen GmbH, Hilden, Germany). A 100ml volume of each culture was grown overnight in LB broth (Appendix D24) containing ampicillin and incubated at 37°C with shaking. The plasmid was purified according to the manufacturer's instructions and sequenced using an Autoread sequencing kit (Pharmacia Biotech), and the A.L.FTM (Automated Laser Fluorescent) DNA Sequencer (Pharmacia Biotech) with a few modifications.

2.2.2 Sequencing as a Tool to Confirm Cloning

Initially the A.L.FTM DNA Sequencer was used in this study to confirm that the insert was cloned into the correct reading frame of the pGEX 1 expression vector, thereafter, the ABI PRISM 310 Genetic analyser (P.E. Biosystems) was used for subsequent analysis of recombinant clones. Because of this, both methods will be briefly discussed.

2.2.2.1 The A.L.FTM DNA Sequencer and the ABI PRISM 310 Genetic Analyser

The A.L.FTM DNA Sequencer employs a non-radiochemical approach to sequencing. A Fluore-dATP Labelling Mix (fluorescein-15-dATP in solution with dCTP, dGTP and dTTP) is used in combination with the AutoRead Sequencing Kit and Automated Laser Fluorescent A.L.F. DNA Sequencer from Pharmacia Biotech. This mix allows primers that are not fluorescein labelled to be used in the sequencing reactions, since fluorescein-labelled dATP is incorporated during the labelling reaction (Voss *et al*, 1992). Using standard dideoxy sequencing methods, the fluorescently labelled primer is extended by T7 DNA Polymerase in four separate dideoxy reactions (A, C, G, and T), creating four separate populations of fluorescently labelled chain-terminated fragments. The reactions are then loaded into four adjacent lanes on a sequencing gel and electrophoresed. As the DNA fragments in each lane migrate through the gel, they pass a fixed laser beam that excites the fluorescent signals that are stored automatically for subsequent sequence analysis. Frederick Sanger and Andrew Coulson of the Medical Research Council Laboratory developed the chain-terminating method for Molecular Biology at Cambridge (Sanger *et al*, 1977). Walter Gilbert and Allan Maxam of Harvard developed an alternate method of sequencing, the chemical degradation method. Both methods were first published in 1977 (Brown, 1994).

Dideoxy sequencing has become the preferred method of sequencing. Instead of the use of one type of fluorescein label and four separate dideoxy reactions, four different fluorescent labels, one for each dideoxy reaction, which is all mixed in one tube, are then electrophoresed in one lane. As each band passes the detector, the imaging system can determine the wavelength of the fluorescence, thereby identifying which family the band belongs (Prober *et al*, 1987). This method is utilized in the Perkin Elmer ABI PRISMTM Big DyeTM Terminator Cycle Sequencing Ready Reaction Kit (P.E.Biosystems.).

2.2.2.2 The ALF™ DNA Sequencer

2.2.2.2.1 The Autoread Sequencing Reaction

One microgram per kilobase of plasmid/insert DNA was sequenced using the pGEX 5' and pGEX 3' sequencing primers (2 pmole/ μ l) and a Fluoro-dATP mix (Pharmacia Biotech). To the tubes containing the template DNA and primers, 1.5 μ l of freshly prepared 1M NaOH was added in a total volume of 12.5 μ l. The tubes were vortexed, centrifuged briefly and incubated at 70°C for 4 min. The tubes were spun briefly and transferred to a 37°C incubator for 15 min. During this time, 1.5 μ l of 1M HCl was added and the contents mixed. The annealing buffer (2 μ l) was added, samples were mixed and the tubes left to incubate for 15 min at 37°C. Meanwhile the A, C, T, and G termination mixes were prepared. For each sample 3 μ l of each termination mix, 1 μ l of DMSO and 0.5 μ l of extension buffer was added and left at 4°C. After the incubation at 37°C, 1 μ l fluorescein-15-dATP, and 1 μ l of T7 DNA polymerase was added to the template tube and this tube was incubated for 10 min at 37°C. 3.5 μ l of template was added per termination mix (4 μ l) that had been warmed to 37°C for 3 min. These reactions were transferred to a nunc plate and incubated for 15 min at 40°C. The plate was sealed to prevent evaporation of samples. Stop solution (4 μ l) was added to end the reaction. The reactions were stored on ice until they could be loaded into the gel. Sequencing was performed using the Automated Laser Fluorescent (ALF) DNA Sequencer. Sequencing data was analysed using the DNASIS software (Hitachi Software Engineering USA) and Genbank data bank sequences. Internal primers were used to confirm sequence in both directions.

2.2.2.2.2 Preparation of Gel and Sequencing Apparatus

A 6% Long Ranger gel was prepared as follows: 42g of Urea (ALF grade) was dissolved in 12ml of 10X TBE (Appendix D26), and 12ml of 50% Long Ranger concentrate (FMC Bio Products, USA). This gel mix was made up to 100ml with filtered distilled water and placed on a magnetic stirrer at slow speed for 30 min. The solution was filtered with a 0.45 μ m filter into an Erlenmeyer flask, degassed for 15 min and then transferred to a Pharmacia Biotech gel mix bottle that was suitable for pouring the gel. Meanwhile the gel plates, spacers, combs, upper and lower buffer chambers were cleaned with water. The gel plates were further cleaned and polished using water, absolute ethanol and lint free tissue

wipes. The first 5cm of the notched plate where the comb wells will be positioned was treated with bind silane for a few minutes and then rinsed with water to remove any acetic acid present. The gel cassette was assembled and ready for the gel. Before pouring the gel, 50µl Temed (BioRad Laboratories, Inc, USA) and 500µl of 10% (w/v) ammonium persulfite (Appendix D25) was added to the gel solution and gently mixed. The gel solution was applied by moving the nozzle of the bottle in an even movement back and forth along the lower edge (furthest edge) in the glass plate using even pressure. Capillary action drew the solution front upwards to fill the entire space between the plates. The gel was left for 2 hours at room temperature to polymerise. The gel cassette was mounted on the sequencing apparatus according to the manufacturer's instructions. The upper and lower buffer chambers were filled with 0.6% TBE. The system was connected to the water circulation and the laser was activated. The A.L.F™ Sequencer is linked to a computer terminal where the data is captured and processed using specific software packages. A new file was opened for the run and named accordingly. The optimum settings selected was an operating voltage of 1900V, current of 65mA, power at 35W, sampling interval of 2.5 sec, laser power at 4mW, temperature between 45° to 48°C and running time of 600 minutes. Once the temperature was reached, the comb was removed and the wells washed thoroughly with buffer to remove any acrylamide or urea that may be present in the wells. Before loading the samples onto the sequencing gel, they were heat denatured at 85°C for 3 min. The electrodes were connected to the sequencer and the lid was closed. The run was started once the temperature and laser values were stable. The sequencing results were saved to file on to a stiffy disk as well as being printed for analysis.

2.2.2.3 The ABI PRISM 310 Genetic Analyser

The ABI PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit provide dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, rTth pyrophosphatase (a component in AmpliTaq DNA Polymerase, FS), magnesium chloride, and buffer that are premixed into a single tube of ready reaction mix and is ready to use. The enzyme used is a variant of *Thermus aquaticus* DNA polymerase that contains a point mutation in the active site that results in less discrimination against dideoxynucleotides. Further, the second mutation in the amino terminal domain eliminates the 5'→3' nuclease activity of the enzyme. The set of dye terminators are labelled with novel, high-sensitivity

dRhodamine acceptor dyes and each sequencing reaction is a single tube reaction with all four terminators.

The reagents are suitable for performing fluorescence-based cycle sequencing reactions on double-stranded DNA templates or on polymerase chain reaction fragments. The template to be analysed had to be of good quality. Since plasmids were being analysed, they needed to be purified using a Qiagen plasmid preparation kit. The plasmid was run on a 1% agarose gel together with a molecular weight marker to ascertain the concentration of plasmid. About 500ng of template DNA was used in a cycle sequencing reaction using the ABI PRISM® BigDye™ Terminator Ready Reaction Kit (PE Applied Biosystems). The reaction was carried out according to the manufacturer's instructions. After the sequencing reaction, the extension products were purified. The reaction tubes were centrifuged so that the products could be collected easily. The reactants were transferred to 1.5ml micro-centrifuge tubes and the volume determined (20µl). Sterile water was added to make up the volume to 36µl and 64µl ethanol (95%) was added to precipitate the extension products. The contents of the tubes were vortexed and left at room temperature for 15 min. The tubes were centrifuged for 15 min at 12000 rpm. Every drop of supernatant was removed as all unincorporated dye terminators are dissolved in them. Freshly prepared 70% ethanol (100µl) was added to the pellet, tubes vortexed and centrifuged for 10 min as before. After a second spin with 70% ethanol, the supernatant was removed. The pellet was dried at 90°C for 2 min. Each pellet was re-suspended in 20µl of Template Suppression reagent that is supplied with the polymer. The tubes were vortexed, centrifuged, and heat denatured for 2 min at 100°C. The samples were chilled on ice for 2 min and then transferred to 0.5ml sample tubes and stored at 4°C in the dark until ready for sequencing on the ABI Prism 310 Genetic Analyser. The ABI Prism 310 Genetic Analyzer is a laser-induced fluorescence capillary electrophoresis system. DNA samples are loaded onto the autosampler. These samples are then automatically introduced into a polymer-filled capillary for electrophoresis. The dye-labelled DNA fragments electrophorese through the polymer, and separate according to size. As the labelled samples travel through the capillary and into the window, they are illuminated. The fluorescent dyes attached to the fragments are excited by the laser and emit light at a specific wavelength for each dye. The light is collected and separated by a spectrograph according to the wavelength. It is collected onto a charge-coupled device (CCD) camera; so all four types of fluorescent emissions can be detected simultaneously. The data collection software collects the light

intensities using software filters and stores them as electrical signals that is further processed. At the end of the run, the computer automatically analyses the collected data and electropherograms are printed. Correct colour interpretation of the collected data was dependent on the use of matrix files (Sequencing Analysis), which were created for each specific separation and dye chemistry. The analysed data was then edited and printed (Anon, 1998).

2.2.3 Optimization of Protein Expression

pGEX recombinant clones were screened for fusion protein expression prior to large scale purification to check clones for expression of the desired fusion protein. Several colonies of *E.coli* transformed with pGEX recombinants were picked into separate 15ml green-capped tubes containing 2ml of LB broth with ampicillin. A separate control tube with bacteria transformed with the parenteral pGEX was also inoculated. The cultures were grown overnight at 37°C with shaking. The next day a 1/10 dilution was made of the overnight culture and grown to an A_{600} of between 0.6-0.8 (2 to 2.5 hr) with vigorous agitation at 37°C. Fusion protein expression was induced, by adding 1µl of 1M IPTG (final concentration 0.5mM—Appendix D27). Incubation was continued for an additional 4 hr. The liquid culture was transferred to a clean micro-centrifuge tube that was centrifuged for 10 min at 3000 rpm and the supernatant discarded. Each pellet was resuspended in 150µl of ice-cold 1% PBS (Appendix C28). The cells were lysed using the Branson Sonnicator that was equipped with a fine bore probe used for small volumes. The cells were sonicated at 50% output, and at 5-6 for 10 sec. Triton X-100 (20%) was added to a final concentration of 1% and left on ice for 30 min before centrifugation (5 min x 10,000 rpm). Lysis was complete when the cloudy cell suspension became translucent without frothing which can denature the protein. The supernatant was transferred to a clean tube and 10µl of this supernatant was added to an equivalent amount of 2% SDS sample buffer (Appendix D29) and heated at 95°C for 5 min before being cooled on ice. The samples together with a protein calibrator were run on 12% SDS-PAGE (Appendix D31) at a constant voltage of 130V on ice for 1 hr. After the electrophoresis was completed, the gel was stained with Coomassie© Brilliant Blue G 250 (BIORAD) to visualize the polypeptide bands.

2.2.4 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The term electrophoresis is defined as the separation of molecules based on their mobility in an electric field. For SDS-PAGE analysis, proteins need to be disrupted to their primary structures by using the right detergents. SDS is an anionic detergent that binds to hydrophobic regions of protein molecules and causes them to unfold into extended polypeptide chains. As a result, the individual proteins are dissociated from other proteins and are freely soluble in the SDS-solution (Wu *et al*, 1997). The protein sample is denatured with heat in the presence of SDS and a reducing agent (2-mercaptoethanol– which breaks any S-S bonds present in proteins). The SDS coats the proteins, providing them with a net negative charge proportional to their length. When the coated sample is run on an SDS page gel, the proteins separate by charge and migrate towards the positive electrode (anode) and by the sieving effect of the gel matrix. Sharp banding of the proteins is achieved using a discontinuous gel system that has stacking and separating gel layers that differ in salt concentration, pH, acrylamide concentration or a combination of these (Anon, 1993).

The effective range of separation of SDS-page gels depends on the concentration of polyacrylamide used to cast the gel and on the amount of cross-linking. Cross-links formed from bis-acrylamde add rigidity and tensile strength to the gel and form pores through which the SDS- page complexes must pass. The size of these pores decreases as the bis-acrylamide/acrylamide ratio increases reaching a minimum when the ratio is approximately 1:20. Most page gels are cast with a molar ratio of bis-acrylamide:acrylamide of 1:29, which is capable of resolving polypeptides that differ in size by as little as 3%. Sieving properties of the gel are determined by the size of the pores (determined by the bis-acrylamide/acrylamide ratio). Table 2.4 shows the linear range of separations obtained with gels cast with concentration of acrylamide that range from 8% to 14% (Sambrook *et al*, 1989c).

TABLE 2.4 Effective Range of Separation of SDS-PAGE Gels

Protein size	8%	10%	12%	14%
Optimal size range KDa	25-150	15-100	10-70	5-50
Resolvable size range KDa	15-300	5-200	5-150	2-100

The primary uses for page are the determination of the size of a protein component, the estimation of protein purity in a solution, the purification of a protein species for other procedures and the fractionation of a complex protein mixture before immunoblotting (Western blots). After electrophoresis, the gel is stained with Coomassie Blue R-250 to visualize the polypeptide bands. The molecular weight of the polypeptide is inversely proportional to its mobility. The molecular weights of the polypeptide can be estimated by the use of calibration proteins of known molecular weights (Sambrook *et al*, 1989c).

2.2.4.1 Assembly of the Glass Plate Sandwich

The first step in SDS-PAGE is the casting of a discontinuous page gel that consists of a resolving or separation (lower) gel and a stacking (upper) gel. This study made use of the Mini-Protean® II Dual Slab Cell (Bio-Rad Laboratories, Inc, USA). Glass plates were wiped clean with ethanol and allowed to air dry. The gel cassette was assembled according to the manufacturers' instructions. Briefly, the glass plate sandwich was assembled, by placing the longer glass plate down first. Two spacers of equal thickness were placed along the short edges of the glass plate. The shorter plate was placed on top of the spacers so that the bottom ends of the spacers and the bottom of the glass plates were aligned. The spacers were left sticking up above the long glass plate about 5 mm. The glass plate sandwich was held in such a way that the longer plate was facing away from me, so that I could slide it gently into the clamp assembly. The two top screws of the clamp assembly were tightened, but not too tightly. The clamp assembly was placed into the alignment slot on the casting stand with the clamp screws facing away from me. The two top screws were loosened and the glass plates and spacers set flush against the casting stand. Both pairs of screws on the clamp assembly were gently tightened. The clamp assembly was transferred to the casting slot on the casting stand. Since two gels were always cast, the clamp assembly was placed on the side opposite the alignment slot to make aligning the next sandwich easier. Placing the bottom first and gently pushing the assembly by pressing against the white portions of the clamps until it snapped into place attached the sandwich.

Combs were placed into the assembled gel sandwich until the teeth were about $\frac{2}{3}$ into the sandwich. A mark was made 1cm below the teeth of the comb on the glass plate. The separating gel was poured to this level. The combs were then removed (Anon, 1989).

2.2.4.2 Casting the Discontinuous Polyacrylamide Gel

The formulations for the preparation of the stock solutions are included in the Appendix. A 12% separating gel monomer (Appendix D31) was prepared by combining 6.7ml of distilled water, 5ml of a 1.5M TrisHCl pH 8.8 (Appendix D33), 200 μ l of 10% (w/v) SDS stock solution (room temperature), 8ml acrylamide/bis-acrylamide (30% stock Appendix D30), and 8 μ l of Temed in a clean beaker or Erlenmeyer flask and mixed thoroughly. This percentage gives good separation of the proteins in the 10,000 to 70,000dalton range. Gloves were worn at all times during the handling of the reagents because of the toxicity of acrylamide and Temed. Finally 200 μ l of ammonium persulfite (APS-10% solution prepared fresh Appendix D25) was added to the solution and mixed. The concentration of APS that was used was high to eliminate the need for degassing the acrylamide. Degassing means the removal of any dissolved oxygen from the acrylamide, which can retard polymerisation. The resolving gel solution was poured between the glass plates until the mark on the glass plate was reached. Water-saturated n-butanol (Appendix D32) was layered along the surface of the gel solution with a pipette. This prevented the oxygen from diffusing into the gel, thereby inhibiting polymerisation. The gel was allowed to polymerise, the n-butanol was removed and that space rinsed completely with distilled water. The excess fluid was removed with a blotting paper.

A 5% stacking gel (Appendix D35) was prepared by combining 2.57ml water, 0.83ml acrylamide/bis-acrylamide mix, 1.26ml of a 1M TrisHCl pH 6.8 (Appendix D34), 0.05ml SDS and 0.005ml Temed. The solution was mixed thoroughly, before adding 0.05ml APS. The stacking gel solution was poured on top of the resolving gel using a pipette. The 10-well comb was inserted and more gel solution added to fill the cassette completely. The gel was allowed to polymerize for 30-40 min at room temperature. After polymerisation, the combs were removed and the wells rinsed with distilled water and the gel was ready for loading (Anon, 1989).

2.2.4.3 Assembling the Upper Buffer Chamber

After the stacking gel polymerised, the clamp assembly/ gel sandwich was released from the casting stand. The inner cooling core was laid down on the bench. The clamp assembly with the screws was facing out while the wedges faced towards the top of the cooling core.

The clamp assembly wedges were slid underneath the locator slots on the cooling core until the inner plate of the sandwich was against the notch in the gasket. The clamp assembly was snapped until the cooling core latch engaged each side of the clamp assembly. The other clamp assembly/ gel sandwich was attached to the other side of the cooling core. The cooling core was placed into the lower buffer chamber of the electrophoretic cell. The upper and lower chamber was filled with 1% electrode buffer (Appendix D38). The samples were prepared for electrophoresis by diluting the sample with an equal volume of 2% SDS sample buffer and heating at 95°C for 5 min in a heating block. A protein molecular weight marker (Combithek Cat. no. 1317474 Boehringer Mannheim, GmbH, Mannheim, West Germany) was loaded into lane 1 using a gel-loading tip (Eppendorf, Netheler-Hinz. GmbH, Germany). Empty wells were loaded with an equal volume of 1% sample buffer to prevent spreading of adjacent samples during electrophoresis. The voltage was set at a constant of 130V for 1 hr and run under cold conditions. The electrophoresis was stopped once the tracking dye reached the bottom of the resolving gel. The protein gel cassette was removed from the apparatus and the buffer was discarded. The two plates were separated carefully and the orientation of the gel marked by trimming a corner of the gel so that sample order was not lost during the staining step. The gel was removed from the glass plate and stained for protein detection only or kept for transfer onto a nitrocellulose membrane (for immunoblotting without staining). The gel was placed in a staining gel dish containing Coomassie Blue staining solution (Appendix D36) for 30 min. The gel was destained with the destaining solution (Appendix D37) from 1 hr to 3 hr. The gel was then photographed. The gel was soaked in destaining solution containing glycerol and dried onto Whatman paper for long-term storage.

2.2.5 Preparation for Large Scale Protein Expression and Purification

Before proceeding with large-scale expression and purification, a small pilot experiment was run to establish optimal conditions for expression. A single colony of *E.coli* containing a recombinant pGEX plasmid was used to inoculate 150ml of LB broth containing ampicillin. The culture was incubated for 16 hr at 37°C with vigorous shaking. The next day the culture was diluted 1/100 into fresh pre-warmed LB broth and grown at 37°C with shaking until the culture reached an A_{600} of 0.6 to 0.8. For 1L of culture, a 2L flask was

used for proper aeration of the culture. Once the appropriate optical density was reached, 10ml of the culture was removed and growth was allowed to continue. To the remaining culture 1M IPTG (Sigma-Appendix D27) was added to a final concentration of 0.5mM to induce expression of the recombinant protein. The culture was transferred to appropriate centrifuge containers and centrifuged (8000g x 10 min) in a Beckman JA 21/20 rotor to sediment the cells. The supernatant was discarded and the pellet drained onto a paper towel. The pellet was re-suspended in 1/20 of the original culture volume with ice-cold PBS. The solution was placed on ice. The suspended cells were disrupted using a Branson sonicator with a suitable probe. The cells were disrupted with short bursts of 30 sec at 30 sec intervals at a Duty cycle of between 70-80% and an output of between 4-5 for a total of 3 min. Triton X-100 was added to a final concentration of 1% and the sonicate kept on ice for 30 min to aid solubilisation of the protein. Pefabloc (Pharmacia Biotech, UK-Appendix D39) was added to a final concentration of 5mM. The sonicate was centrifuged (10,000g) for 30 min at 4°C and the supernatant transferred to a clean container. An aliquot of the supernatant and the cell debris (pellet) was saved for analysis by SDS-page as described in 2.2.4.

2.2.6 Chromatography

Chromatography refers to the technique used in the separation and purification of one or more biological compounds from a mixture of such compounds. The selection of a chromatographic technique is based on the particular material to be isolated. In this study, the technique used was based on affinity chromatography, as proteins were the macromolecules that required purification and the unique property of biological specificity was being exploited (Williams and Wilson, 1979). All chromatographic separations depend upon the differential partition of solutes between phases, a mobile phase and a stationary phase. Such partition between two phases is described by the so-called partition coefficient or distribution coefficient. The distribution coefficient describes the distribution between any two phases, be it a liquid/ solid or gas/ liquid phase. In chromatography there is always a distribution between two such phases, one kept stationary while the other, the mobile phase, flows over or through it. In the situation with affinity chromatography, the stationary phase is the immobilised biomolecule that is packed into a tubular column and the mobile phase is liquid that flows through the packed column. Affinity chromatography exploits a specific relationship between a protein and a ligand, to select out the desired

protein from a crude mixture, in a single step. The ligand in question is glutathione, which is immobilized by conjugation to an insoluble matrix being agarose beads or resin, in a manner that does not interfere with its interaction with the protein. The resin or agarose beads have three parts to it, namely, the matrix that is linked to a ligand via a spacer arm of about six to ten carbon atoms. The sample solution is passed through the column and by its specific interaction with the immobilised ligand the protein of interest is retained, while all other proteins pass straight through the column (Dennison, 1999). The protein is subsequently eluted by a free competing ligand that is 50mM Tris HCl (pH 8.0) (Appendix D40) containing 10mM reduced glutathione (Sigma) (final pH 7.5 freshly prepared (Appendix D41) (Smith and Johnson, 1988). The protein of interest is usually immobilised in a small volume of agarose beads, therefore affinity chromatography columns are quite small. The volume of solution in which the protein occurs, is quite large and to pass this volume through the column in a reasonable time, while maintaining the linear flow rate within limits, the column is thus relatively wide [15mm in diameter] (Dennison, 1999).

2.2.6.1 Preparation of Glutathione Agarose for Purification of Fusion Protein

Fifty percent pre-swollen slurry of glutathione agarose beads (Sigma catalogue number G4510) was prepared by allowing the lyophilised powder (200ml/g) to swell for 2 hr (90-95% swelling occurs during this period). The gel was packed into an affinity chromatography column and washed with 10 volumes of water. The column was equilibrated with several column washes of ice-cold PBS. The suspension was allowed to pass through the 50% pre-swollen slurry of glutathione. The supernatant was passed through twice. The column was washed three times with 50ml ice-cold PBS. The fusion proteins were eluted with 1ml elution buffer (50mM TrisHCl pH8, 10mM reduced glutathione) with gentle mixing for 2 min. The elution step was repeated four times and the eluates pooled. The glutathione agarose gel was regenerated with 3M NaCl (Appendix D46) for re-use. The purity and relative molecular size of the recombinant proteins were analysed by SDS-PAGE (section 2.2.4). Yields of fusion protein were calculated using the Bio-Rad DC Protein Assay Kit II (Bio-Rad Laboratories: Life Science Group, USA) and bovine serum albumen as a protein standard.

2.2.6.2 Estimation of Protein Concentration

The Bio-Rad DC Protein Assay (Bio-Rad DC Protein Assay Kit II. Bio-Rad Laboratories, Life Science Group, USA) is a colorimetric assay for protein concentration following detergent solubilisation. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. Two steps lead to colour development: The reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein. Colour development is primarily due to the amino-acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine and histidine. Proteins effect a reduction of the Folin reagent by loss of 1, 2, or 3 oxygen atoms, thereby producing the characteristic blue colour with a maximum absorbance at 750nm and minimum absorbance at 405nm.

The protein standard was obtained in a lyophilized form. Sterile water was added to the bovine serum standard to obtain a concentration of 1µg/ml. Whenever the concentration of an unknown protein was required; it had to be measured against a known standard, and this standard was diluted with water to obtain a concentration of 100ng/µl. Dilutions of the protein standard were made starting from zero concentration (0µl) of BSA, 10µl, 20µl, 40µl, 60µl, 80µl and 100µl or 10,000ng. Dilutions of the unknown protein were made in a similar manner. 500µl of reagent A, an alkaline copper tartrate solution, was added to each tube. Reagent B, a dilute Folin Reagent (4ml) was thereafter added. The tubes were mixed and incubated in the dark at room temperature for 15 min. The absorbance of the protein samples were read at 750nm using a Beckman spectrophotometer. A standard curve of absorbance versus wavelength was drawn from which the concentration of the unknown protein was obtained.

2.2.7 Western Blot Analysis of Fusion Proteins

The major principle of Western Blotting is the interaction between a specific antibody and its target protein on the membrane. An antibody is a Y-shaped molecule containing two heavy chains; two light chains, and two identical antigen-binding sites or bivalency at the tip of two arms. These binding sites are specific for a particular antigen. It is the antigen-binding sites that allow an antibody to cross-link the antigenic determinant of the target antigen or protein in immunoblotting. Many antigens have multiple antigenic determinants

or epitopes that enable the antigen to form linear chains or cyclic complexes with the antibody (Wu *et al*, 1997).

Western blotting (Towbin *et al*, 1979) is a technique whereby proteins that have been electrophoretically separated, are transferred from a gel to an insoluble matrix (nitrocellulose membrane) and probed with reagents that are specific for particular sequences of amino acids. Probes that are utilized for the detection are antibodies that react specifically with antigenic epitopes displayed by the target protein attached to the nitrocellulose. Electrophoretic separation of proteins is always carried out under denaturing conditions whereby problems of solubilisation, aggregation and co-precipitation of the target protein with adventitious proteins are eliminated. Western blotting is extremely useful for the identification of specific proteins in complex mixtures and simultaneously determining its molecular weight.

2.2.7.1 Method

Fusion proteins that were separated using SDS-page in section 2.2.4 were electrophoretically transferred from the gels onto nitrocellulose membranes (MSI-Micron Separations Inc. Catalogue number NJOHY00010) using a trans-blot semi-dry electrophoretic transfer cell (Bio-Rad Laboratories, Inc, USA). A piece of nitrocellulose membrane and six pieces of 3mm Whatman paper, the size of SDS-page gel was cut and equilibrated in transfer buffer (Appendix D42) for 10 min. One corner of the filter paper was marked with a soft lead pencil. The gel and the nitrocellulose membrane were sandwiched between the layers of the filter paper. This sandwich was placed between graphite plate electrodes, with the nitrocellulose filter on the anodic side. A glass roller was used to squeeze out any air bubbles or lumps in the sandwich. The area of the gel was calculated and a current of $5\text{mA}/\text{cm}^2$ was applied for 1 hr. During the transfer, ice packs were placed on top of the transfer apparatus to prevent it from overheating. After transfer, the gel was stained with Coomassie Brilliant Blue G 250 as described previously in section 2.2.4.3 and de-stained to ensure that the transfer was complete.

2.2.7.2 Immunoblot Detection

For immunoblot detection of the transferred membrane, the nitrocellulose membrane was blocked in 5% w/v solution of Elite non-fat milk powder in 0.1% PBS-Tween (Appendix D43) with gentle rocking for 1 hr. Blocking of membrane is to seal potential binding sites to reduce background of non-specific binding of irrelevant proteins with antibody. The membrane was washed twice with phosphate buffered saline containing 0.05% (w/v) Tween 20 (Appendix D44). It was then incubated overnight with the primary antibody (goat anti-GST or Mason Pfizer Monkey Virus (MPMV) diluted 1/1000 in PBS-Tween 20 with gentle agitation on a rocking platform at room temperature. This antibody binds directly to the antigen. The membrane was washed as before (2 x 15min) with PBS-Tween and incubated with the secondary antibody, an enzyme conjugate, anti-goat peroxidase conjugate (Promega Corporation, Madison, USA) diluted 1/1000 for 1 hr at room temperature in blocking buffer. This secondary antibody interacts with the first antibody. The membrane was washed as before and then immersed in a substrate solution (4-chloro-1-naphthol in methanol and peroxidase-Appendix D45). This substrate detects the complex formed, that is, the protein-primary antibody-secondary antibody which is formed on the blotted membrane (Wu *et al*, 1997). The membrane was incubated in the dark for 20 min or until bands were clearly visible against a lightly stained background. Once the bands developed, the membrane was rinsed with deionised water, and dried and mounted onto Whatman paper and then photographed.

2.2.8 Enzyme-linked Immunosorbent Assays (ELISAs)

ELISAs are heterogenous assays that were first described in 1971 by Engvall and Perlman and Van Weemen and Schuurs to detect antigen (Ag) or antibody (Ab) presence in variety of biological samples (Engvall and Perlman, 1971; Van Weemen and Schuurs, 1971). Many variations in the methodology of the ELISA have evolved since its inception in the 1960's, but the basic concept is still the immunological detection and quantification of a single or multiple Ag or Ab in a patient's sample (usually serum). The ELISA technique employs the sensitivity of a spectrophotometric assay of an enzyme and the specificity of antibodies. For this purpose, a stable enzyme that can be easily measured and which has a high turnover number is covalently linked to an immunoreactive molecule (antibody or antigen). Thus, when an Ab binds with its target molecule, it carries with it an enzyme

“label”. By measuring the amount of enzyme associated with the target molecule, a measure of the amount of target molecule present is available (Barker *et al*, 1993).

A number of methods are available for the performance of ELISAs, and are classified as indirect, competitive, sandwich or capture assays. The assays are conducted in microtitre plates containing 96 wells or strips. A typical assay consists of the antigen or antibody attached on a solid phase (support), and conjugate and substrate detection system. The solid support can be 96 well microtitre plates, strips, plastic beads, polyvinylchloride or polystyrene surfaces. Polyvinylchloride microtitre plates are considered a more suitable surface for protein binding in the standard EIAs, competitive and sandwich assays that have been used in this study. Microparticles and beads have increased surface areas and can therefore absorb larger quantities of antigen

2.2.8.1 Indirect Enzyme-linked Immunoassay

The most often used assay is the standard indirect ELISA. In the standard assay GST-JSp26 protein was bound overnight at 4°C onto Nunc microorb strips ((Nunc-Immuno Module MaxiSorp F8 Frame, catalogue no. 469949 NUNC Brand products, Denmark). A 1/5 dilution (125µg) of the antigen in question was made in 0.05M carbonate bicarbonate buffer pH 9.6 (Sigma) and added in a serial dilution starting from 1/5 (25µg) to 1/640 (0.19µg). The following day the excess antigen was shaken off, and washed three times with one times wash buffer (20% Genelavia wash buffer used at a 1/10 dilution). The wells were blocked with 200µl of blocking buffer (5% Elite milk powder in 1% washing buffer (20 X Genelavia wash buffer) for 30 min at 37°C. Excess blocking buffer was removed and 100µl of a 1/5 and 1/10 dilution of each antibody sample were added to each vertical row. The reaction was incubated at 37°C for 1 hr and washed as before using the PW40 (Sanofi Diagnostics Pasteur, France)) microtiter plate washer. Horseradish peroxidase labelled Protein G conjugate (100µl-Sigma-Aldrich, USA) diluted at a concentration of 1/4000 was added. After washing, 50µl of OPD substrate (10 mg in 0.1M sodium citrate buffer pH 4.5 and 6µl H₂O₂) was added and incubated in the dark for 5 min. 0.05M Sulphuric Acid was added to stop the reaction, which was read at a wavelength of 492 nm and 630 nm using a Bio-Rad 580 Plate reader (Bio-Rad Laboratories, Inc, USA).

2.2.8.1.1 Use of Indirect ELISA to Determine Specificity of the GST-JSp26 Fusion Protein

Microtitre plate wells were coated with GST-JSp26 (0.250 μ g) and GST-HIVp24 (0.650 μ g) antigens in a serial dilution starting at 1/10 (12.5 μ g) down each vertical row and ending at 1/1280 (0.097 μ g). Adsorption of the antigen occurred by immobilization onto the walls of the plate. The enzyme-linked immunoassay was conducted in a manner similar to that outlined in 2.2.8.1, except serum from a naturally affected sheep named Bruwer was used at a dilution of 1/10 and titrated across the plate, with the most concentrated solution to the left of the plate and the most dilute to the right (1/320).

2.2.8.1.2A Competitive Enzyme-linked Immunoassay

Two strips of microtitre plate wells were each coated with a 1/10 dilution (12.5 μ g) of GST-HIVp24 and GST-JSp26 antigen, respectively. The ELISA protocol adopted here was the same as in section 2.2.8.1, except for the antibody step using antiserum from a sheep naturally infected with JSRV (Bruwer anti serum), where there were a few changes. To the first well of each strip 100 μ l of a 1/5 dilution of antibody was added. The rest of the wells had 100 μ l of blocking antibody added. A 1/20 dilution of GST-HIVp24 antigen (10 μ l of 0.65 μ g/ μ l antigen in 190 μ l of blocking buffer) was added as a competitor in a serial dilution vertically down the rows.

2.2.8.1.2B Competitive Enzyme-linked Immunoassay

In this experiment, five rows of microtitre plate wells were coated with a 1/10 dilution of GST-JSp26 (0.25 μ g/ μ l) and five with a 1/30 dilution of GST-HIVp24 antigen (0.65 μ g/ μ l) as outlined in section 2.2.8.1. Two different tests were carried out. The only difference is in the antibody stage of the ELISA test.

1. GST-HIVp24 was added as a competitor at a 1/100 dilution (0.65 μ g/ μ l) to only one row each of the antigens (100 μ l). To the same row Bruwer antiserum (100 μ l) was added serially starting at a 1/5 dilution till 1/640. The second row of each antigen had only 100 μ l of blocking buffer added.

2. Two different antibodies, namely, MPMV and R3 were used. MPMV used at a starting dilution of 1/2000 till 1/256000. R3 was used at a dilution starting at 1/10 and going down to 1/1280.

Both the above two ELISA tests were completed as in section 2.2.8.1.

2.2.8.2 Preparation and Use of an In-house Conjugate Using GST-JSp26

2.2.8.2.1 Preparation of In-house Enzyme-labelled Conjugate

The second assay used in the study was the antibody capture assay. For this assay, an in-house enzyme-labelled conjugate was prepared using Pierce's E-Link™ Plus activated Peroxidase Kit (31487). One mg of affinity purified GST-JSp26 protein was added to 0.5ml of conjugation buffer (0.2M Buph Carbonate Bicarbonate Buffer pH 9.4). The protein sample was added to 1mg of lyophilised E-Z link™ Plus activated Peroxidase that had been reconstituted with 100µl of water. The solution was incubated at room temperature for 1 hr. To this mix 10µl of Reductant Solution (Sodium cyanoborohydride- Na-CNBH_3) was added to the protein solution with a further incubation of 15 min at room temperature. Quenching Buffer Solution (20µl) was added and the protein solution incubated as before.

The prepared conjugate solution was desalted using the Pharmacia desalting columns. The top cap of the column was removed and the excess liquid poured off. The bottom cap was removed and the column was supported using a special rack. The gel was equilibrated with three column washes of water (3% x 5ml). The equilibration buffer was allowed to completely enter the gel bed. The sample was added to the column in a maximum volume of 1ml (sample was made up to 1ml with distilled water). The conjugate was diluted with 1.5ml-distilled water. To this an equal volume (1.5ml) of 2x PBS was added. For long-term storage at -20°C , glycerol to a final concentration of 50% (3ml) was added to the conjugate.

2.2.8.2.2 Indirect ELISA to Determine Working Dilution of Conjugate

Before using the conjugate (JSp26-HRP), it was titrated to obtain that dilution that gave the best signal to noise ratio. A 1/50 dilution was made and titrated 50-fold from 1/50 to 1/3906250. GST-JSp26 protein ($0.25\mu\text{g}/\mu\text{l}$) was titrated from 1/5 to 1/640 in coating buffer and incubated overnight at room temperature. The plates were washed as before and then blocked with $200\mu\text{l}$ of blocking buffer at 37°C for 30 min. The washing procedure was followed as before (section 2.2.8.1). The MPMV antiserum was used at a dilution of 1/50 and incubated at 37°C for 1 hr. After washing the plates, $50\mu\text{l}$ of substrate (HRPO substrate-one tablet to 10ml of 0.03% H_2O_2 substrate) was added to each well. The plate was incubated for 5 min at room temperature in the dark. The reaction was stopped with 0.05M Sulphuric Acid and the plates read at 492 nm.

2.2.8.2.3 Antibody Capture ELISA

The principle behind the antibody capture assay or the antibody sandwich assay is that an antigen is coated onto the microtitre plate; this is followed by the addition of the antibody to the plate. Should there be a reaction between the antigen and the antibody this complex is detected by an antigen that is labelled with for example horseradish peroxidase. Addition of the substrate would light up the wells positive containing the antigen/antibody/antigen complex. The principle is that one arm of the antibody binds tightly to the antigen on the microtitre plate whilst the other arm is free to be bind to another antigen that has been labelled with an indicator enzyme. In this way one by passes the need to use an indirect conjugate step.

Once the working dilution of the in-house conjugate (JSp26-HRP) was determined, an antibody capture ELISA technique was used against infected and non-infected JSRV samples. The wells were coated with $100\mu\text{l}$ of GST-JSp26 antigen in 0.05M carbonate bicarbonate pH 9.6 at a dilution of 1/80 ($0.39\mu\text{g}/\text{well}$). After an overnight incubation at room temperature, the plates were washed and blocked as before for 30 min at 37°C . Eight samples negative for JSRV, and eight positive for JSRV were tested. All samples were tested at a 1/50 dilution. The conjugate (JSp26-HRP) was also used at a 1/50 dilution. Horseradish peroxidase substrate was used and the reaction stopped with 0.05M Sulphuric-acid and the test read at a wavelength of 492nm.

This antibody capture assay was utilized against a batch of 39 JSRV positive sera and 41 JSRV negative sera and the test performed as in section 2.2.8.1. These positive and negative sera were obtained from the Onderstepoort Veterinary Research Institute. The positive sera were obtained from sheep diagnosed with OPA upon post-mortem.

2.2.8.3 Indirect ELISA Utilising Monoclonal Antibodies

Nunc microsorp microtitre plates were used. The wells were coated with different antigens at a 1/10 and 1/100 dilution. These antigens were lung lavage samples from affected and normal sheep that were hundred times concentrated and used at two different concentrations to cover a range of dilutions. The dilutions were made in 0.05M Carbonate bicarbonate buffer and the plates incubated overnight at room temperature. The following day the strips were washed three times with BioWhittaker 20% wash buffer used at a 1% final concentration. Blocking buffer (wash buffer with 5% Elite fat free milk powder) was used as a blocking reagent to block the non-reactive sites on the plate, membrane or tissue and reduce non-specific binding of proteins during subsequent steps in the assay. Blocking buffers improve the sensitivity of the assay and reduce background interference (Pratt and Roser, 1984). Further they have no active part in the specified immunochemical reaction of a particular assay. The plate was blocked at 37°C for 30 min. Excess blocking buffer was removed and the monoclonal antibody 59G11 was diluted at 1/50, of which 100µl was added to each well. The plate was incubated at 37°C for 1 hr. After the plate had been washed, a goat anti-mouse HRP conjugate (Sigma) was used at a dilution of 1/3000. 100µl of conjugate was added per well and the plate incubated as before. The substrate (OPD) was added and incubated at room temperature for 30 min. Later the reaction was stopped as above and read at 492 nm using a Bio-Rad 580 plate reader.

2.2.8.4 Gold Conjugates

An additional method utilised in this study, was gold labelling. Following the use of gold colloids as a marker in 1971, gold labelling has become an important tool for the detection and quantification of proteins, antigens, and nucleic acids. Gold labels can also be used with numerous techniques such as blotting (nitrocellulose or polyvinylidene difluoride). Recently, gold conjugates have been incorporated into rapid test immunoassays. In these techniques the unique red colour of the accumulated gold label, when observed by lateral or transverse

flow along a membrane on which the antigen is captured, or by observation of the red colour intensity in solution, provides an extremely sensitive method for detecting sub-nanogram quantities of proteins in solution. Gold conjugates act as markers for molecules that are otherwise invisible by eye or through other detection systems. A colloidal gold conjugate consists of a suspension of gold particles coated with a selected protein or macromolecules (such as an antibody). The gold particle may be manufactured to any chosen size from 1-250nm. This gold conjugate when incubated with a specific target will reveal the target through the visibility of the gold particles themselves. For detection by eye, gold particles will reveal immobilized protein on a solid phase, such as a membrane, through the accumulation of the gold at the reaction site. This is observed as an increasingly intense red band. The protein bands are easily separated. It appears silver enhancement of this gold precipitate can also further enhance the sensitivity of detection (Anon, 2000).

The conjugation of selected proteins to gold particles depends upon at least three physical phenomena: charge attraction of the negative gold particles to positively charged protein, hydrophobic absorption of the protein to the gold particle surface and binding of the gold to sulphur (dative binding) where this may exist within the structure of the macromolecule. The condition under which a gold conjugate is manufactured affects its performance and stability. Firstly, all antibodies and proteins used for conjugation should be affinity purified and of the highest quality. They must possess very strong affinity for the specific antibody or antigen and have high avidity to withstand severe incubation and washing conditions. Cross-reactivity must be the lowest possible. Gold conjugates should be consistent from batch to batch and give the same high performance time after time. The gold conjugate should produce a strong specific signal together with a low background. For this to occur, the conjugation of the antibody to gold particles must be complete and stable under a variety of incubating conditions. The gold particle must have the minimum effect on the activity of the antibody to which it is conjugated, but must be strongly enough absorbed to the surface to remain stable for years. Gold conjugates should be capable of being frozen without loss of the antibody or antigen activity (Anon, 2000).

The question is, why use gold? Well, there are several advantages. Less than 10pg of protein is detected with each gold conjugate. 1nm gold conjugate produces even higher sensitivity. Primary antibodies can therefore be used at high dilutions. The optical density

of 20nm gold conjugate at 520 nm is 4 OD. For 1nm gold conjugate, the optical density is not measurable as it is a clear liquid. The 1nm gold conjugate has a protein concentration of 50µg/ml. A low background is obtained and crisp well-defined bands can be seen which ensures accurate identification. The specific labelling produced in this way can be compared and quantified with a total protein stain obtained from a duplicate sample analysed in parallel. This is a much more accurate technique for protein identification on membranes compared with Coomassie blue method for staining gels where distortion can occur between the gel and the membrane during protein transfer (Anon, 2001).

While fluorescent labels and enzyme based colour reactions may fade with time and light exposure, gold particles do not disappear, but give a permanent label (Moeremans *et al*, 1984).

The conjugation of the rabbit viral capsid antibody to gold was performed in an outside laboratory (MDS Medical Diagnostic Services, Westville, KwaZulu-Natal).

2.2.9 Antibody Production

Antibodies are proteins made by the immune system of animals as part of a defence mechanism against infection by foreign organisms. Antibodies play a role in being able to distinguish 'self' and 'non-self'. Because of their specificity and versatility, antibodies are useful reagents in identifying and analyzing proteins (Dennison, 1999).

The injection of a foreign molecule, usually a protein into an animal will elicit an immune response that includes the products of antibodies that react with the foreign protein and target it for removal from the system. The foreign protein is called the immunogen and the antibody produced in response to this protein will react with a molecule called the antigen. The foreign protein used to be known as the antigen, but some molecules that are antigenic (react with antibody) may not be immunogenic (that is, they will not elicit an antibody response when injected into an animal). A single injection of an immunogen is not usually effective in eliciting an immune response. In the case of a natural infection, molecules from the infecting agent will diffuse from the site of an infection to become exposed to the immune system in small amounts, but over a prolonged period. This natural process has to be copied to elicit an antibody response. Making an emulsion of the aqueous antibody

solution with adjuvant oil does this. An adjuvant releases immunogen slowly in small amounts for exposure to the immune system over a period of time. The emulsion is made by a process known as trituration and is injected into the breast muscle of a chicken or subcutaneously in a rabbit. The injected emulsion will exist at a focal site, and as it gradually breaks down, it will be released slowly and thus be exposed to the immune system. This is the principle of Freund's incomplete adjuvant.

2.2.9.1 Immunisation of Chickens to Produce IgY JSRV p26 Antibodies

In this study we decided to use hens for antibody production because it is not necessary to bleed them in order to harvest the antibodies. Further, in a study by Polson *et al* (1980), he showed that the production of antibodies in rabbits did not differ from that in hens when the titre of IgY was examined. This was observed when the two species were immunised and hyperimmunised simultaneously. It was also shown that the molecular weight of the antigen is important in stimulating antibody production as well as in the visibility of the reaction between antigen and antibody. The term IgY denotes immunoglobulin in hen serum as well as in yolk (Polson *et al*, 1980). Antibody transfer occurs via the egg yolk, which provides a convenient source from which antibody may be isolated.

2.2.9.1.1 Preparation of Immunogen for Immunisation

The immunogen used was GST-JSp26 that had been affinity purified and the concentration of which had been determined using the Biorad DC Protein Assay (Bio-Rad Laboratories, Inc, USA). 100µg of GST-JSp26 equivalent to 200µl was made up to 1ml with sterile PBS (phosphate buffered saline) and this was pipetted into a 10ml beaker. An equal amount of Freund's complete adjuvant, an attenuated *Mycoplasma* spp, live, but not virulent, (Sigma Aldrich, USA) was added to the immunogen/PBS mix to form an emulsion by a process referred to as trituration. Briefly, a sterile syringe was used, the plunger of which is removed and the base lubricated with some of the adjuvant. The inside of the syringe is also lubricated so that the adjuvant does not adhere to the sides of the syringe when it is used to emulsify the immunogen-mix. Drawing the sample up into the syringe and then releasing it mixed the sample. This is continued until the mixture is emulsified sufficiently in the 10 ml beaker. Once the sample is ready, as much as possible is drawn into the syringe. The syringe is turned in such away that the sample tends to move in the direction

of the plunger. The plunger is moved slowly so that the sample is moved to the top until all the air bubbles have been released. The sample is now ready for immunisation (Polson et al, 1980).

2.2.9.1.2 Immunisation of Chickens

Hens of between 12-14 weeks were kept in isolation for immunisation purposes and egg laying production at the Ukulinga Research Farm that is part of the Faculty of Agricultural Sciences of the University of KwaZulu-Natal, Pietermaritzburg. The breed of hens used was Hyaline Brown. Prior to the initial immunization, eggs were collected on a daily basis. IgY antiserum from these eggs constituted the baseline IgY antibodies and has no reaction to the immunogen in question that is GST-JSp26. This baseline antiserum is equivalent to pre-bleed sera in rabbits.

The prepared emulsion was used immediately by injecting 50µg of immunogen intramuscularly on either side of the breastbone of two hens Y3 and Y4. Hens Y3 and Y4 were immunised with about 0.900ml of sample per hen. The area that lies to the left and right of the breastbone that is free of feathers is the muscular part of the body of the hen. Before injecting, the area is swabbed with a disinfectant and then the needle is plunged into the muscle. The needle is not pulled out directly, but turned round and round about five times, so that it could be pulled out easily from the muscle. The chicken is returned to the cage and fed. Thereafter eggs were collected on a daily basis and kept at 4°C. These eggs provided the 2-week post-inoculation antisera.

After two weeks, a booster injection was given using a similar amount of immunogen together with Freund's incomplete adjuvant. Booster injections are essential to obtain antisera of high titre and avidity (good fitting antibody).

Two weeks after the first injection, chicken Y3 developed swellings on either side of the breastbone, but this was not considered a problem. Chickens were immunised a month later, as well as a further month and 10 days thereafter. Eggs were collected on a daily basis from the time of immunisation.

2.2.9.1.3 Procedure for the Isolation of IgY from Chicken Egg Yolks

Eggs were separated from the egg whites and washed carefully under running water to remove all traces of albumen. The yolk sac was punctured and the yolk released into a measuring cylinder, so that the volume of yolk could be determined. Two volumes of 100mM Na-phosphate buffer pH 7.6 was added to the yolk and thoroughly mixed. Solid PEG (Mr 6000) was added to 3.5% (m/v) and dissolved by gentle stirring. PEG was used for the fractionation of the egg yolk (Polson *et al*, 1980). The precipitated vitellin fraction was removed upon centrifugation at 5000rpm for 30 min at room temperature by filtering the supernatant through absorbent cotton wool to remove the lipid fraction. The PEG concentration was increased to 12% by adding 8.5% (m/v) to the supernatant. The solution was mixed thoroughly and centrifuged at 10,000 rpm for 15 min at room temperature. The supernatant was discarded and the pellet dissolved in PBS buffer in a volume equal to that obtained after filtration. The final PEG concentration in the solution was increased to 12% (m/v). The solution was mixed thoroughly by stirring and centrifuged as before. The supernatant fluid was discarded and the final antibody pellet dissolved in 1/6 of the original egg yolk volume using PBS pH 7.6 (Polson *et al*, 1980; Polson *et al*, 1985).

2.2.9.1.4 Collection and Storage

The antibody was stored in the presence of 0.02% Sodium Azide at 4°C. The Sodium Azide was used to preserve the IgY.

2.2.9.2 Polyclonal Antiserum used in Enzyme-linked Immunoassays

2.2.9.2.1 Effectivity of Polyclonal Antiserum in an Immunoblot Assay

SDS-PAGE gel was prepared as in section 2.2.4. GST-JSp26 (100µg) was run on a prepared 12% SDS-PAGE gel. The following antisera were used:

Y3 (BASELINE 1/11/1998)

Y3 (WEEK 1-7/11/1998)

Y3 (WEEK 2-12/11/1998)

Y3 (WEEK 3-21/11/1998)

Y3 (WEEK 4-29/11/1998)

Y3 (WEEK 5-06/12/1998)

Y3 (WEEK 6-13/12/1998)

Y3 (WEEK 8-25/12/1998)

MPMV

ANTI-GST

The immunoblot assay was performed as described in section 2.2.7.2. Anti-chicken antibody (Sigma Aldrich, USA) was used as the enzyme-labelled conjugate for the antiserum prepared in chicken and Protein-G (Sigma Aldrich, USA) for the others.

2.2.9.3 Use of Polyclonal Antiserum used in Enzyme-linked Immunoassays

2.2.9.3.1 Indirect Enzyme-linked Immunoassay

Wells of nunc microtitre plates (Nunc-Immuno Module MaxiSorp F8 Frame, catalogue no. 469949), NUNC Brand products, Denmark) were first coated with GST-JSp26 antigen ($9\mu\text{g}$ or $22.5\mu\text{l}$ in $1777.5\mu\text{l}$ of 0.05M carbonate-bicarbonate buffer pH 9.6 starting with $1\mu\text{g}/\text{well}$ in the first row and serially diluted across the plate, with the most concentrated antigen to the left of the plate and the least concentrated towards the right. After blocking, antiserum from the eighth week after inoculation was used as antibody at a $1/10$ dilution. The antibody was added in a serial dilution down the plate with the most concentrated in the top row and the most dilute at the bottom ($1/10$ - $1/1280$). The protocol followed was that of section 2.2.8.1.

2.2.9.3.2 Use of the Different Antisera Extracted against GST-JSp26 Fusion Protein

Wells of nunc microtitre plates were coated at $10\text{ng}/\text{well}$ (results obtained from indirect immunoassay section 2.2.9.3.1). Wells were blocked, antisera from section 2.2.9.2.1 were diluted $1/5$ and added in a serial dilution down the plate. The method thereafter was as per section 2.2.8.1.

2.2.9.3.3 To Determine the Effect if any upon the addition of GST to the Antiserum in an ELISA

Two rows of wells of nunc microtitre plates were coated at 10ng/well for both GST-JSp26 and GST-HIVp24 and blocked as in section 2.2.8.1. Antiserum (IgY GST-JSp26) extracted from the fifth week (6/12/1998) was used. The antiserum was treated as follows and added to the test wells as presented in Table 2.5.

Table 2.5 Different Concentrations of GST (1.6µg/µl) Added to a Constant Amount of GST-JSp26 Antibody (IgY)

ROW	A	B	C	D	E	F	G	H
AB ¹	1/50	1/50	1/50	1/50	1/50	1/50	1/50	Blocking buffer
GST	10 µl	5 µl	1 µl	1 µl of 1/5 dil.	1 µl of 1/25 dil.	1 µl of 1/125 dil.	1/50 of AB only	Added only
Total volume	500 µl	500 µl	500 µl	500 µl	500 µl	500 µl		

1: GST-JSp26Antibody-fifth week after immunization (6/12/1998).

The above preparation was incubated at 37°C for 10 min. This was to allow the GST to bind to the GST-JSp26 antiserum. 100µl of this preparation was added to the wells and the protocol for ELISA tests followed as in 2.2.8.1.

2.3 RESULTS

2.3.1 Extraction of DNA from Sheep Tissue

There are a number of protocols available for the isolation of genomic DNA as mentioned in 2.2.1.1. In this study the two protocols that were used (FastPrep and Salting Out methods), were selected for their ease of use and availability of reagents. Genomic DNA was isolated from normal lung (obtained from the KwaZulu-Natal Abattoir based in Cato Ridge) using the Salting out method. The FastPrep method was used in the isolation of genomic DNA from sheep muscle tissue of three Australian sheep (LPW, MSCU and 230909). The yield of genomic DNA obtained for the different tissue is presented in the following Table 2.6. A 1/50 dilution (10µl of DNA in 490µl water) of the DNA was added to a glass quartz cuvette and measured using a spectrophotometer (Beckman Du® 5

spectrophometer from Beckman Instruments Inc. Fullerton, California). Water was used first to blank the spectrophotometer. Readings were made in duplicate and the average taken as the corrected optical density reading. A value of 1.8 –2.0 obtained as a 260/280 ratio was indicative of pure genomic DNA.

Table 2.6 Genomic DNA extracted from four sources of sheep tissue

Tissue	Extraction	Starting amount	OD 260	OD 280	Ratio 260/280	Concen.µg/ml or ng/µl	Total Yield(µg)
LPW	Fast Prep	8x200mg	0.182	0.094	1.94	455	182
MSCU	Fast Prep	8x200mg	0.038	0.021	1.8	95	38
230909	Fast Prep	8x200mg	0.112	0.060	1.6	280	112
Normal lung	Salting Out	2x100mg	0.153	0.082	1.86	382.5	153

The genomic DNA isolated was used as template DNA in PCR reactions with JSp26 and JSorf-X primers. Other sources of DNA were also used such as JS 382 plasmid (cloned gag segment of a South African strain of JSRV used as a positive control), JS DNA 7 (A cell line derived from a JS infected Scottish sheep provided by Professor J. De Martini) and JS 4844 (lung tissue from a natural case of exogenous OPA). These samples were obtained from Dr D.F. York (Department of Virology, University of KwaZulu-Natal; Nelson R. Mandela School of Medicine). The cloned DNA was used as positive controls for exogenous JSRV. 3 Australian sources of sheep muscle DNA viz. LPW, MSCU and 230909 were also included as templates. In 1997 South Africa imported full sheep carcasses without the offal from Australlia and this was the only available tissue at that time. DNA was isolated from these tissues to investigate for the presence of endogenous JSRV.

2.3.2 Amplification JSp26 gag gene

DNA from JS 382, JS DNA 7, JS 4844, Normal sheep lung, and 3 Australian sources of sheep DNA viz. LPW, MSCU and 230909 were used as templates together with forward primer JSp26*Bam*H1 (5'tataggatccccgtcttcgaaaataacaac 3'-position, 1035-1053) and reverse primer JSRTp26*Bam*HI (5' ggatcctgcatagcaatgcctgca 3', position 1699-1679) in an optimised PCR reaction to amplify a 653 bp fragment. The amplified fragments are presented in Figure 2.1. The primers used here, amplified a highly conserved region

corresponding to the capsid region (CA) of the *gag* gene of JSRV. This protein is recognised by anti-MPMV p27 serum (York *et al*, 1992). The sequences of the capsid proteins obtained from amplification of endogenous JSRV (Normal lung, LPW, MSCU and 23909) and exogenous JSRV (JS 382, JS 7 and JS 4844) DNA were analysed and aligned with the published sequence of a South African strain of JSRV (York *et al*, 1992).

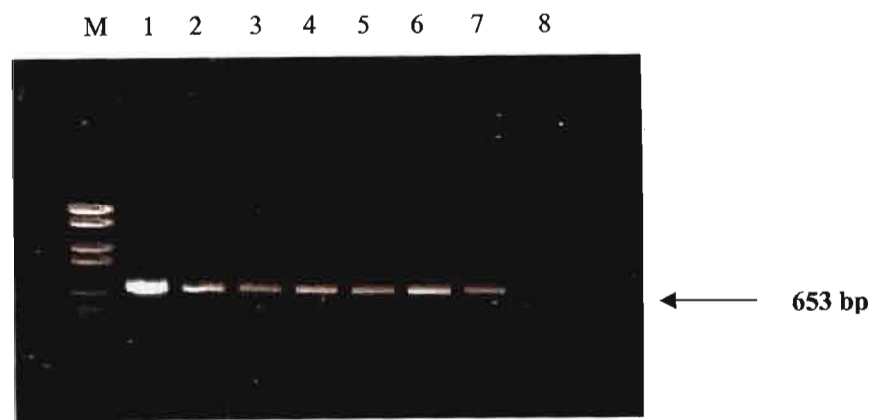


Figure 2.1 An ethidium bromide stained agarose gel (0.8%) analysis of JSp26 PCR products (653bp) from endogenous and exogenous sources of DNA

Lanes M. Molecular weight marker VI (Boeringher Mannheim), 1. JS 382 plasmid, 2. DNA 7, 3. sheep 4844, 4. Normal lung, 5. Normal Australian Sheep DNA LPW, 6. Normal Australian Sheep DNA 230909, 7. Normal Australian Sheep DNA MSCU and 8. Negative

2.3.3 Cloning of JS p26 fragments into a T-A Cloning Vector

The PCR amplicons were purified using either the Wizard DNA PCR purification (Promega) or Qiagen DNA PCR purification system. These fragments were purified to remove all traces of Taq DNA Polymerase as well as any other contaminants. The purified products were cloned into the pMOSS T-A cloning vector based on the assumption that Taq DNA Polymerase adds a single 3'A nucleotide onto each amplicon. This 3'A overhang is used to ligate the amplicon to a compatible single T nucleotide overhang present on the vector. Once ligated, the recombinant plasmids were then transformed into the highly efficient pMOSS Blue competent cells. As mentioned in section 2.2.1.11 the 653 bp fragment amplified from plasmid JS 382 was used as an example throughout this study.

2.3.3.1 Selection of Recombinant Clones

A quick assay used to screen for recombinants is by direct colony PCR. A typical direct colony screen of JSp26 is presented in Figure 2.2. This reaction was performed according to the manufacturer's protocol (Amersham Pharmacia Biotech).

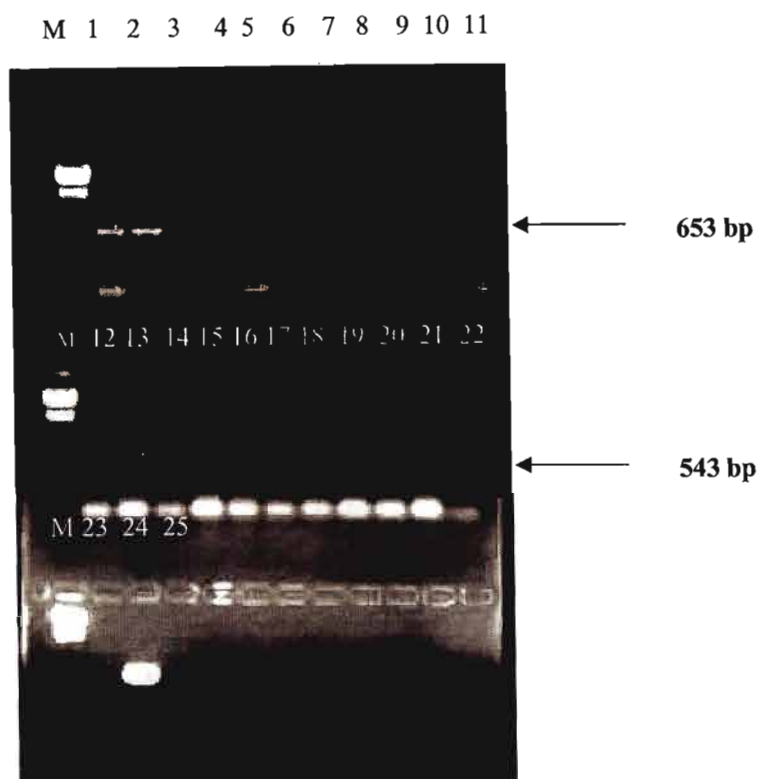


Figure 2.2 An ethidium bromide stained agarose gel (0.8%) analysis of 24 transformed colonies that have been screened to identify those that contain the JSp26 recombinant fragment

Lanes M. Marker II (Boehringer Mannheim), 1-23. Clones screened to amplify fragment, 24. Positive PCR product and 25. Negative colony

2.3.3.2 Screening of Clones by Alkaline Lysis Method

Those recombinant plasmid colonies that were positive upon amplification were screened further, using the alkaline lysis method. The purified plasmid DNA was subjected to restriction digest using *Bam*HI restriction enzyme (see Figure 2.3). The pMOSS linearized vector is 2887 base pairs in length. The cloned fragment is 653 base pairs in size (with *Bam*HI restriction sites at each end). To ensure that this is the correct recombinant clone and more importantly that the insert was in frame with the vector, the plasmid DNA was

sequenced. The sequence obtained in text format has been compared to that published by York et al, 1992 and stored in the Genbank (data not shown).

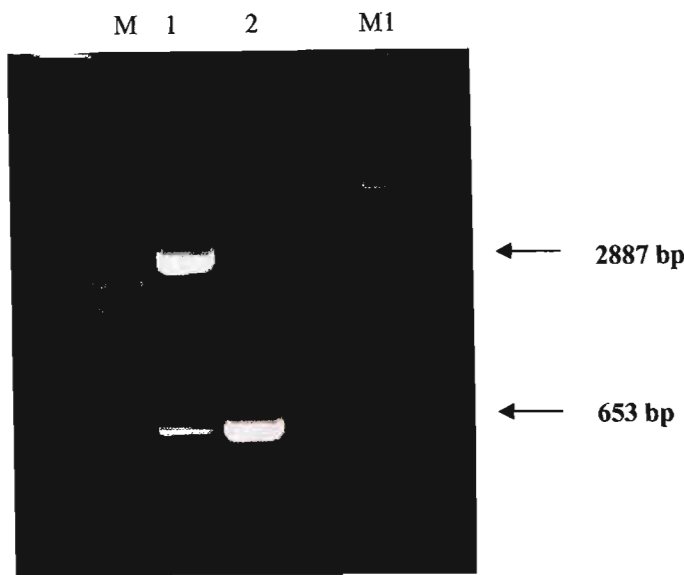


Figure 2.3 An ethidium bromide stained agarose gel (0.8%) analysis of the products obtained from the *Bam*HI restriction digest of pMOSS JSp26 plasmid showing the pMOSS linearized vector (2887bp) and the digested p26 fragment (653 bps)

Lanes M. Molecular Marker V1 (Boehringer Mannheim), 1. Restriction Digest of pMOSS JSp26 with *Bam*HI, 2. JSp26 PCR fragment, and M1. Molecular Marker 11 (Boehringer Mannheim)

2.3.4 Large Scale Isolation of pMOSS JSp26 DNA and Restriction Digest

A large-scale plasmid culture was purified using the Qiagen Maxi-Plasmid isolation kit (Southern Cross Biotechnology) (methods 2.2.1.11.1). To prepare the insert for cloning, the purified plasmid DNA (10ug) was digested with *Bam*H1 overnight in a 37°C incubator and run on a 0.8% agarose gel. The result of this electrophoretic run is not shown here.

2.3.5 Cloning of the Digested JSp26 Capsid Protein into pGEX-1 Expression Vector

The circularised expression vector was linearised by digesting the plasmid with restriction enzyme *Bam*H1. This linearised pGEX-1 vector was dephosphorylated to ensure that the vector does not re-circularise in any ligation reaction. A series of ligation experiments with

the vector ensured that the vector was able to participate in a ligation reaction without re-circularising. The linearised vector together with the insert DNA (JSp26) were added to a 0.5µl Eppendorf microcentrifuge tube with 1x ligase buffer and 10 units (1µl) of ligase enzyme and left overnight at 4°C to incubate. The ligation samples were transformed with strain JM105 *E.coli* competent cells. Screening for recombinant clones was followed according to section 2.2.1.11 Recombinant pGEX-1 JSp26 clones can be seen in Figure 2.4. This photograph displays pGEX-1 JSp26 recombinant clones obtained by direct colony PCR. The next picture (Figure 2.5) displays recombinant clones that have been isolated by alkaline lysis and thereafter digested with *Bam*H1.

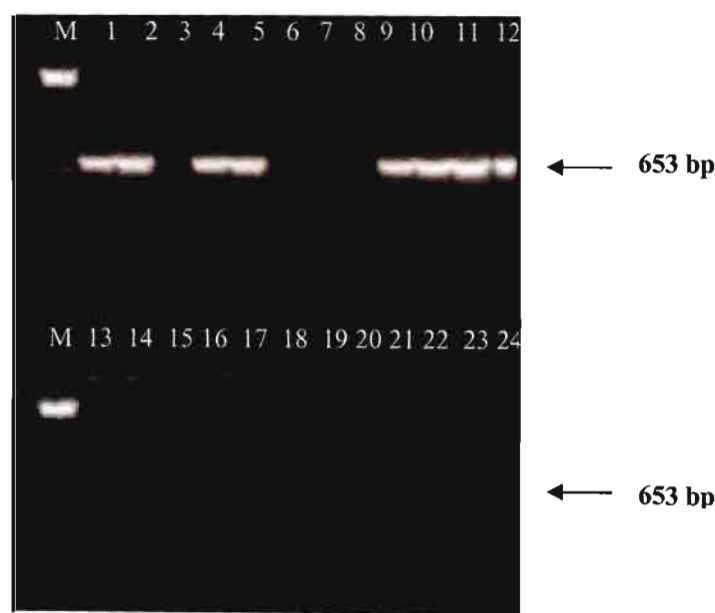


Figure 2.4 Ethidium bromide agarose stained gel (0.8%) depicting the PCR screened pGEX-1 JSp26 clones

Lanes M. Molecular Marker 11 (Boehringer Mannheim), 1-23. Colonies amplified by direct PCR and 24. Negative colony

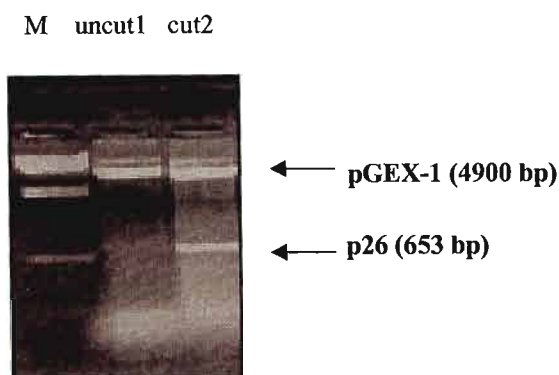


Figure 2.5 pGEX-1 JSp26 clones isolated using alkaline lysis and thereafter digested with *Bam*HI restriction enzyme

Lanes M. Molecular Marker 11 (Roche/Boehringer Mannheim), 1. Uncut (not digested) pGEX-1JSp26 recombinant clone and 2. Cut (digested) pGEX-1JSp26 clone

2.3.5.1 Confirmation of Successful Cloning of JSp26 into pGEX-1

The positive pGEX-1 p26 recombinant clones, which were obtained, were subsequently sequenced using the ALF DNA Sequencer. This was done to ensure that the fragment was inserted into the correct reading frame and aligned in the correct direction as the JSp26 capsid gene had *Bam*H1 restriction sites at both the 5' ends. The sequences obtained upon sequencing of the recombinant clones in text format can be seen in Appendix A (only the 5' end of DNA sequence of pGEX-1JSp26). This was then analysed by comparison with the published South African strain of JSRV that had been deposited in Genbank under accession number M80216 (York *et al*, 1992) using DNASIS software. The sequencing confirmed that the fragment cloned was identical to the original sequence. The comparison displayed 100% homology with no amino-acid changes.

2.3.6 Optimisation of Expression

Mini-protein expression experiments were carried out as described in section 2.2.3 to ascertain the presence of expression. The presence or absence was confirmed by running the expressed products on a 12% SDS-PAGE gel. The samples were run alongside a protein calibration marker (Combithek-Boehringer Mannheim Biochemica, Cat.No. 1317474) so that the correct size of the expressed protein would be known. The molecular size of Glutathione-S-Transferase (GST) is 26 kDa and that of JSp26 also 26 kDa and that

of the combined fusion protein 52 kDa. The expected size of the fusion protein to be seen on a gel is therefore 52 kDa and this is exactly what is shown in Figure 2.6.

Once expression was confirmed, a large-scale expression of the fusion protein was performed as described in section 2.2.5 to establish optimal conditions for expression, such as concentration of IPTG, length of time for induction of expression of protein, sonification time and condition of sonicator itself. Once this was established, a large-scale expression was done.

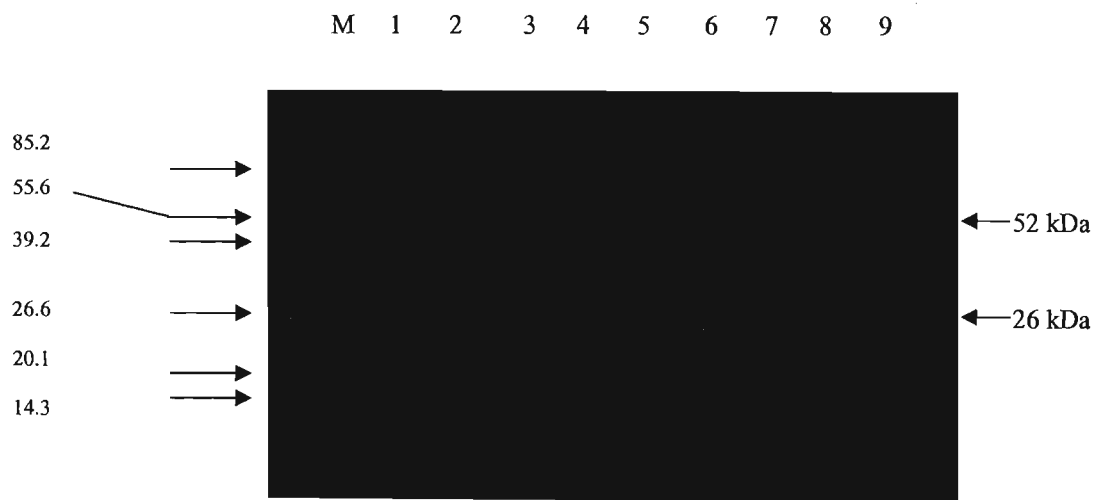


Figure 2.6 12% SDS-PAGE analysis of the purification of a GST-JSp26 expressed fusion protein

- Lane M: Calibration Protein for SDS-gel Electrophoresis (Combithek®-Boehringer Mannheim, Germany)
- Lane 1: Sonicate of *E.coli* JM105 cells containing a pGEX-1 plasmid that codes for GST-JSp26.
- Lane 2: Pellet of sonicate of *E.coli* JM105 cells containing a pGEX-1 plasmid that codes for GST-JSp26.
- Lane 3: Eluate following purification of sonicate through a glutathione agarose column
- Lane 4: Flow through after washing with 10mls of PBS. Note some of the sonicate.
- Lane 5: Flow through that is clear of any sonicate
- Lane 6: JSp26 fusion protein eluted with 10mM reduced glutathione
- Lane 7: Bovine Serum Albumin (68 kDa) reference marker
- Lane 8: Sonicate of *E.coli* JM105 containing a pGex-1 plasmid that codes for GST-JSp26 that has not been induced with IPTG.
- Lane 9: Purified GST

2.3.6.1 Large-scale Expression and Purification of pGEX-1JSp26 and pGEX-1

A culture of 1L of pGEX-1-JSp26 was expressed according to the method outlined in section 2.2.5. Purification of GST-JSp26 was carried out using an affinity chromatography column packed with glutathione agarose. Figure 2.6 shows the lysate expressing GST-p26 that was loaded onto an immobilised glutathione column and the various washes that were carried out, as well as the purified protein that was thereafter eluted from the column.

2.3.6.2 Estimation of Protein Concentration

The concentration of fusion protein eluted from the gel was determined using the Bio-Rad DC Protein assay as described in section 2.2.6.2. A protein standard was set up against which the concentration of the unknown fusion protein was estimated. The protein used for the standard being Bovine Serum Albumin. The concentration of the standard protein (known) used, the unknown amount used for GST, and that of the unknown GST-JSp26 fusion protein is shown in Table 2.7. A standard curve was drawn for this as can be seen in Figure 2.7 and from this curve; the approximate concentration of the fusion proteins was extrapolated. From the Standard Curve (not shown) the concentration of GST at an absorbance of 0.0338 is equivalent to 8000ng. Therefore a volume of 4µl of GST contains 8000ng of protein and 1µl has a volume of 1600ng protein. Figure 2.8 shows a SDS page analysis of GST-JSp26 in relation to GST alone.

Table 2.7 Absorbance and Concentration of BSA and GST Protein

X	Absorbance at 750nm	0.0015	0.0038	0.0129	0.0232	0.0537	0.0714
Y	Volume of BSA in µl (100ng/µl)	0	10	20	40	80	100
	Volume of water in µl	100	90	80	60	20	0
	Absorbance of GST	0.0042	0.0307	0.0338	0.0591		
	Unknown concentration of GST µl	0	1	2	5		
	Volume of water in µl	100	99	98	95		
	Absorbance of JSp26	0.038	0.054				
	Unknown conc.of JSp26 in µl	20	40				
	Volume of water in µl	80	60				

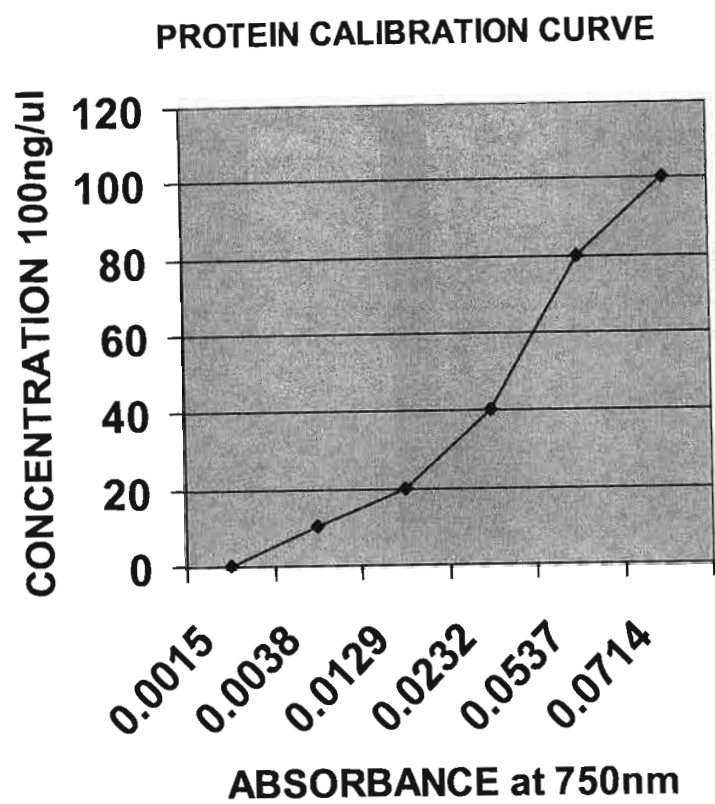


Figure 2.7 Protein Calibration Curve for Bovine Serum Albumen

From the Standard Curve of BSA, the fusion protein GST-JSp26 has a concentration of 200ng/μl.

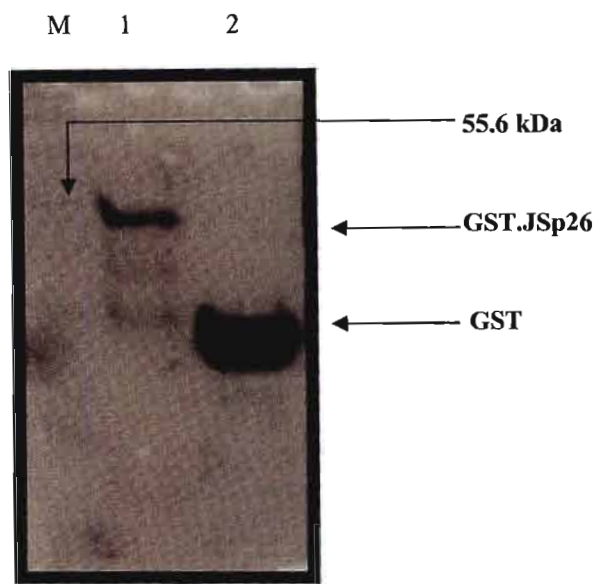


Figure 2.8 12% SDS-PAGE Analysis of the GST-JSp26 Affinity Chromatography purified fusion protein

Lanes M. Protein Calibration Marker, 1. GST-JSp26 Fusion Protein-52 kDa and 2. GST-26 kDa

2.3.7 Immunoblot Detection

The antigenicity of the purified GST-p26 fusion protein was evaluated by means of an immunoblot detection assay as described in section 2.2.7. The purified GST-p26 fusion protein, GST and Bovine Serum Albumin (negative control) was fractionated on a 12% SDS-page gel using a Mini-Protean®II Dual Slab Cell (Biorad) apparatus and transferred to a nitrocellulose membrane using a Trans-blot electrophoretic transfer cell (Biorad). The SDS-page gel was run in duplicate. The membrane was processed using anti-MPMV and anti-GST antibody and horseradish-peroxidase Protein G (anti-goat IgG horseradish-peroxidase conjugate) and 4-chloro-1-Naphthol substrate. The results for the immunoblot detection of GST-p26 fusion protein using anti-MPMV are shown in Figure 2.9A and that of Figure 2.9B is an immunoblot assay displaying the reaction between GST-JSp26, GST and BSA with anti-GST being used as antibody.

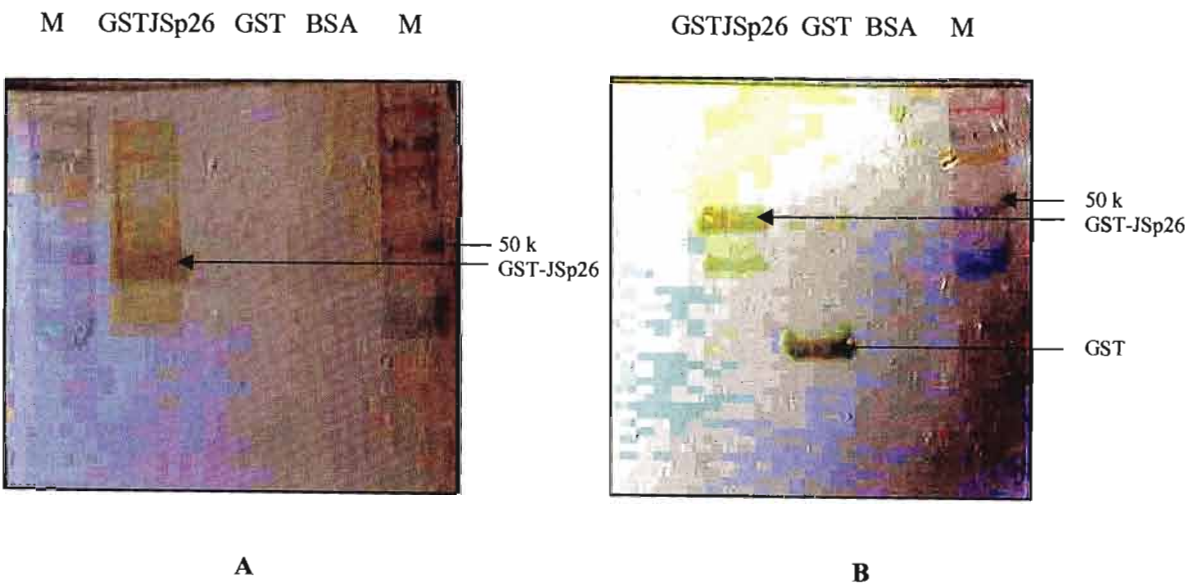


Figure 2.9 A Western blot analysis of GST-JSp26 fusion protein using: (A) anti-MPMV sera and (B) anti-GST

Lanes: M. Rainbow Protein Marker (Amersham Life Sciences, UK), 1. 3µg of GST-JSp26 fusion protein, 2. 3µg of GST and 3. 3µg of BSA

2.3.8 Use of JSRVp26 fusion protein in an ELISA

2.3.8.1 Enzyme-linked Immunosorbent Assay (ELISA)

Once the antigenicity/immunogenicity of the JSRVp26 fusion protein was confirmed to cross-react with anti-MPMV antibody in an immunoblot assay, further experiments were performed to test whether this antigenicity was also true for sera obtained from sheep positive for OPA using a microtitre plate format. Most of the sera used in the following experiments were obtained from Onderstepoort Veterinary Research Centre in Pretoria. Diagnosis of the sheep was based upon pathological examination of the lungs of the sheep during post-mortem. Antibody to Mason-Pfizer monkey virus was used as a positive control and negative sheep serum as negative control.

2.3.8.1.1 Indirect or non-competitive Enzyme-linked Immunosorbent Assay

The JSRVp26 fusion protein was coated onto the wells of microtitre plates (Nunc maxisorb) starting with a concentration of 125µg (500µl) in 2000µl of 0.05M carbonate bicarbonate buffer. The antigen was coated in serial dilution down the plate starting with 1/5 (25µg) and ending at 1/80 (0.19µg) with the most concentrated solution in the top row and the most dilute in the bottom row. Sheep sera used as antibody was obtained from Onderstepoort as well as a Merino sheep farm in Uitenhage. Five of the sera (Bruwer, O5, O13, and R3) were used at both a 1/5 and 1/10 dilutions. Two sera (JS784 and JS1943) were used at a 1/5 dilution. MPMV antibody was used at 1/5000. The negative sheep serum was used at a dilution of 1/10 in blocking buffer. Protein G at a dilution of 1/4000 was used as the conjugate that was linked to peroxidase. Following the addition of the substrate (OPD), ten-minute incubation and stopping of the reaction, the plates were read at 492 nm using the Biorad microtitre plate reader. The absorbance readings are presented in a tabulated form in Table 2.8.

TABLE 2.8 Investigation into the use of the GST-JSp26 fusion protein as a possible antigen (125µg in buffer) to detect the presence of JSRV antibodies in sheep sera using an indirect ELISA (enzyme linked immunosorbent assay)

DILUTION OF DIFFERENT SHEEP SERA USED													
SERA →		BR	BR	O5	O5	O13	O13	R3	R3	784	1943	MP/MV	N
[AG] µg/well		1/5	1/10	1/5	1/10	1/5	1/10	1/5	1/10	1/5	1/5	1/5000	1/10
5	25	105	133	158	131	127	118	134	087	160	520	245	048
10	12.5	208	194	123	103	103	103	120	082	107	529	210	068
20	6.25	107	154	063	063	092	065	106	054	049	407	148	046
40	3.13	041	057	050	055	052	068	064	061	058	390	090	063
80	1.57	045	042	056	052	062	063	089	064	044	456	059	055

Pos/Neg Cut-off values greater than 101 (ave. of negative sera x 1.8) regarded as positive.

The negative control showed no reaction to the fusion protein as is expected. The antibody used as a positive control (MPMV) was used at 1/5000 and showed lower absorbance values than expected. Background absorbance was taken as 101 (calculated by multiplying the mean of the negative by a constant factor of 1.8). One of the concerns that were expressed was whether the expressed protein was purified of all bacterial proteins. To assess this, a control protein also expressed in pGEX was purified using the same method and microtitre wells coated at a similar concentration.

2.3.8.1.2 Use of Indirect ELISA to Determine the Specificity of the GST-p26 Fusion Protein.

The following experiment was designed to assess the specificity/purity of the GST expressed JSp26 protein. Equivalent concentrations of GST-JSp26 (0.250µg/µl) and GST-HIVp24 (0.250µg/µl) antigen were coated onto microtitre plates starting with a 1/10 dilution of each antigen. Serum from Bruwer sheep was used as the antiserum against both antigens (see Methods 2.2.8). The results are presented in Table 2.9 and Table 2.10.

TABLE 2.9 ELISA results showing absorbance readings when Bruwer (JS positive sheep serum) serum was used as antibody at a dilution of 1/10 to 1/320 against antigen GST-JSP26

AB (BRUWER) →		1/10	1/20	1/40	1/80	1/160	1/320
[Antigen] in µg/well							
1/10	12.5	0.400	0.352	0.231	0.208	0.098	0.134
1/20	6.25	0.394	0.182	0.263	0.280	0.094	0.101
1/40	3.13	0.264	0.161	0.157	0.193	0.114	0.126
1/80	1.56	0.223	0.160	0.168	0.176	0.129	0.192
1/160	0.78	0.221	0.129	0.123	0.165	0.156	0.165
1/320	0.39	0.247	0.158	0.153	0.157	0.132	0.197
1/640	0.20	0.267	0.186	0.140	0.127	0.129	0.192
1/1280	0.10	0.232	0.165	0.155	0.126	0.127	0.245

500µl (125µg) in 2000ul of buffer, see 2.3.8.1.1

TABLE 2.10 ELISA results showing absorbance readings when Bruwer serum was used as antibody at a dilution of 1/10 to 1/320 against antigen GST-HIVP24

AB (BRUWER) →		1/10	1/20	1/40	1/80	1/160	1/320
[Antigen] in µg/well							
1/10	12.5	0.509	0.377	0.360	0.204	0.138	0.097
1/20	6.25	0.381	0.246	0.190	0.142	0.131	0.102
1/40	3.13	0.390	0.238	0.195	0.133	0.127	0.113
1/80	1.56	0.293	0.271	0.181	0.152	0.122	0.122
1/160	0.78	0.468	0.256	0.168	0.162	0.128	0.117
1/320	0.39	0.384	0.306	0.162	0.138	0.120	0.126
1/640	0.20	0.519	0.304	0.163	0.154	0.119	0.143
1/1280	0.10	0.422	0.282	0.153	0.129	0.106	0.121

125µg in 2000ul of buffer

The results presented in both Table 2.9 and 2.10 revealed that the antibody (Bruwer serum) is reactive to both GST-JSp26 and GST-HIV p24 antigens and showed no difference. Various experiments were made in an attempt to compete out or absorb out antibodies that were non-specifically present in the Bruwer serum to establish if indeed there were specific antibodies to the JS p26 capsid proteins. The conclusion was that the concentration of antibodies to the JSp26 in the Bruwer serum was low. A comparison is shown in Table 2.11 where the Bruwer serum is used against both JSp26 and HIVp24 expressed and purified proteins following an attempt to absorb out the non-specific proteins using the HIVp24 protein. Notice a drop in the intensity of the reaction however it is still clearly not specific.

Table 2.11 **A comparison between the JSp26 and HIV p24 expressed proteins using the sera from a sheep with JSRV before and after absorbing out the sera with the HIV p24 proteins.**

AB → 1/5		BRUWER AB		BRUWER AB ABSORBED OUT WITH HIV P24 AG	
[Antigen] in µg/well		JSp26	HIVp24	JSP26	HIVP24
1/10	12.5	0.400	0.509	0.110	0.306
1/20	6.25	0.394	0.381	0.131	0.245
1/40	3.13	0.264	0.390	0.106	0.166
1/80	1.56	0.223	0.293	0.092	0.134
1/160	0.78	0.221	0.468	0.092	0.106
1/320	0.39	0.247	0.384	0.098	0.107
1/640	0.20	0.267	0.519	0.106	0.141
1/1280	0.10	0.232	0.422	0.099	0.222

From the results in Table 2.11, it can be seen that the addition of the competitor did lower absorbance readings obtained for both OPA and HIV.

The conclusion that was arrived at was that the Bruwer antiserum (as an example of serum from a naturally infected sheep with OPA) clearly does not contain any antibodies to JSp26 or that they were present at too low a concentration for the sensitivity of this assay.

The question was then asked as to whether the antibody response might be localised to the lungs of infected sheep. For this purpose an experiment was planned to look for the presence of JS anti-p26 antibodies in lung wash pellets. The only source of viral material that was available for analysis, were lung wash samples that had been concentrated by ultra centrifugation. A few samples of nasal fluid from JS affected sheep were also available. There were a few problems that needed to be overcome however. Firstly, a p26 peroxidase-conjugate, using the purified GST-JSp26 protein needed to be produced. The use of an anti-sheep conjugate was not feasible, as it would react directly with all sheep antibodies, irrespective of their specificity. Secondly, the lung wash pellets were fairly old. A method needed to be used to demonstrate the presence of antibodies in these samples. This was demonstrated using Mab 59G11 (as described in the following results section 2.3.9).

2.3.9 The Use of MAb 59G11 to Assess for the Presence of Antibodies in the Lung Wash and Nasal Fluid Pellets

2.3.9.1 Indirect Antibody Enzyme-Linked Immunoassay

One of the concerns that arose from the above studies was that we could not detect any anti-p26 antibodies in the sera of JSRV infected sheep. We therefore decided to look for the presence of anti-p26 antibodies in the lung lavage of JS affected and JS negative sheep. We used MAb 59G11 (MAb 59G11 is a sheep IgA specific monoclonal antibody) to establish that antibodies (most specifically IgA) were present in the lung lavage samples.

Two representative JS and NL wash pellets were diluted (1/5) and then titrated as a five fold dilution with 0.05M carbonate bicarbonate buffer (pH 9.6), and bound to microtitre plates and tested for the presence of antibodies using MAb 59G11 (see methods 2.2.8.1). The results are present in Table 2.12.

TABLE 2.12 Indirect ELISA Using Mab 59G11 (Monoclonal antibody specific for sheep IgA) against two representative JS and NL Pellets. Both MABs were used at a dilution of 1/50

	MAB 59G11	MAB 59G11
Dilution of Antigens	NL	JS
1/5	1.191	0.830
1/10	1.155	0.637
1/20	1.194	0.658
1/50	1.309	0.699
1/100	1.195	0.626
1/200	0.895	0.490
1/400	0.675	0.300
1/800	0.244	0.192

The results presented in Table 2.12 indicated that 59G11 was detecting antibodies in both NL and JS lung. A series of JS and NL wash pellets were diluted at 1/10 and 1/100 with carbonate buffer and bound to microtitre plates and tested, as before, for the presence of antigen with which the MABs reacted (see methods 2.2.8.1). The results are presented in Table 2.13.

TABLE 2.13 ELISA Test Showing Reaction to MAB 59G11, When JS AND NL Pellets Were Diluted At 1/10 and 1/100 and Used As Antigen

Sheep tested	Number of samples	%+VE 59G11	%-VE 59G11
JS	16	100	0
NL	3	100	0

The results presented in Table 2.13 confirm that all lung wash pellets (both JS and NL) contain sheep IgA antibodies. The samples could then be analysed for the presence of anti p26 antibodies using an antigen sandwich assay. To this end a p26 peroxidase conjugate would need to be prepared.

2.3.9.2 Preparation of an In-house GST-JSp26 Conjugate Labelled with Peroxidase

2.3.9.2.1 Titration of Peroxidase Labelled GST-JSp26 Conjugate

Wells of a microtitre plate were coated with the peroxidase labelled GST-JSp26-HRP in-house conjugate. The conjugate was titrated down the plate with the most concentrated solution (1/100) top and the most dilute (1/7812500) in the bottom row. One row was also titrated with GST-JSp26 fusion protein (see methods 2.2.8.2.2 for concentration of Ag) only. The results of this titration are presented in Table 2.14.

TABLE 2.14 Direct titration of in house GST-JS p26 horseradish peroxidase labelled conjugate

Dilution of conjugate and antigen	GST-JSp26-HRP CONJ	GST-JSp26 AG (12.5µg)
1/100	1.334	0.079
1/500	0.829	0.089
1/2500	0.367	0.085
1/12500	0.109	0.089
1/62500	0.091	0.097
1/312500	0.094	0.137
1/1562500	0.093	0.116
1/7812500	0.130	0.108

From the absorbance values presented above in Table 2.14 it was concluded that the conjugate could be used at a dilution as high as 1/2500 and still provide a good positive to negative ratio. It was however decided to use the conjugate GST-JSp26-HRP at a dilution of 1/50 for the antigen capture assay.

2.3.9.2.2 Use of the Conjugate GST-JSp26-HRP in Two Antibody Capture Assays

2.3.9.2.2A Use of the Conjugate against Lung Wash and Nasal Fluid Samples from JS Affected and Negative Sheep

A preliminary assessment of the conjugate against seven positive and seven negative lung wash and nasal fluids, revealed that the optimal conditions for the conjugate to be used was 1/80 for the antigen and 1/50 for the conjugate. The data confirming this was not included in the thesis.

TABLE 2.15 Presentation of Data for Antibody Capture ELISA Using In-House Conjugate GST-JSp26-HRP

	JSRV NEGATIVE SERA	JSRV POSITIVE SERA
	0.091	0.435
	0.083	0.221
	0.105	0.266
	0.099	0.203
	0.080	0.122
	0.094	0.250
	0.228	0.401
	0.098	0.346
No of True Positives	0	7
No of True Negatives	7	0
No of False Positives	1	0
No of False Negatives	0	1
Percentage	87.50	87.50

Cut-off is 0.197 (average of negative OD times 1.8)

The data presented in Table 2.15 show that the antibody capture ELISA using the in-house conjugate GST-JSp26-HRP was encouraging and prompted an investigation of a larger number of samples.

2.3.9.2.2B Use of Antibody Capture ELISA against a Larger Sample Size

Eighty samples were tested using the in-house conjugate GST-JSp26-HRP. The data from the experiment are presented in Table 2.16.

TABLE 2.16 Absorbance Values Obtained for Antibody Capture Assay Evaluated against 80 Samples

	1	2	3	4	5	6	7	8	9	10
A	1.039	0.362	0.156	0.138	0.133	0.097	0.126	0.081	0.151	0.073
B	0.408	0.155	0.071	0.104	0.071	0.067	0.080	0.063	0.189	0.081
C	0.189	0.076	0.081	0.114	0.144	0.092	0.084	0.067	0.137	0.071
D	0.102	0.059	0.066	0.063	0.062	0.095	0.135	0.074	0.066	0.064
E	0.192	0.062	0.073	0.064	0.066	0.066	0.081	0.082	0.076	0.067
F	0.235	0.069	0.101	0.068	0.065	0.065	0.066	0.066	0.065	0.069
G	0.409	0.070	0.098	0.070	0.073	0.074	0.067	0.072	0.073	0.080
H	0.073	0.065	0.065	0.070	0.067	0.073	0.068	0.068	0.068	0.067

ALL OD FOR POSITIVE SERA IN BOLD

OD FOR NEGATIVE SERA NOT IN BOLD

A positive cut-off value of 0.132 was calculated from the average absorbance values obtained for the three negative samples times a constant value of 1.8.

The cut-off is therefore equal to the average of the absorbance values of calf serum plus a known negative (0.068+0.073+0.080) divided by 3 times 1.8, which is 0.132. In the test there were 39 positive and 41 negative samples. A summary of the results is presented in Table 2.17. The positive samples were from field cases obtained from the Onderstepoort Veterinary Research Institute (Pretoria) and were diagnosed with OPA at post-mortem and histologically confirmed.

TABLE 2.17 Summary of Results

	NO.OF POS. (39)	NO.OF NEG. (41)	% POSITIVE	% NEGATIVE.
True Positive	12	0	20	0
True Negative	0	37	0	80
False Positive	0	4	25	0
False Negative	27	0	0	42.18

Sensitivity = 12 /39 (31%)

Specificity = 37/41 (90%)

From the data presented in Table 2.17, it can be seen that although the in-house conjugate was able to detect a few positive samples the assay clearly lacked sensitivity. The specificity was a little better but is still not acceptable as a diagnostic tool for OPA.

2.3.9.3 Applications of GST-JSp26 Polyclonal Antibodies to Detect Antigen in JS Positive Samples

All investigations designed to look for antibodies to JSp26 in sera and lung fluid revealed that very few anti-p26 antibodies, if at all in some cases, were being produced. There was now a need to develop a method to look for the presence of the JSRV p26. The tool required was an anti p26 antibody. This was achieved by making anti-p26 antibodies in a chicken.

2.3.9.3.1A Assessment of the chicken anti-p26 Polyclonal serum using an Indirect ELISA

To test for the presence of anti-p26 antibodies the purified GST-JSp26 antigen was diluted 1/10 with carbonate bicarbonate buffer (pH 9.6), so that 1 μ g of antigen was bound to each well of the first row of a microtitre plate, The antigen was then titrated across the microtitre plate, with the most concentrated antigen being towards the left of the plate and the less concentrated on the right of the plate. The antiserum (Y3 of 25/12/98-8 week after immunisation) was used at a 1/10 dilution and titrated down the plate, with the highest concentration at the top and weakest towards the bottom. The checkerboard titration of antigen against antiserum is presented in Table 2.18.

TABLE 2.18 Titration of Antigen (GST-JSp26) and Antibody (Y3)

Ab ↓	Ag GST- JS-p26 → concentration of GST-JSp26 (nanograms/well)											
	1000	500	250	125	62.5	31.25	15.62	7.81	3.90	1.95	0.97	0.49
1/10	>3	>3	>3	>3	>3	>3	>3	>3	>3	>3	2.38	0.82
1/20	>3	>3	>3	>3	>3	>3	>3	3.13	1.62	1.07	0.91	0.63
1/40	>3	>3	>3	>3	>3	>3	>3	2.50	1.30	0.94	0.92	0.68
1/80	>3	>3	>3	>3	>3	>3	>3	2.31	1.21	0.63	0.53	0.32
1/160	>3	>3	>3	>3	>3	>3	2.61	1.26	0.65	0.67	0.38	0.28
1/320	>3	>3	>3	>3	>3	2.75	2.19	1.24	0.56	0.67	0.36	0.22
1/640	>3	>3	>3	>3	0.44	1.554	0.99	0.98	0.89	0.63	0.45	0.17
1/1280	>3	>3	>3	2.62	1.07	0.63	1.23	1.35	1.41	1.26	0.67	

The results in Table 2.18 show that no end point was reached for the antibody titration. There is still a strong response even at 10ng/ μ l of antigen.

2.3.9.3.1B Indirect ELISA Utilising the Different Antisera Extracted Against Both GST-HIVp24 and GST-JSp26

Eggs were collected from a number of time points following immunisation. To determine which collection point had the highest titre of antibodies all collection points were tested against 10ng of JSp26 protein.

Using the results obtained from Table 2.18, 10ng of antigen per well was diluted in carbonate buffer and bound to the microtitre plate and tested for the presence of antigen using the extracted antisera. The antisera were used as follows:

- 1: Baseline antiserum 31/10/98
- 2: 7 days 8/11/98
- 3: 12 days 12/11/98
- 4: 22 days 22/11/98
- 5: 29 days 29/11/98
- 6: 35 days 6/12/98
- 7: 42 days 13/12/98
- 8: 54 days 25/12/98

The antisera were diluted starting at a 1/5 dilution and going down the plate till 1/390625. The results are presented in Table 2.19 (for GST-JSp26) and Table 2.20 (for GST-HIVp24).

TABLE 2.19 TITRATION OF DIFFERENT ANTISERA AGAINST A CONSTANT AMOUNT OF GST-JS p26 ANTIGEN
Time points →

Ab ↓	1	2	3	4	5	6	7	8
5	0.981	0.416	1.511	>3.00	>3.00	>3.00	1.626	>3.00
25	0.267	0.110	0.079	>3.00	3.023	>3.00	>3.00	3.331
125	0.092	0.051	0.146	3.054	1.649	>3.00	3.219	2.997
625	0.055	0.040	0.060	1.666	0.558	2.614	1.879	1.211
3125	0.031	0.057	0.048	0.739	0.153	1.258	0.712	0.123
15625	0.045	0.045	0.042	0.255	0.068	0.425	0.230	0.260
78125	0.060	0.061	0.069	0.176	0.235	0.435		
390625	0.249	0.240	0.318	0.264	0.311	0.381		

The results in Table 2.19 show that the antiserum extracted at 22 and 35 days after immunization gave the best set of absorbance values when compared to the antisera extracted at other times. When observing the absorbance values in column four and six, the

values in six are better with the antiserum giving a relatively good titration. The choice of antibody titre was between 1/5 and 1/125. So an in between titre of 1/50 was used.

To confirm that the antibodies were specific to the JSp26 an equivalently expressed control protein was used as antigen instead of the JSp26 protein.

TABLE 2.20 TITRATION OF ANTISERA AGAINST A CONSTANT AMOUNT OF GST-HIVp24ANTIGEN

	1	2	3	4	5	6	7	8
5	1.023	0.682	1.488	2.271	1.262	3.220	2.692	3.074
25	0.575	0.168	0.654	1.706	0.610	2.315	2.262	2.141
125	0.113	0.068	0.259	1.193	0.093	0.939	0.779	0.972
625	0.097	0.056	0.089	0.252	0.059	0.241	0.266	0.207
3125	0.640	0.050	0.046	0.067	0.043	0.110	0.077	0.099
15625	0.114	0.037	0.036	0.036	0.048	0.110	0.055	0.054
78125	0.082	0.038	0.057	0.058	0.032	0.039	0.048	0.062
309625	0.217	0.082	0.093	0.055	0.055	0.060		

The results presented in Table 2.20 indicated that there was a cross-reaction of the antisera to the GST portion of the fusion protein.

2.3.9.3.2 Effect of Adding GST to the Antiserum in an ELISA Immunoassay

In an attempt to reduce the non-specific cross-reaction to the fusion portion of the protein, the sera was absorbed against the GST fusion protein. The JSp26 antiserum from the 35-day collection was selected for this blocking out experiment. The procedure simply involved incubating the sera in nunc microtitre wells coated with 10ng/well of GST-JSp26 and GST-HIVp4 antigen. Incubation was at 37°C for 10 min. The antibody was added as presented in Table 2.21. A comparison is made using pure GSTp26 and GSTp24.

TABLE 2.21 Absorbance Values Presented After Addition of GST to p26 antibody

		GST-JSP26 AG	GST-HIVP24 AG
A	10ul GST	3.300	1.254
B	5ul GST	3.140	0.906
C	1ul GST	2.985	0.915
D	1ul of 1/5	3.248	1.075
E	1ul of 1/25.	3.114	0.946
F	1ul of 1/125	3.179	0.987
G	AB 1/50	3.088	0.827
H	Blocking buffer	0.143	0.055

The results presented in Table 2.21 indicate that the addition of GST had very little effect on the reaction between antibody alone, and antibody and GST together. It is indicative of a reaction to the GST. Since the GST-JSp26 antigen was used to immunise the chickens, antibody was probably produced to the GST component of the fusion protein as well as to the whole antigen. At this stage we believed that we needed to produce the antiserum again, but this time against a JSp26 protein only.

2.3.10 Polyclonal Rabbit Antiserum to JSRV Viral Capsid Antigen

At about this time we were made aware of the fact that Professor De Martini of Fort Collins, USA, had produced a high-titred antiserum against the JSRV viral capsid antigen (JSp26) in rabbits. This antibody became available at a time that I was planning on re-cloning the JSRVp26 viral capsid gene into an expression vector with a thrombin cleavage site. I envisaged that once I had achieved this, I could then immunise a new set of chickens with the JSp26 protein only. Due to the non-specificity of the polyclonal antiserum that had been produced in chickens, a decision was made to test the potential of this rabbit antiserum in both an ELISA and immunoblot assay as a diagnostic tool.

2.3.10.1 Use of Rabbit polyclonal viral capsid antiserum in an immunoblot assay

The sensitivity of the antiserum was tested by means of an immunoblot detection assay as described in section 2.2.7. Purified JSRV-GSTp26 was used as antigen. The purified JSRV-GSTp26 (500ng and 250ng), 1ug of bovine serum albumen and 1ug of GST-alone was fractionated on a 12% SDS-polyacrylamide gel as described in section 2.2.4 along with a protein calibration marker (Rainbow marker-Amersham). After electrophoresis, the

proteins were transferred to a nitrocellulose membrane using a Trans-blot electrophoretic transfer cell (Biorad). The SDS-PAGE gel was run in duplicate on two sets of gels. One SDS-Page gel was stained with Coomassie blue and this is shown in Figure 2.10. The membrane was processed using the polyclonal antiserum at a dilution of 1/250, anti-GST at 1/1000 and a gold- labelled antibody at 1/50 (not shown). The secondary antibody was horseradish peroxidase Protein G (anti-goat IgG horseradish peroxidase) and 4-chloro-1-naphthol as substrate. The results are shown in the following Figures 2.11 and 2.12.

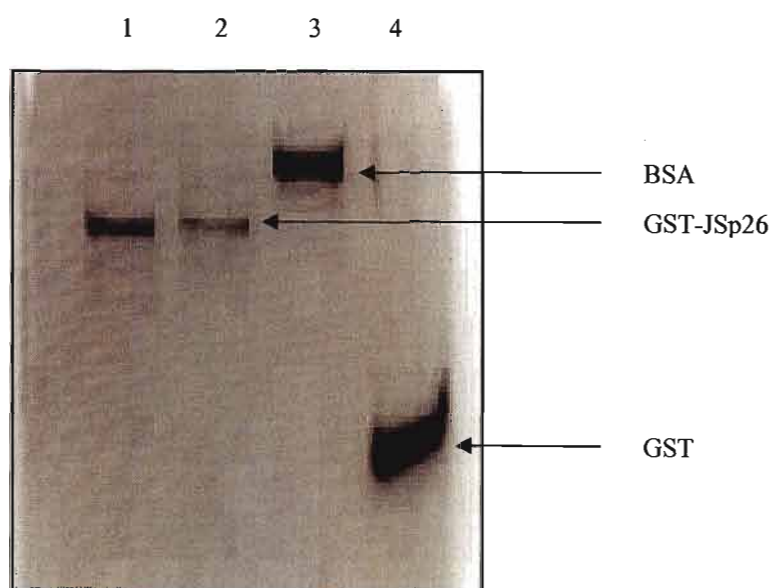


Figure 2.10 **SDS-PAGE GEL ANALYSIS OF GST-JSp26 fusion proteins**
Lanes 1. 500 ng GST-JSp26 fusion protein, 2. 250 ng GST-JSp26 fusion protein,
3. 1 µg of Bovine Serum Albumen and 4. 1.6 µg GST-alone

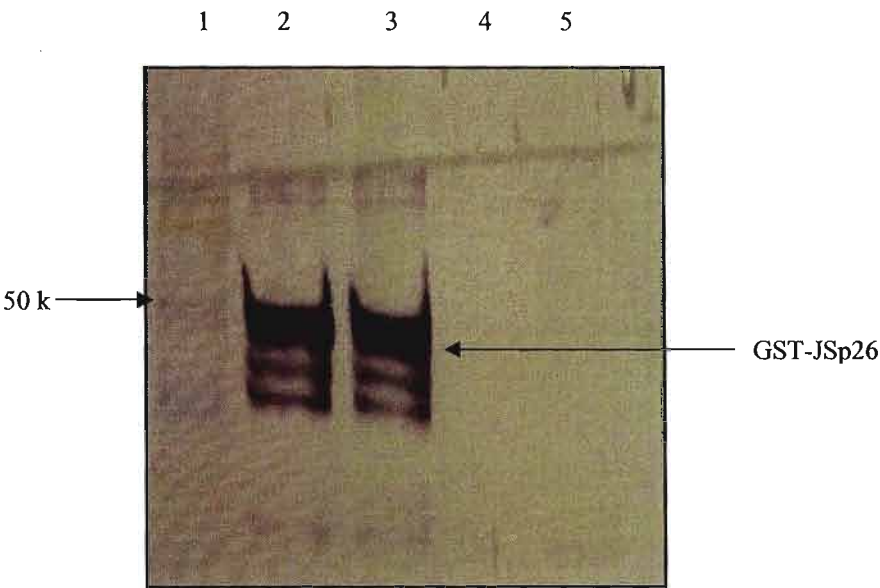


Figure 2.11 **Immunoblot Analysis of pGEX-JS p26 Fusion Protein using the Rabbit Viral Capsid as antiserum**
1) Protein Calibration Marker (Amersham Life Sciences, UK)
2) and (3) pGEX-JSp26 Fusion Protein Detected by the Rabbit Viral Capsid Antiserum
4) BSA
5) GST-alone. Note that the Rabbit Viral Capsid Antiserum does not detect it

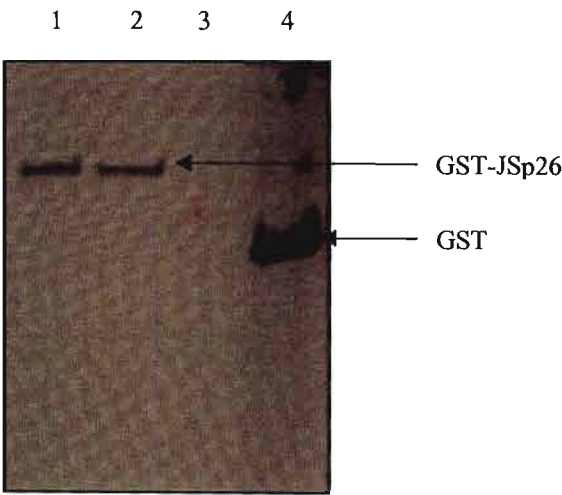


Figure 2.12 **Immunoblot Detection of pGEX-JSp26 Fusion Protein using Anti-GST as antiserum**
Lanes 1. and 2. pGEX-JSp26 detected by anti-GST antiserum, 3. BSA not detected and 4. GST detected by Anti-GST Antiserum

2.3.10.2 Use of Viral Capsid Antibody in an Indirect Enzyme Immunoassay

In an attempt to investigate the reactivity of the rabbit antisera to JSRVp26 an indirect ELISA test was performed as described in section 2.2.8.1 but a slight change in the method was the use of a biotinylated labelled affinity isolated goat anti-rabbit and goat anti-mouse that was complexed to conjugated strepavidin horseradish peroxidase as well as Protein G as the secondary antibody or conjugate. The wells were coated with different affected JS lung and NL antigens at dilutions of 1/5 in 0.05M carbonate bicarbonate buffer (pH 9.6) and tested for the presence for antigens using rabbit polyclonal viral capsid antibody. The results are presented in Table 2.22

Table 2.22 ELISA Test Showing Reaction to the Rabbit Polyclonal Viral Capsid Antibody When JS and NL Pellets were Diluted at 1/5 and Used as Antigens

Antigen	1												
Ab	Rab anti Capsid												
Conjugate	Protein G and												
	Goat anti mouse Biotin												
Ag 1/5	VCp25			VCp25		PreBleed		PreBld		Blk Buf		Blk Buf	
Conj	Anti Rab Biotin			Protein G		Anti Rab Bio		Protein G		Anti Bio	Rab	Protein G	
NL 3310	407	JS5926/5	2109	175	111	465	287	449	115	264	199	524	220
NL3253	331	JS4133/9	258	195	80	256	149	278	85	161	99	260	78
NL7	223	JS4811/0	1474	57	128	130	154	73	181	102	115	69	148
JS 5818/4	1523	JS4844/1	543	419	70	248	133	569	85	163	92	487	102
JS4546/4	1625	JS3685/9	781	156	86	213	172	216	118	134	120	179	91
JS3330/5	1719	JSp26 1/5	3000	117	2901	219	165	132	55	113	72	119	50
JS4084/3	1813	JSp26 1/10	3000	94	2829	197	138	132	52	112	75	142	46
JS3224	2626	JSp26 1/100	3000	178	2414	257	226	181	45	159	186	189	46
Pos/Neg Cut off	576.6			191.4		354		228.6		247.8		267.6	

1 Positive and negative lung wash pellets

Sensitivity: 8/10 (80%)

Specificity: 3/3 (100%)

There was a strong reaction of the rabbit antisera against nearly all the JS positive samples (8/10). There was a noticeable difference between the Protein G conjugate and the Anti-Rabbit Biotin-Streptavidin conjugate. The rabbit prebleed sera results were used to confirm the non-specific reaction of the normal lung antigens.

The above result confirmed the specificity of the rabbit anti-JSRVp26 sera. It also confirmed the fact that the JSp26 antigen was being expressed in the lungs of JSRV affected sheep, something that we had been aware of.

2.4 DISCUSSION

For years (early 1980's) there has been much debate as to whether circulating antibodies to JSRV are present in the sera of sheep with OPA. M. Sharp and Herring in 1983 were the first to report on an immunological cross-reaction between the 27 000 molecular weight major core protein (p27) of MPMV and a 25 000 molecular weight polypeptide of JSRV. However, the p27 used as an antigen in an ELISA to screen sheep, failed to detect any circulating antibodies to this cross-reacting protein. It was only when the JSRV genome was isolated, cloned and sequenced, did it become apparent that there was an endogenous form of the exogenous infectious JSRV. It was then theorised that the reason why sheep did not make antibodies to the exogenous JSRV was because the sheep recognised the JSRV as self-antigens, due to the expression of the endogenous virus. The hypothesis is that during T-cell ontogeny there is a clonal elimination of all T-cells that recognise self-antigens, hence, the inability of sheep to develop antibodies to the viral antigens (York *et al*, 1992). Whilst we were aware of this hypothesis, once the virus had been cloned and sequenced, the tools became available to clone and express some of the actual JSRV antigens and investigate their potential as antigens.

This study focused on the cloning and expression of three genes, namely, the JSRVp26, JSRVTM (envelope) and JSRVorf-X. The object was then to purify the expressed proteins and investigate their potential as diagnostic proteins. To achieve this, the cloning and expression of the JSRVp26 capsid protein, the ORF-X protein (of unknown function) and the JSRV transmembrane protein (part of the envelope gene) were therefore undertaken. The cloning and subsequent expression of the JSRVp26 capsid protein was successful. This is illustrated in figures 2.5, 2.6 and 2.8 and can be seen in Appendix A where the JSRV p26 gene was shown to be in frame with the GST-multicloning site. This was achieved by sequencing the resultant recombinant GST-JSRVp26 fusion protein (Sharp and Herring, 1983; Ortin *et al*, 1998; Palmarini *et al*, 1995; Kwang *et al*, 1995).

In this study the JSRVorf-X and TM proteins were successfully cloned. Attempts were made to express both the fusion proteins, but difficulties were encountered, due to the highly hydrophobic characteristics of these proteins (Bai *et al*, 1999). Hydrophobicity usually leads to incorrect folding and aggregation of the expressed protein with *E.coli* host proteins and co-aggregation of the fusion protein with the bacterial membranes (Marston,

1986). The JSRVorf-X and JSRVTM were cloned into the correct reading frames, but failed to express the fusion proteins due to their extreme hydrophobicity. Even though optimised expression conditions were used, and denaturants such as 8MUrea, BPer (Pierce) and N-Lauroyl sarcosine, we were unable to successfully express these proteins.

For purposes of this study it was decided to concentrate on the JSRV p26 fusion protein. One of the first things that we wanted to establish was whether the endogenous JSRVp26 was the same as the exogenous JSRVp26 gene. To investigate this, the gene coding for the JSRVp26 capsid protein was isolated from three separate Australian sheep genomic DNA (LPW, 23909 and MSCU). The three genes were isolated, cloned sequenced and expressed in a pGEX-1 vector. Since JSRV has never been identified in Australia, this gene could only be from an endogenous form of JSRV (York *et al*, 1992). The cloning and expression of this gene was achieved with little difficulty and sequence analysis revealed that in all cases the exogenous JSRVp26 gene was identical to all three endogenous isolates. It was therefore not unexpected to find that the endogenous JSRVp26 gene was efficiently expressed and cross-reacted with the MPMV antiserum. As there are 15 to 20 endogenous JSRV loci in each sheep genome it might be argued that the primers and amplification conditions had favoured the isolation of the equivalent gene in all cases. Whilst this is a valid criticism we nevertheless established that there was an equivalent endogenous gene that was present in normal sheep. This was further supportive evidence for the theory that sheep do not produce antibodies to the JSRVp26 because it is recognised as a self-antigen.

We nevertheless wanted to complete our investigation using a purified viral protein such as the JSp26 antigen, which we had not been able to achieve in the past. The purified GST-p26 fusion protein proved to be immunogenic by its cross- reactivity with the anti-MPMV antibody (which is the OPA-associated p27 marker protein) in a Western immunoblot assay, Figure 2.9. The purified GST-p26 recombinant antigen was used in an ELISA to screen sheep sera for the presence of serum antibodies. The GST-p26 fusion protein was applied in an indirect ELISA to screen eight sera (MPMV as positive antiserum, sera from six known positive sheep, and one negative serum). Preliminary results were encouraging in that six out of eight sera were positive and the negative was not reactive (see Table 2.8). Unfortunately, when the capsid gene of another retrovirus i.e. HIV was used as a negative control it turned out that the same sheep sera were positive against this antigen (Table 2.9 and 2.10). It was likely that the sheep had antibodies to some of the co-purified *E.coli*

proteins or to the fusion protein that was derived from *S. japonicum*, which is a nematode that could have infected these sheep. Kwang in 1995 and Ortin in 1998 made a similar observation when they claimed that the reactivity reflected the presence of antibodies to the GST fusion partner of the recombinant antigen used in the assay (Sharp and Herring, 1983; Ortin *et al*, 1998; Kwang *et al*, 1995).

The non-specificity of the reaction was confirmed when we showed that the reaction could be competed out using the HIV expressed fusion protein. We concluded that the non-specificity of the reaction might be due to interactions between antibody in the sera and proteins that contaminate the antigen preparation, such as *Escherica coli* proteins as well as the GST component of the fusion protein that is derived from the parasitic helminth, *S. japonicum* (Smith and Johnson, 1988). Observations made show that although the sheep sera were cross-reacting with the GST portion of the two expressed proteins GST-JSp26 and GST-HIVp24, it was also possible that there could be some cross-reaction between GST-HIVp24 and other sheep retroviral proteins that also cross-react with the GST-JSp26 protein that is being investigated. The presence of antibodies against GST in the sheep sera could also be explained if the sheep had been exposed to helminths such as nematodes or trematodes. The Bruwer serum was also cross-reactive with the control HIV p24 that contained the GST fusion protein. We take into account the possibility that there may be cross reactive retroviral proteins that cross react to HIV p24. It is also possible that there may be unpurified E coli proteins in the purified preparations of the expressed proteins that account for the cross reacting proteins. The fact that there was no difference between the JS p26 and the HIV p24 antigens does suggest that the reaction was not specific, this is in line with the contention that there are no circulating antibodies to JSRV in sheep (Ortin *et al*, 1998).

All evidence so far supported the theory that JSRVp26 did not stimulate a humoral immune response in sheep infected with the exogenous JSRV. We therefore decided to investigate whether the antibody response was localised to the lungs of the infected sheep. Earlier experiments by York 1984 had investigated and reported on the presence of JSRV specific antibodies in the lungs of JS affected sheep that were not present in the lung lavage of normal unaffected sheep. The predominant antibodies were of the IgA sub class. A criticism of these early experiments was that the virus was not pure therefore the positive reactions could be due to cross reaction of the antibodies with non-viral proteins that had co-purified with the virus. Since we now had relatively pure viral antigens these

experiments could be repeated. To investigate this, lung lavage samples from infected and normal sheep lungs were analysed for the presence of JS anti-p26 antibodies. To control for the integrity (presence and reactivity of the antibodies in these samples which were over 10 years old), a monoclonal antibody Mab 59G11 (Mab 59G11 is specific sheep IgA) (De Martini and York, 1992) was used. An in-house JS p26 peroxidase conjugate and antibody capture assay (Table 2.15) was used to demonstrate that there were anti-p26 antibodies in the lung wash samples from seven out of eight affected sheep. Only one of eight unaffected sheep lung wash was positive. Subsequently, a larger sample size was investigated using this assay and it was found that the assay was very specific but lacked sensitivity (Tables 2.16 and 2.17). The low sensitivity (31%) of the assay could be due the fact that the lung samples were about 15 years old or that there was a low titre of antibodies in the lungs. Nevertheless, the results were encouraging and in line with earlier findings that suggested that there was a specific localised immune response to JSRV in the lungs of JS affected sheep. However, in view of the finding that quite a few JS affected lung wash samples were negative, we felt we needed to investigate alternate diagnostic options further.

One of the earliest and most useful facts from a diagnostic perspective was the observation that MPMV antisera detected JSp26 in all lung wash samples and nasal fluids. We therefore decided that we should develop an antigen capture assay. Ideally we required a JS p26 specific antibody or polyclonal antisera. Extensive work went into the production of high titred JSp26 antisera in chickens. Unfortunately, a more detailed analysis of the antisera revealed that a significant proportion of the antisera were against the fusion portion of the JSp26 protein. The fusion protein was also 26 kDa (based on literature, Smith and Johnson, 1988)), the identical size to the JSRVp26 protein. Attempts to absorb out the antibodies to the fusion protein were partly successful. Subsequently consideration was given to cloning the JSp26 gene into another expression vector so as to remove the fusion protein. At this stage, we fortunately received an antiserum to the JSp26 protein from Professor DeMartini (University of Colorado, Fort Collins). This enabled us to investigate the use of this antiserum in an antigen capture assay. We confirmed the specificity of the antiserum using a Western immunoblot assay and went on to show that the antigen capture assay detected JS antigen in the lung fluid of JS affected sheep lung lavage and not in normal lung lavage. Even though only a limited number of sheep lung lavage were available for testing (Table 2.22) the results were very encouraging and

showed that it was possible to use the JSp26 antigen capture assay to detect JSp26 in lung lavage and nasal fluid (sensitivity of 80% and specificity of 100%).

Using the JSRVp26 expressed protein and antisera; we essentially confirmed earlier observations. The conclusions from this study can be summarised as follows:

1. That there is a poor humoral response to the viral proteins. However this could be restricted to the JSp26 protein as it was shown that an identical gene is present in normal Australian sheep not believed to have Jaagsiekte.
2. That there is a localised immune response in the lungs of JS affected sheep.
3. That there is a lot of virus present in the lungs and nasal fluids of affected sheep and that a JSp26 antigen capture assay could be used to identify JS affected sheep

In view of the present findings, it was felt that a serological/antigenic approach to diagnosing JSRV was probably not the best method. More emphasis needed to be given to investigate the molecular diagnostic approach.

The following chapter, therefore, focused on a molecular approach to detect the exogenous JSRV in OPA affected sheep.

CHAPTER 3

CHAPTER THREE

OPTIMIZATION AND ASSESSMENT OF A MOLECULAR APPROACH TO IDENTIFY THE EXOGENOUS INFECTIOUS FORM OF JSRV

3.1 INTRODUCTION

The development of a molecular diagnostic assay to identify sheep infected with JSRV/OPA should have been straightforward once the infectious JSRV genome was sequenced (York *et al*, 1992). Unfortunately, the finding that there are as many as 15 to 20 endogenous JSRV loci in all sheep genomes (York *et al*, 1992; Hecht *et al*, 1994), made the designing of a molecular assay for the exogenous JSRV more challenging. Studies were thereafter designed to assess the presence of any sequence differences between the endogenous and exogenous JSRV-related viruses that could be used to differentiate the two viral forms (York *et al*, 1992; Palmarini *et al*, 1996a; Bai *et al*, 1996 and Palmarini *et al* 1996b). Initially the *gag* and LTR regions of both exogenous and endogenous JSRV were sequenced and compared (Bai *et al*, 1996). One of the first useful differences that were identified was a unique *Sca*1 restriction site (at position 1729) that was present in exogenous JSRV but not in endogenous forms of the virus. This *Sca*1 site was found to be exclusively present in the cDNA of OPA-affected sheep and not in unaffected control sheep (Palmarini *et al*, 1996a).

Other regions of the genome that have been studied include the U3 region of the long terminal repeats [LTR's] (Palmarini *et al*, 1996b) and the Variable one and two regions found in the matrix (MA) of the *gag* gene (Hallwirth, 2004).

A comparison of the LTR region of the exogenous JSRV with three endogenous JSRV loci revealed some conserved differences between the endogenous and exogenous genomes, in this region. These differences in the nucleotide sequences could be responsible for a reduction in the pathogenicity of exogenous JSRV compared to the endogenous JSRV

(Palmarini *et al*, 1996b) but for our purposes they could be used to design a molecular assay that could distinguish the two viruses.

To accentuate these differences in the LTR region of both endogenous and exogenous JSRV, a long range PCR whose primers were based on the exogenous JSRV sequences, was developed to amplify and characterize six endogenous retroviral loci (Bai *et al*, 1996). These loci could be differentiated based on their restriction enzyme profile and proviral genomic size. The endogenous restriction map profiles revealed that the full-length endogenous loci could be distinguished from their exogenous JSRV counterparts in the same tumour cell by the following restriction sites [*Nde*I, *Hind*III, *Sca*I, *Kpn*I and *Eco*R1] (Bai *et al*, 1996). Exogenous JSRV from different breeds had a similar restriction map profile. A more detailed analysis at the nucleic acid sequence level revealed that the endogenous LTR sequences were 94 to 98% identical amongst endogenous JSRV from different breeds particularly in the R and U5 region between the endogenous loci and the exogenous JSRV, due to deletions and many point mutations in the U3 of the LTR (Bai *et al*, 1996). The endogenous LTR's are longer (442-445 bp) than the exogenous JSRV (York *et al*, 1992) sequence (397 bp) and there are two large deletions of 30bp and 16 bp at positions 198 and 261, respectively (Palmarini *et al*, 1996b).

The differences identified in the LTR's, *gag*, *env* and *orf-X* genes of some endogenous JSRV loci have been useful in distinguishing the endogenous viruses from the exogenous viruses, but these data were obtained from PCR amplifications of portions of endogenous proviruses and not from full length sequences in the case of the endogenous viruses (Bai *et al*, 1996; Palmarini *et al*, 2000). In 2000 Hallwirth *et al* cloned and sequenced three full-length endogenous JSRV loci that have been called enJS5.6A1, enJS5F16, and enJS5.9A1. This was the first time that it was possible to compare the full-length genomes of endogenous and exogenous JSRV's. When compared to the exogenous JSRV, the exJSRV isolates were consistently distinguishable from the enJSRVs in the structural genes in three variable regions called VR1, VR2 and VR3. VR1 and VR2 are found in the matrix (MA) region of *gag*, whereas VR3 is located in the transmembrane region of the *env* gene (Palmarini *et al*, 2000). VR1 covers nucleotides 624 to 661 (based on JSRV 21) and is a proline rich area. Downstream of VR1 (50 amino-acid residues), another region of polymorphism between the exogenous and endogenous viruses is located and termed VR2. It is fairly rich in proline for both enJSRV and exogenous JSRV (Palmarini *et al*, 2000).

Based on the differences in the VR1 and VR2, a molecular assay using specifically designed primers was developed to amplify exogenous specific isolates (Hallwirth, 2004).

In this chapter, use will be made of the restriction enzyme and sequence differences that have been identified between JSRV and JSRV-related endogenous sequences to specifically amplify exogenous JSRV that may be present in a background of endogenous virus and to detect it in OPA affected animals (Palmarini *et al*, 1996a; Palmarini *et al* 1996b and Palmarini *et al*, 2000).

The first molecular assay that is investigated targets the LTR-*gag* region. A hemi-nested LTR-*gag* PCR was optimized to amplify a region in the *gag* gene of both the exogenous JSRV and enJSRV related virus present in affected and unaffected tissue, and to differentiate them by restriction enzyme digestion using *Sca1* (second *Sca1* site) present only in the exogenous form at nucleotide position 1729 (York *et al*, 1992). This is a definite molecular marker that can be used to identify possible reservoirs of OPA infection (De Martini and York, 1997).

The second assay focused on variable regions 1 and 2 (VR1 and VR2). This assay uses a one step PCR that should only amplify the exogenous virus (Hallwirth, 2004).

The third molecular assay targets a region that is known to have major differences between the endogenous and exogenous viral sequences of JSRV i.e. the U3 region of the JSRV LTR. This assay uses a sensitive hemi-nested (hn) PCR approach. The outer primers amplify both the endogenous and exogenous forms of JSRV and an internal hemi-nested primer that is specific for the exogenous forms of JSRV. This approach is both sensitive and specific (Palmarini *et al*, 1996b).

In summary, this chapter will investigate, assess and modify (when needed) three molecular assays designed to identify the exogenous JSRV but equally important to differentiate the exogenous from the endogenous form. These three molecular assays are summarized as:

1. A LTR-*gag* based PCR that flanks a unique *Sca1* restriction site that is only present in the exogenous form of JSRV.

2. A PCR based single step assay that targets the exogenous variable regions 1 and 2 (VR1 and VR2) and
3. A hemi-nested PCR assay that targets the U3/LTR region of JSRV.

These assays will be assessed for sensitivity and specificity against exogenous and endogenous cloned DNA templates as well as formalin preserved sheep lung tumor samples from positive and negative sheep.

3.2 MATERIALS AND METHODS

3.2.1 Source of DNA

3.2.1.1 Cloned DNA

Three endogenous proviral clones penJS5.6A1, penJS5F16 and penJS5.9A1 were obtained by screening a lambda phage library from a sheep with JSRV induced OPA (Palmarini *et al*, 1999). The lengths of the clones were 6915 bp for enJS5F16, 7939 bp for enJS5.6A1 and 6695 bp for enJS5.9A1 (Palmarini *et al*, 2000). Clones provided by Dr. D.F. York.

Plasmid mini-preps (QIAprep®Spin Miniprep Kit, Qiagen, GmbH, Germany) were used to purify plasmid DNA for each of the clones. OD ratios at 260nm and 280nm were used to determine DNA purity and concentration.

3.2.1.2 JSRV21

pJSRV21 is a full length JSRV proviral clone isolated from a genomic DNA library derived from a natural case of OPA. The full-length provirus is 7834 bp in length and represents the exogenous infectious form of JSRV (Palmarini *et al*, 1999). The sequence of JSRV21 is 7455 bp in length. The cytomegalovirus promoter-driven infectious expression construct pCMV2JS21 is 11,537 bp in length.

3.2.1.3 JS7 DNA

The JS7 cell line originated from a culture of OPA tumour tissue obtained from an adult sheep (Scottish Blackface) with naturally occurring disease in Scotland in 1985 (Jassim *et al*, 1987) was provided by Professor J. De Martini. The JS7 cell line has a single integrated copy of the exogenous JSRV in each cell. An infectious and pathogenic JSRV provirus called JSRV_{JS7} was isolated and cloned from this cell line. The full-length provirus is 7841 bp in length. (De Martini *et al*, 2001). Please note that DNA extracts of these cells will also contain endogenous JSRV DNA.

3.2.1.4 JSRV 382 plasmid

JSRV 382 (position 953-3029) was one of the cDNA clones that was isolated from Lambda Zap 11 vector (Stratagene). The cDNA library was made from virus purified from a South African sheep lung that was infected with JSRV. The cloning into a Bluescript vector was performed at the Veterinary Research Institute, Onderstepoort, Pretoria by Dr D.F.York. The *gag* and *pro* genes of the JSRV genome formed the JSRV 382 plasmid (York *et al*, 1992).

3.2.1.5 Lung Tumour DNA

Dr. J. Vorster, who had been based at the Onderstepoort Research Institute in Pretoria, donated the 22 sheep lung tumours that had been used in this study. The tumours were preserved in formalin. The tumour samples were examined macroscopically and histologically to confirm the diagnosis of OPA in the sheep (Vorster, personal communication). To extract the DNA, 200mg of tissue was sliced from each section using aseptic conditions. The scalpel blade was dipped into 99% ethanol and flamed between different samples to prevent cross- contamination. The tissue was sliced finely into pieces and then subjected to genomic DNA isolation as described earlier (see section 2.2.1.1).

3.2.1.6 Australian Sheep DNA

Genomic DNA was isolated from three unaffected Australian sheep referred to as LPW, MSCU, 230909, and lung (normal lung-from a normal South African sheep). Australia has

not had any reports of JSRV and it is believed the Island is JSRV free. As all sheep have the endogenous virus, the Australian sheep are a good source of negative material. (See section 2.3.1. for source of tissue).

3.2.1.7 Other sources of DNA

DNA also used in the assays included:

- Genomic DNA from sheep 4844 (South African sheep with natural OPA).
- Genomic DNA from whole blood of three local unaffected merino sheep (Claus Hallwirth, Univ of KZN)
- Plasmid DNA from clone pJSRV120 (concentration of 2ng was used) obtained from Dr. D.F. York. (see York *et al* 1992).

3.2.2 DNA Extraction Methods

Genomic DNA was extracted from lung tissue using the BIO 101 Fast DNA Kit (B10 101, Inc., La Jolla, California, USA) with the Fast Prep™ Instrument as mentioned in Methods (section 2.2.1.1.1). The DNA obtained with this method was suitable for PCR purposes. The DNA purity and concentration were determined as described below (see section 3.2.3).

3.2.3 DNA Quantitation

Extracted DNA was diluted 1/200 with sterile water and the absorbance measured at both 260 and 280nm using the Beckman Du^R-5 Spectrophotometer (Beckman Instruments Inc. Fullerton, California) as described in Methods 2.2.1.2.

3.2.4 The Polymerase Chain Reaction

3.2.4.1 PCR Primers

The nucleotide sequence and positions of the oligonucleotide primers used have been numbered according to the JSRV genome (York *et al*, 1992) and are shown in Table 3.1.

TABLE 3.1: Primers Utilized in the PCR Reaction

LTR-gag with Sca1 digest	Sequences	Positions*
P1	TGG GAG CTC TTT GGC AAA AGC C	7210 – 7224
P2	ATA CTG CAG CCC GAT GGC CAG	1806 – 1826
P5	GCT GCT TTG AGA CCT TAT CGA AA	1600 -1622

(Palmarini *et al*, 1996a)

VR1/VR2 PCR	Sequences	Positions*
JS21-613f	CTA GTT ATG ATC CTC CTC CTC CTC CT	613 – 638
JS21-976r	AAG GCC CTT TGT AGG GGA GAC AT	953 - 976

(Hallwirth et al, 2004; Hallwirth, C., personal communication)

U3/LTR H-nested PCR	Sequences	Positions*
P1	TGG GAG CTC TTT GGC AAA AGC C	7210 – 7224
P3	CAC CGG ATT TTT ACA CAA TCA CCG G	7361 -7385
P3M	CAC CGG ATT TTT ACA CAA TCA CCG	7361 -7384
P4	TGA TAT TTC TGT GAA GCA GTG CC	7316 – 7338

(Palmarini *et al*, 1996a)

*Nucleotide numbering is based on the JSRV sequence published by York *et al*, 1992

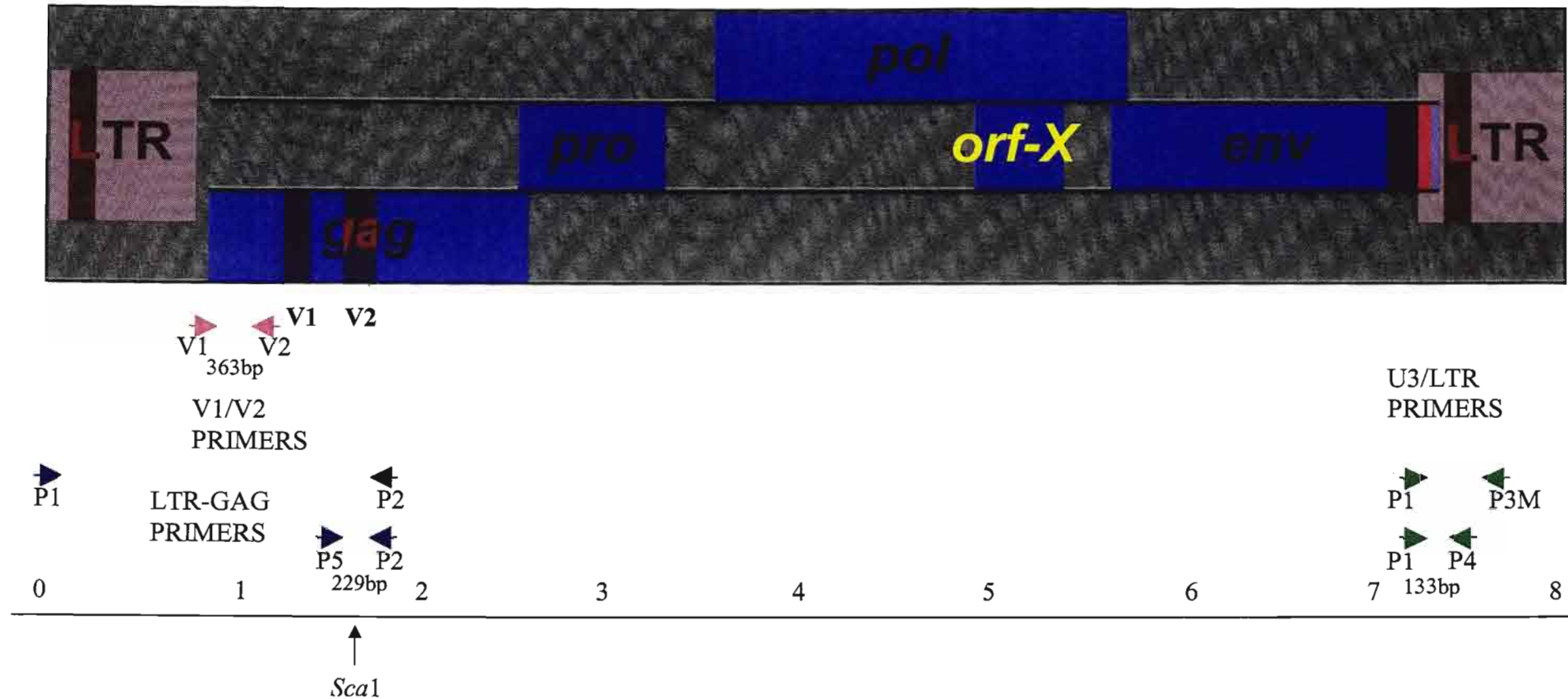


Figure 3.1 Genomic structure of JSRV showing the approximate location of primers used as indicated by arrows for the three molecular assays used in this Thesis: LTR-*gag* hemi-nested (*), V1/V2 (*) and the U3/LTR hemi-nested (*) PCR assays. Also shown are the sizes of the amplified products in bp. In the case of the LTR-GAG product, the position of the *ScaI* site is indicated. The numbered bar at the bottom indicate distances in kb.

3.2.4.2 Single Step PCR

The following reagents (Table 3.2) were used in a one-step PCR.

Table 3.2 Reagents used in the PCR reactions

Reagent	Volume for reaction		
	Variable region 1 and 2 PCR	LTR-Gag PCR	U3/LTR PCR
10X reaction buffer with 2.5mM MgCl ₂	5µl	5µl	5µl
dNTP mix 2.5Mm stock	4µl (200µM)	10µl (500µM)	4µl (200µM)
Forward primer	1µl (20ρmoles)	1µl (15ρmoles)	1µl (6.25ρmoles)
Reverse primer	1µl (20ρmoles)	1µl (15ρmoles)	1µl (6.25ρmoles)
*Template	xµl	yµl	zµl
2% DMSO	0µl	1µl	0µl
DH ₂ O	39µl-x µl	32µl-yµl	39µl-zµl
Taq 1u/µl	2µl	2.5µl	1.5µl
Final volme	50µl	50µl	50µl

*Dilutions of DNA templates from 1ng down to 1fg were used in a total volume of 5µl

x, y, and z refer to volumes of DNA template.

Plasmid DNA from JSRV 21 (100ng/µl), JSRV 7 (813ng/µl), enJSRV5.6A1 (216ng/µl), enJSRV5.9A1 (174ng/µl), enJSRV5F16 (250ng/µl) and JS 382 (6200ng/µl) were diluted to 1ng/µl. Ten-fold dilutions ranging from 100ρg/µl to 1fg/µl were made from the 1ng/µl dilution. These 10-fold dilutions were used such that 5µl contained for example 1ng and this was added to 45µl of PCR master mix containing the reagents as seen in Table 3.2.

Exogenous plasmids DNA from clone JS 120 (1ng/µl) and from the three JSRV endogenous plasmids were also used at concentrations of 2ng in the PCR. Genomic DNA from sheep 4844 infected with JSRV/OPA, and from lung tumours of 22 sheep infected with JSRV were included. Endogenous JSRV genomic DNA templates were provided by three Australian sheep LPW, 230909, and MSCU, three merino sheep, and lung from normal sheep. The genomic DNA templates were diluted such that there were 500ng of

DNA per 10µl added to 40µl of PCR master mix containing the reagents as seen in Table 3.2

The PCR cycling conditions for the PCR assays are presented in a tabulated form in Table 3.3. The Eppendorf Gradient cycler (Eppendorf, Netheler-Hinz. GmbH, Germany) was used for the purposes of the VR1 and VR2 PCR. The Perkin Elmer 9600 thermal cycler (P.E. Biosystems) was used for the other PCR reactions (LTR-*gag* and U3/LTR PCR).

Table 3.3 PCR Cycling conditions

PCR Cycling Conditions								
VR1 and VR2 PCR			LTR- <i>gag</i> PCR			U3/LTR PCR		
1 cycle	94°C	5 min	1 cycle	92°C	2 min	1 cycle	94°C	1 min
35 cycles	94°C	35 sec	10 cycles	92°C	10 sec	35 cycles	94°C	30 sec
	55,6 +/- 0,3 °C	50sec		61°C	30 sec		59°C	1 min
	72°C	25 sec		*68°C	90 sec		72°C	1 min
			*11-30 cycles: progressive increase of 20 sec per cycle for step 3					
extension	72°C	5 min	extension	68°C	7 min	extension	72°C	3 min

3.2.4.3 Hemi-nested PCR

The following PCR assays (LTR-*gag* and U3/LTR) were hemi-nested with differing concentrations of reagents and cycling conditions as shown in Table 3.4. One microlitre of the first round of amplification from the first step (section 3.2.4.2) PCR reaction was added to 49µl of second round PCR master mix. The reagents and volumes for this master mix are presented in Table 3.4.

Table 3.4 **Reagents and volumes (μl) used in PCR reactions**

Reagent	Volume for reaction	
	LTR- <i>gag</i> hn PCR	U3/LTR hn PCR
10X reaction buffer with 2.5mM MgCl ₂	5.5μl	5μl
dNTP mix 2.5mM stock	4μl	4μl (200μM)
Forward primer	1μl	1μl (6.25ρmoles)
Reverse primer	1μl	1μl (6.25ρmoles)
*Template	1μl	1μl
DH ₂ O	37.5μl	38μl
Taq 1u/μl	1.25μl	1.5μl
Final volume	50μl	50μl

Cycling conditions for the hemi-nested PCR differ to that of the one-step PCR. These cycling conditions are presented in Table 3.5.

Table 3.5 **Cycling condition for hemi-nested PCR**

PCR Cycling Condition for Hemi-nested assays					
LTR- <i>gag</i> Hemi-nested PCR			U3/LTR Hemi-nested PCR		
1 cycle	94°C	1 min	1 cycle	94°C	1 min
35 cycles	94°C	45 sec	35 cycles	94°C	30 sec
	57°C	60 sec		57°C	1 min
	72°C	60 sec		72°C	1 min
extension	72°C	2 min	extension	72°C	3 min

3.2.4.4 Restriction Digest of PCR fragments

Half of the PCR product (25µl) obtained from the hemi-nested LTR-*gag* PCR in section 3.2.4.3 was digested with 10u of *Sca*1 (Roche Diagnostics, Mannheim, Germany) enzyme and the appropriate buffer H supplied by the manufacturer at 37°C for one hour in a Labotec incubator. The digested products were run in parallel with the undigested products on a 2% ethidium bromide stained agarose gel together with a DNA molecular standard (Roche Diagnostics, Mannheim, Germany).

3.2.4.5 Sensitivity Titration

To investigate the sensitivity of the assays, five plasmids (see section 3.2.4.2) were titrated starting at 1ng and going down to 1fg to determine which of the three PCR assays (see sections 3.2.4.2 and 3.2.4.3) was specific and sensitive enough to be used to test further infectious tumour samples.

3.2.4.6 Agarose Gel Electrophoresis

Amplified products were detected by electrophoresis of 10µl of sample through a 2% agarose gel containing 0.5µg/ml ethidium bromide (Chapter 2: Methods-section 2.2.1.8.1). A more concentrated agarose is used here because the final amplified product was between 90 bp to 250 bp in size. The amplified products were run alongside molecular weight marker 1X (Roche Diagnostics, Mannheim, Germany).

3.2.4.7 Viewing and Photography of Gels

Agarose gels were visualized using a SYNGENE Gene Genius imaging system that is comprehensive and fully automated gel documentation and analysis system (SYNGENE, a division of Synoptics Ltd, UK) that is attached to a computer. Once the image is captured, it can be stored or improved using the Gene tool software and eventually printed using the HP Laser Jet 1100 printer.

3.3 RESULTS

3.3.1 An Assessment of three Molecular Assays to determine their sensitivity and ability to distinguish the endogenous from the exogenous infectious form of JSRV

Three endogenous plasmids (penJSRV5F16, penJSRV5.6A1 and penJSRV5.9A1), an infectious JSRV clone JSRV21 and JS7 DNA were diluted 10-fold from 1ng down to 1fg and used as templates for the three molecular assays, the LTR-*gag* hemi-nested PCR, Variable region 1 and 2 and the U3/LTR hemi-nested PCR. The genomic structure of JSRV showing the approximate locations of primers used in the three molecular assays mentioned can be seen in Appendix B.

3.3.1.1 Titration of Plasmids using the LTR-*gag* hemi-nested PCR and *Sca1* enzyme digestion

Primers P1 and P2 (see methods Table 3.1) were used in the first round PCR to amplify both the endogenous and exogenous target gene that flanked the unique *Sca1* site. To improve the sensitivity, a hemi-nested second round of amplification was performed using primers P5 and P2 (see Table 3.1). The second round amplified a *gag* product of 229 base pairs (see Fig. 3.2) for JS 7 (exogenous and endogenous JSRV), JS 21 (exogenous), JS382 (exogenous), JS5.6A1 (endogenous), JS5.9A1 (endogenous) and JS5f16 (endogenous). The amount of DNA used was the same for each of the clones and the lanes at which the DNA is detected are shown in bold.

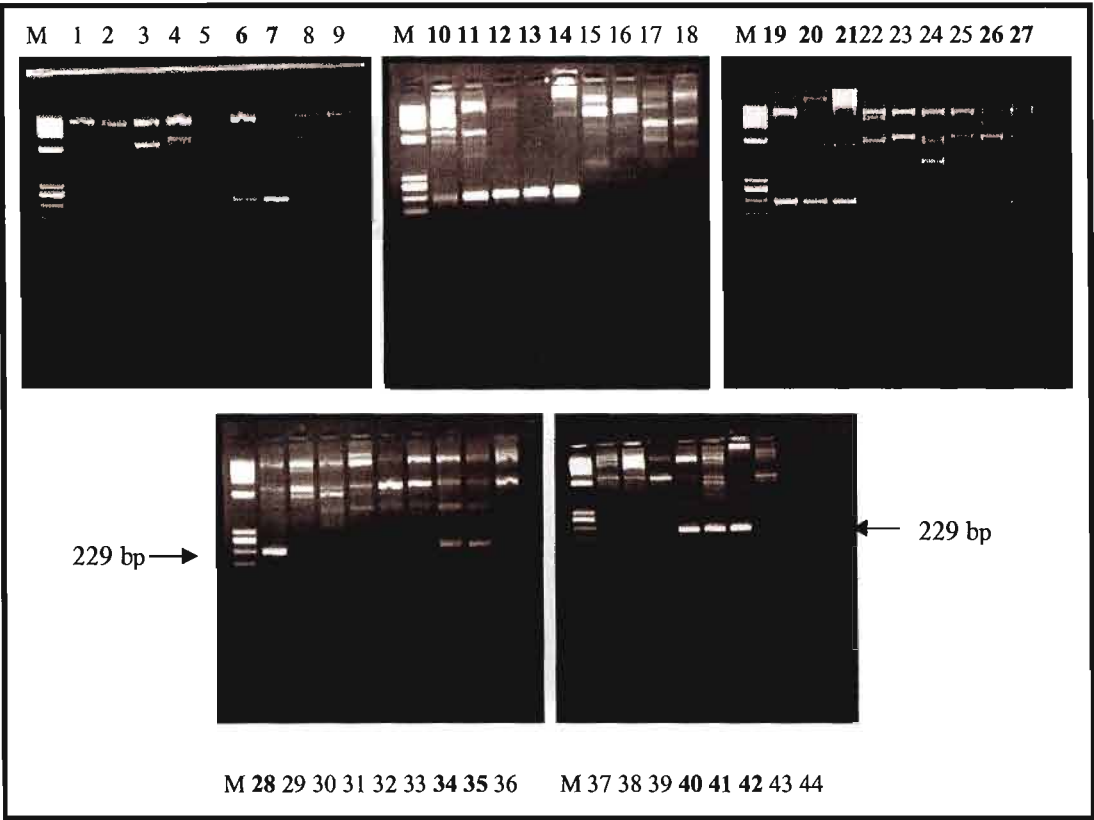


Figure 3.2 Ethidium bromide agarose gel (2%) analysis of the hemi-nested LTR-gag PCR showing different dilutions of exogenous and endogenous JSRV plasmids to determine the specificity and sensitivity of the test

Lanes M. Marker 1X, **Lanes 1-7** dilutions of en+exJS 7 from 1 fg, 10 fg, 100 fg, 1 μ g, 10 μ g, 100 μ g and 1 ng; **Lanes 8-14** dilutions of exJSRV 21; **Lanes 15-21** dilutions of exJS 382 from 1 fg to 1 pg; **Lanes 22-28** of enJS5.6A1; **Lanes 29-35** of enJS5.9A1; **Lanes 36-42** of enJS5F16 showing a product size of 229 bp before *Sca*1 digestion. **Lane's 43-44** negative controls. The lanes in bold indicate those concentrations at which the 229 bp product was detected.

On digestion of the 229 bp products with *Sca*1, only the exogenous infectious clones were digested to two products of sizes 131 bp and 98 bp (see Fig. 3.3). This *Sca*1 restriction site in the *gag* gene represents an important molecular marker for the exogenous form of JSRV. The result of this hemi-nested LTR-gag PCR titration is presented in Figure 3.2 and that of the *Sca*1 restriction enzyme digest in Figure 3.3. Note the two products of 131 bp and 98 bp that are only present in the exogenous form. Figure 3.3 also shows lane 4, lane 5 and lane 6 as uncut fragments because these are endogenous JSRV clones and do not have the second *Sca*1 site.

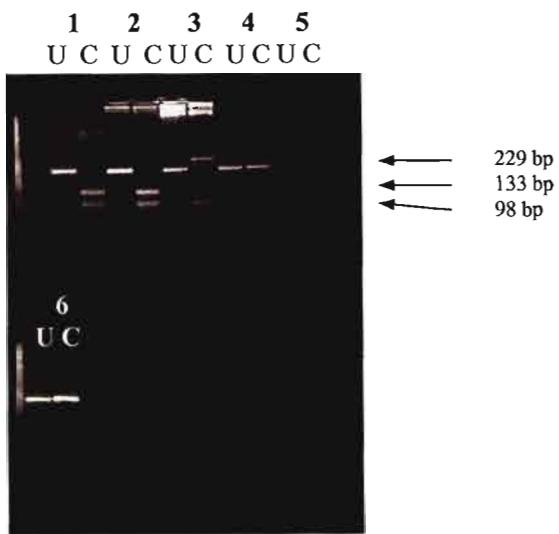


Figure 3.3 Ethidium bromide stained agarose gel (2%) analysis of JSRV LTR-gag PCR products digested with restriction enzyme *ScaI*

Lane 1: 1ng ex+enJS7, Lane 2: 1ng exJSRV21, Lane 3: 1ng exJS382, Lane 4: 1ng enJSRV5.6A1, Lane 5: 1ng enJSRV5.9A1 and Lane 6: 1ng enJSRV5F16. U represents uncut PCR product and C-cut PCR product.

3.3.1.2 An investigation into the sensitivity and specificity of the Variable Region 1 and 2 PCR

The variable region 1 and 2 (VR1 and VR2) PCR targets and amplifies a product of approximately 363 bp that should only be amplified if the exogenous form is present. Primers 613f (primer that binds upstream of VR1) and 976r (primer that binds downstream of VR2) were used in a one-step PCR reaction on a titration of the cloned DNA. The sequence alignment for the V1 and V2 regions of the exogenous JSRV 21, JSRV 382 or JSRV-SA and the endogenous enJS5.9A1, enJS5.6A1 and enJS5F16 is shown in Appendix B. The results of this titration can be viewed in Figures 3.4.

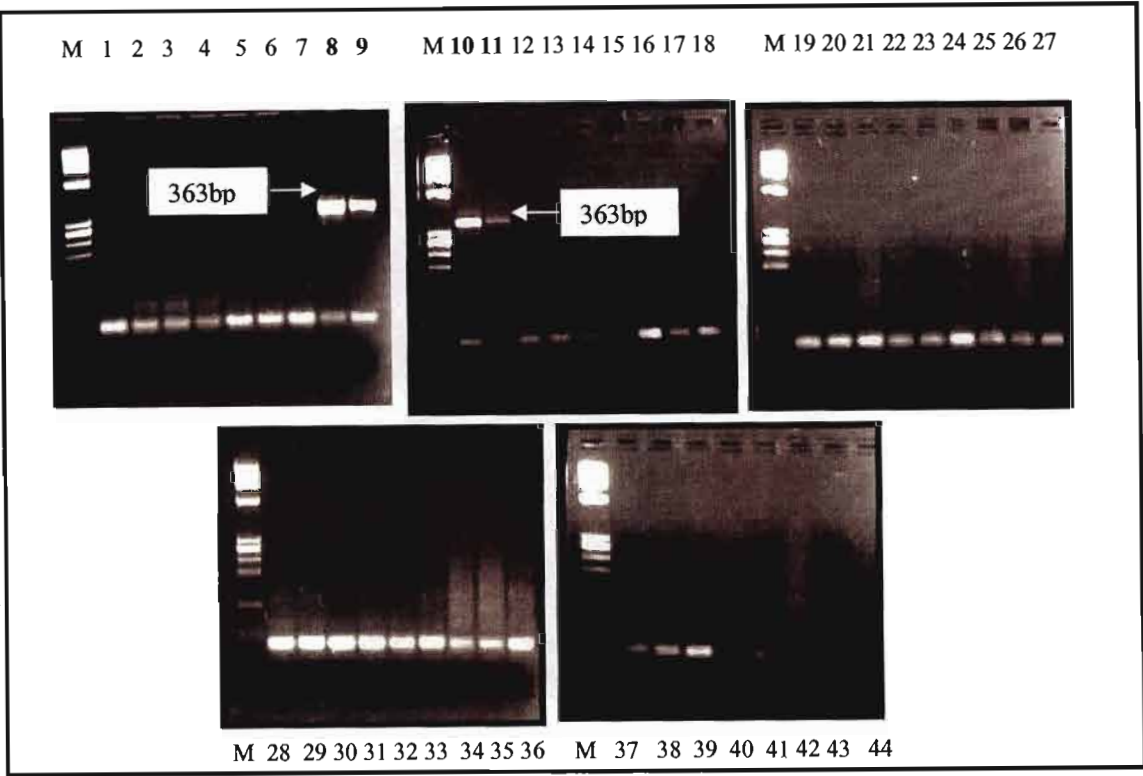


Figure 3.4 Ethidium bromide stained agarose gel (2%) depicting the results of the Variable region 1 and 2 PCR. The templates were exogenous and endogenous JSRV plasmids ranging from 1 ng to 1fg.

Lanes M: Marker IX, Lanes 1-7: ex+enJS 7 DNA, Lanes 8-14: exJSRV 21, Lanes 15-21: exJS 382, Lanes 22-28: enJSRV5.6A1, Lanes 29-35: enJSRV5.9A1, Lanes 36-42: enJSRV5F16 and Lanes 43-44: Negative controls.

3.3.1.3 An assessment of the JSRV U3/LTR hemi-nested PCR

The sensitivities of the JSRV U3hn PCR were improved using a hemi-nested PCR. A primer internal to the PCR product, primer 4 (see Table 3.1) was paired with primer 1(see Table 3.1) in a second round hemi-nested PCR (JSRV U3hn PCR). The specificity of the assay was determined by the inclusion of three endogenous plasmid controls as part of the investigation. The hypothesis being to use an outer primer pair that amplified both the endogenous and exogenous viral genomes, but in the second round, only the exogenous forms would be amplified and detected due to the one internal primer being exogenous specific. Obviously, being a hemi-nested PCR had the disadvantage of amplicon contamination. The outcome of this titration using the JSRV U3-and hemi-nested PCR is presented in Figure 3.5. An amplicon of 176 bp was expected after the first round, but only if the exogenous template is present should one detect a 133 bp product.

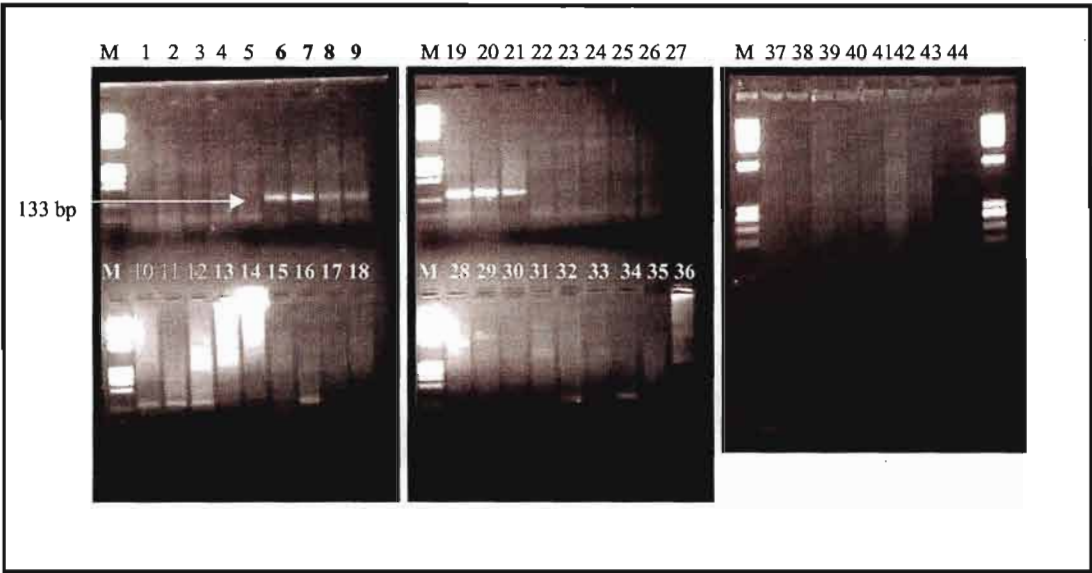


Fig 3.5 Ethidium bromide stained agarose gel (2%) showing titration of JSRV plasmids with the JSRV U3/LTR hemi-nested PCR

Lanes M. Marker 1X, Lanes 1-7: 1fg to 1ng of ex+enJS7, Lanes 8-14: 1fg to 1ng exJSRV21, Lanes: 15-21: 1fg to 1ng exJS382. Lanes 22-28: 1fg to 1ng enJSRV5.6A1; Lanes 29-35: 1fg to 1ng enJSRV5.9A1, Lanes 36-42: 1fg to 1ng enJSRV5F16 and Lanes 43-44: Negative controls. Note the partial amplification of products in lanes 32-35 (enJSRV5.9A1)

3.3.2 An Investigation into the use of an exogenous specific *Sca1* site to differentiate between endogenous and exogenous LTR-*gag* sequences using lung tumour samples

The products obtained upon amplification using primers P1 and P2 (Table 3.1) were subjected to a second round of amplification using primers P5 and P2 (Table 3.1). The second round amplification amplified a gag product of 229 base pairs. Restriction enzyme digest of this product using *Sca1* revealed a *Sca1* site that was present only in the exogenous OPA affected tissue, but was absent in the endogenous unaffected tissues. This restriction site in the *gag* gene represents an important molecular marker for the exogenous form of OPA

3.3.2.1 Amplification of Endogenous LTR-*gag* Sequences

A product of 229 base pairs was detected in clone JSRV382; clone JSRV120, sheep 4844 (South African sheep affected with OPA), DNA7 (Scottish strain of JSRV-Note that this genomic DNA is from the cell line JS7 which also has some endogenous JSRV DNA) that were used as positive controls as well as ten endogenous sheep samples. The results of the hemi-nested LTR-*gag* PCR are presented in Figure 3.6.

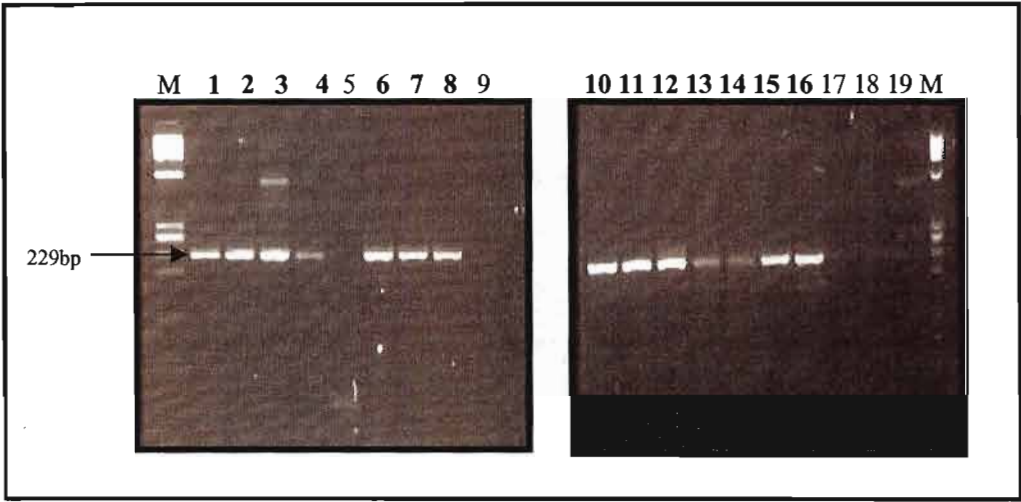


Figure 3.6 An Ethidium Bromide stained agarose gel (2%) showing the hemi-nested LTR-*gag* PCR products of JSRV382, JSRV120, sheep 4844, DNA7 and ten endogenous sheep controls

Lane 1 Sheep 4844 with OPA, Lane 2: JSRV 382, Lane 3: JSRV 120, Lane 4: DNA 7, Lanes 5, 9 and 19: Negative control (water), Lanes 6, 7, and 8: Full-length endogenous JSRV, Lanes 10, 11 and 12: Three Australian tissue samples from unaffected sheep, Lanes 13, 14 and 15:are three unaffected merino sheep, Lane 16; Normal sheep lung, Lane 17 and 18: sheep lung tumourC5 (very faint bands seen). Lane M: Marker 1X (Roche Diagnostics).

The results presented in Figures 3.7 are those of the amplified products followed by digestion with *Sca*1 restriction enzyme to enable differentiation of endogenous from exogenous sheep DNA. The successful digestion is indicated by the presence of two smaller fragments of 131 and 98 base pairs as seen in Figure 3.7

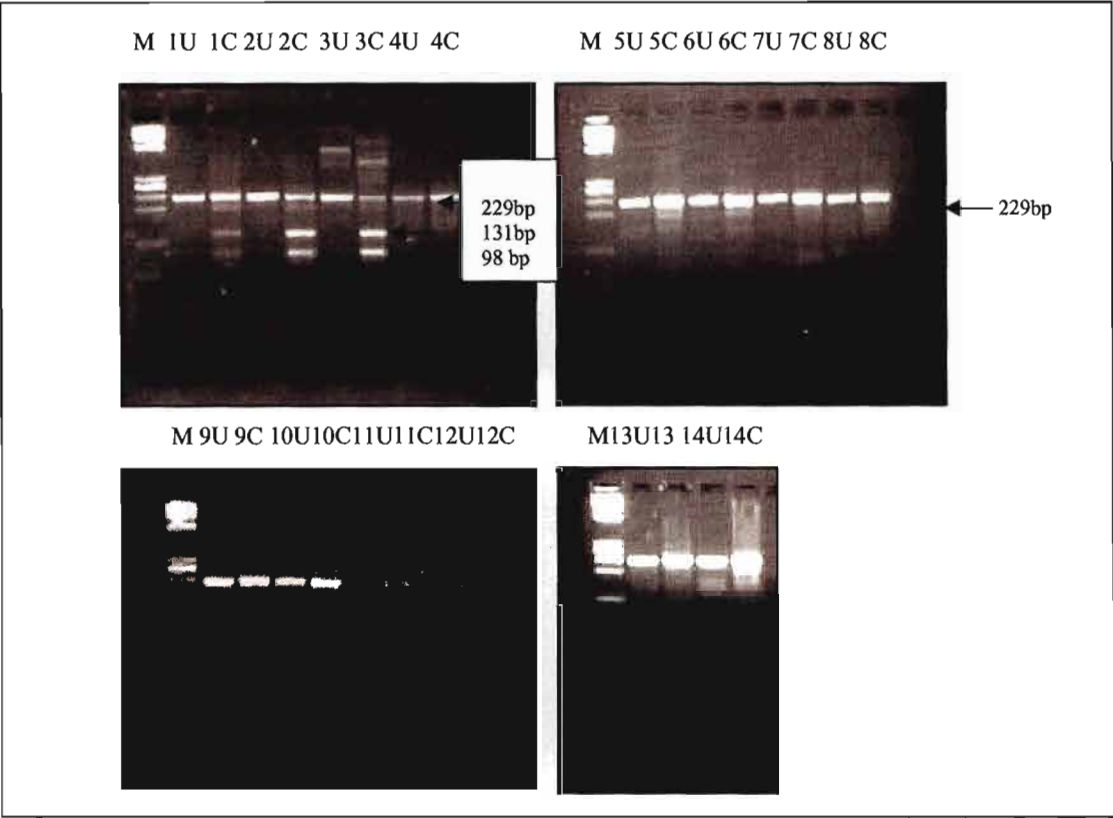


Figure 3.7 Ethidium bromide stained agarose gel (2%) of LTR-*gag* PCR products that have been digested with *Sca1* restriction enzyme together with the undigested PCR products. Two smaller fragments of 131 bp and 98 bp are obtained upon digestion

Lanes 1U and 1C: exogenous sheep 4844, Lanes 2U and 2C: JS 382, Lanes 3U and 3C: JS 120, Lanes 4U and 4C: DNA 7 has detected endogenous JSRV DNA, Lanes 5, 6 and 7 represent endogenous clones JS5 6A1, JS5 9A1 and JS5F16, Lanes 8, 9 and 10: belong to the three Australian sheep LPW, MSCU and 230909 with endogenous JSRV, Lanes 11, 12 and 13: three unaffected merino sheep and Lanes 14: normal sheep lung.

3.3.2.2 Detection of Exogenous JSRV in Tumours of OPA-affected sheep using the LTR-*gag* PCR.

The LTR-*gag* hemi-nested PCR was also employed to amplify the 229 bp products from genomic DNA extracted from 22 sheep lung tumours. The amplified products as well as the results of restriction digest with *Sca1* are displayed in Appendix C, but the figures are not too clear.

A table of the results obtained using the LTR-*gag Sca1* digest can be seen in Table 3.6. The results in Table 3.6 show that the LTR-*gag* hemi-nested PCR only detected 16 lung tumour samples out of 22. There were 6 samples that were not detected by the LTR-*gag*

hemi-nested PCR. Of the 16 lung tumours that were PCR positive, seven did not have the exogenous specific *Sca1* digested fragments of 131bp and 98bp.

Table 3.6 A summary of the results of LTR-gag hemi-nested PCR Amplified Products and Products of Lung tumours that have been restricted with *Sca1* restriction enzyme

Sample No.	PCR	<i>Sca1</i>	Sample No.	PCR	<i>Sca1</i>
269	+	+	211	+	+
195	-	-	250	+	+
3000	-	-	244	+	-
229	+	-	181	-	-
247	+	-	233	+	-
252	+	+	205	+	-
227	+	+	251	+	-
214	+	-	266	+	+
238	+	+	268	+	+
210	-	-	141	-	-
234	-	-	226	+	+

The failure to amplify DNA from all 22 sheep DNA extracts was either an indication that there was insufficient DNA or that there was poor amplification due to inhibitors. The JSRV U3/LTR hemi-nested PCR was therefore investigated to assess its sensitivity. The failure to detect the unique *Sca1* digest products also suggests that the exogenous virus was absent or that it was in too low a concentration for amplification.

3.3.3 An assessment of the Variable Region 1 and 2 PCR on sheep lung extracts

The PCR reaction for the variable region 1 and 2 was used to amplify the exogenous virus from the 22 lung tumour samples. No amplicons were observed when the amplified products were run on a 2% agarose electrophoresis gel (data not shown).

3.3.4 JSRV U3/LTR hemi-nested PCR

An investigation into the reason why the endogenous clone JSRV5.9A1 was amplified with the JSRV U3/LTR hemi-nested PCR revealed that the JSRV5.9A1 sequence, at the region of the exogenous specific internal primer was the only one of the three endogenous clones that had a nucleic acid sequence similar to the exogenous internal primer sequence. An alignment of the U3/LTR sequences of sheep type D retroviruses (exogenous JSRV 21, JSRV-SA and the endogenous enJS5.9A1, enJS5.6A1 and enJS5F16) is shown in Figure 3.8.

The specificity of the JSRV U3/LTRhn PCR was improved by the design of a new internal reverse primer 3 (P3M, see Table 3.1). This primer differed from the earlier by one base pair (see Table 3.1 for modified P3M). New P3 was paired with forward primer 1 in the first round of the hemi-nested PCR (JSRV U3/LTRhn PCR). The new assay was used to amplify DNA from the 22 tumour DNA extracts and against a panel of normal lung DNA that had been extracted from 3 Australian sheep. The total panel consisted of twenty-two samples of lung tumour DNA, one exogenous JSRV sheep genomic DNA, DNA extracts from seven normal sheep lungs, and three full-length endogenous JSRV clones. The PCR used primers P1 and P3M for the first round and 500 η g of DNA from the lung extracts. This assay targeted a 176 bp gene. The hemi-nested PCR used primers P1 and P4. A product of the size of 133 base pairs is expected if exogenous DNA is present. A photo of the amplified products obtained for the endogenous genomic DNA and a few lung tumours are presented in Figure 3.9.

*JSRCG (JSRV)
en56A1
en5F16
en59A1
JSRVSA
JSRV21
P1 F
P4 Rev
P3 REV
P3 REV Modified

```

7190      7200      7210      7220      7230      7240      7250
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
CGACCCG-TGAAGGGTTAAGTCCT-GGGAGCTCTTTGGC-AAA-A-GCCAAAGCCTAGGACAAAT
CGACCCG-TGAAGGGTTAAGTCTT-GGGAGCTCCCTGGC-AGGTATGCCGGGGCCCTAGGACACGT
CGACCCG-TGAAGGGTTAAGTCTT-GGGAGCTCCCTGGC-AGGTATGCCGGGGCCCTAGGACATGT
CTGCCCC-TGAAGGGTCAAGTCTT-GGGAGCTGCTCAGC-AGGTATGCAGAGCCCTAGGACATGT
CGACCCG-TGAAGGGTTAAGTCCT-GGGAGCTCTTTGGC-AAA-A-GCCAAAGCCTAGGACAAAT
CGACCCG-TGAAGGGTTAAGTCCT-GGGAGCTCTTTGGC-AGA-A-GCCAAAGCCTAGGACAAGT
-----T-GGGAGCTCTTTGGC-AAA-A-GCC-----
-----
-----
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```

JSRCG (JSRV)
en56A1
en5F16
en59A1
JSRVSA
JSRV21
P1 F
P4 Rev
P3 Rev
P3 REV Modified

```

7260      7270      7280      7290      7300      7310
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
ACCTAAGCTCCCTGTCCCGCCACCCCTCTAGGATTCTTGAAAGCTCTTAAGGTTT--GGATGTTT-
GCCAAGCTCCCTGTCCCGCCACCCCTCAAGAGTTTTTA-TAACCCCTTAAGGCTCCAAGATGTTT-
GCCAAGCTCCCTGTCCCGCCACCCCTCAAGAACTTTTG-TAACCCCTTAAGGCTCCAAGATGTTT-
TCCAAGCTCCCTGTCCCGCCACCCCTCAAGAATTTTTA-TAGCCCTTAAGACTCCAGGATGTCC-
ACCTAAGCTCCCTGTCCCGCCACCCCTCAAGAATTTTTAAAGCTCTTAAGGTTT--GGATGTTT-
ACCTAAGCTCCCTGTCCCGCCACCCCTCTAGGATTCTTGAAAGCTCTTAAGGCTC--GGATGTTT-
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```

JSRCG (JSRV)
en56A1
en5F16
en59A1
JSRVSA
JSRV21
P1 F
P4 REV (inner)
P3 REV (outer)
P3 REV Modified (outer)

```

7320      7330      7340      7350      7360      7370      7380
...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|...|
GCTGCCGGCATTACTTCACAGAAATGACGGGAAATC-TGATTATGTA-----AG-AA--T--
GGTTTCGGCAACATTTTCATAGAAG-AT-AGATTATC-TTATTGTGTATATTTTCATAG-AAGATAG
GGTTTCGGCAACATTTTCATAGAAG-AT-AGATTATC-TTATTGTGTATATACTTCATAG-AAGATAG
AGTTCTTGCAACATGTCTTAGAAG-AT-AGATTATC-TTATTATGTATAC--AAT-GGTA-A-GG
GCTGCCGGCATTACTTCACAGAAATGACGGGAAATC-TGATTATGTA-----AG-AA--T--
GCTTTTGGCACTGCTTCATAGAAATACCAGGAAATC-TGATTATATA-----AG-AA--T--
-----GGCACTGCTTCACAGAAATATCA-----
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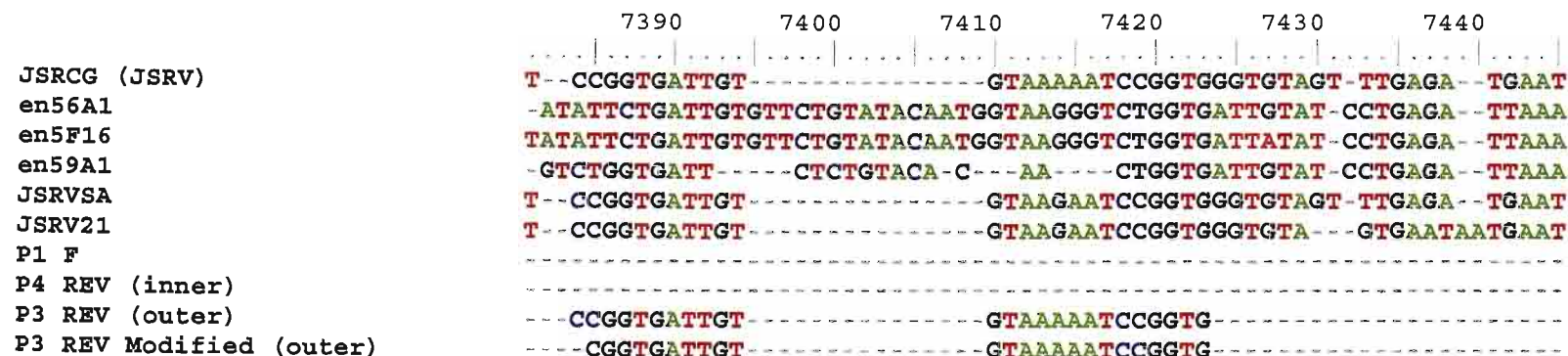


FIGURE 3.8

Showing alignment of the U3/LTR sequences of Sheep type d retroviruses (exogenous JSRV 21; JSRV SA; and three endogenous JSRV loci viz. enJS5.6A1; enJS5F16 and enJS5.9A1).

Dashes indicate lack of sequences. Sequences and position of the U3/LTR hemi-nested primers are also indicated. (P1 and P3 are outer primer pairs while P1 and P4 are the internal pairs).

Nucleotide sequences obtained from GENBANK using accession numbers for JSRV21 (AF105220); JSRV-SA (M80216); enJS5.6A1 (AF153615); enJS5F16 (AF136224) and enJS5.9A1 (AF136225)

*(JSRV): JAAGSIEKTE SHEEP RETROVIRUS

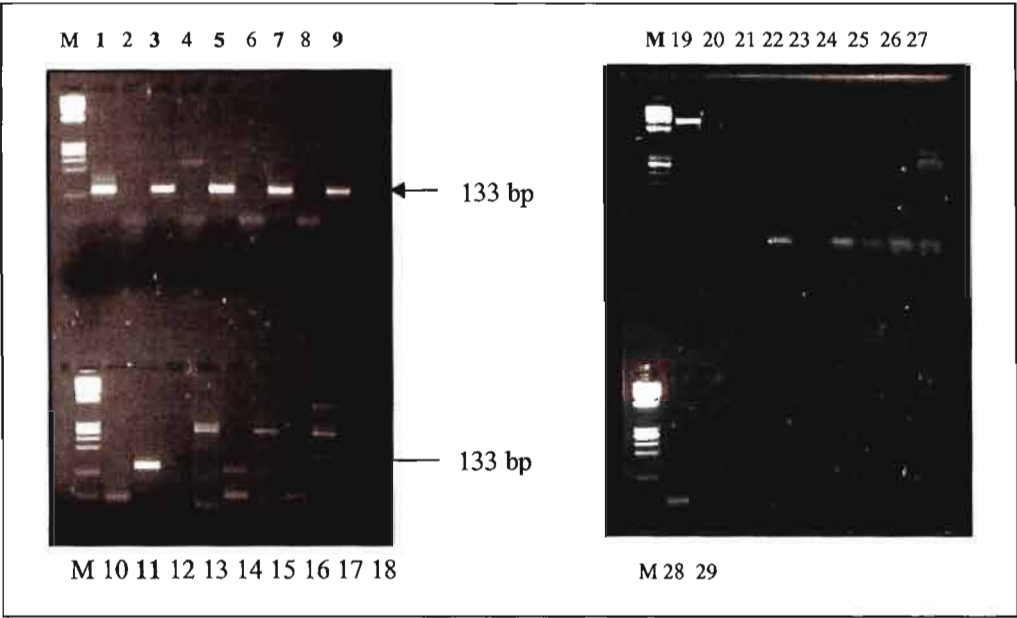


Figure 3.9 Ethidium bromide stained agarose gel (2%) analysis of JSRV U3/LTR hemi-nested PCR. The lanes showing the 133bp product are in bold

Lane 1: Exogenous sheep genomic DNA 4844. **Lanes 3,5,7,9 and 11** Products of amplification of 500ng of genomic DNA from lung tumors of sheep (OPA). **Lanes 2, 4, 6, 8, 10, 12, 14, 16 and 18:** Negative controls (water). **Lanes 13, 15, and 17:** Genomic DNA of healthy merino sheep. **Lane M:** Marker 1X (Boehringer Mannheim)
Lanes 19: plasmid DNA of endogenous JSRV5.6A1. **Lanes 21, 23, 25 and 27:** Genomic DNA belonging to normal sheep (three Australian sheep and normal lung). **Lanes 20, 22, 24, 26, 28 and 29:** Negative controls (water).

Further results of the amplification are presented in Figures 3.10. A summary of all the results obtained from the LTR-gag and U3/LTR hemi-nested PCR for the 22 lung tumours from affected sheep as well as some from normal unaffected sheep controls are shown in Table 3.7.

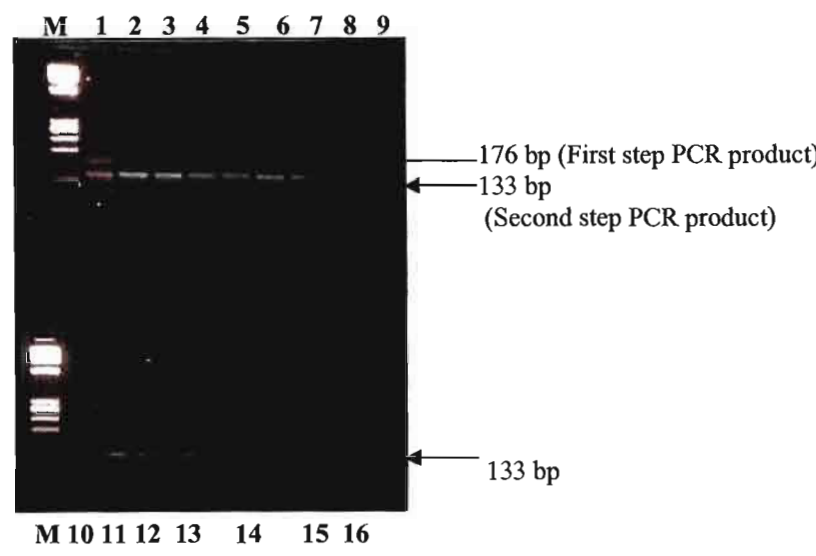


Figure 3.10 Ethidium bromide stained gel (2%) showing amplified products from both the first step (176bp) and second step (133bp) of the U3/LTR hemi-nested PCR

Lane 1: contains both the first step U3 PCR product of 176 bp and the product from the second round of amplification (U3-hnPCR) of 133 bp. The product was amplified from the genomic DNA of a South African sheep 4844 with OPA. This represented the positive control. **Lanes 2 to 16:** Products of amplification of genomic DNA from sheep lung tumours (OPA). **Lane M:** Marker1X (Boehringer Mannheim)

Table 3.7 A summary of the results of LTR-*gag* hemi-nested PCR amplified products and products of lung tumours that have been restricted with *Sca1* restriction enzyme together with those results obtained from theU3/LTR hemi-nested PCR

Sample No.	PCR	Sca1	U3/LTRhn PCR	Sample No.	PCR	Sca1	U3/LTRhn PCR
269	+	+	+	244	+	-	+
195	-	-	+	181	-	-	+
3000	-	-	+	233	+	-	+
229	+	-	+	205	+	-	+
247	+	-	+	251	+	-	+
252	+	+	+	266	+	+	+
227	+	+	+	268	+	+	+
214	+	-	+	141	-	-	+
238	+	+	+	226	+	+	+
210	-	-	+	Normal lung	+	-	-
234	-	-	+	*LPW	+	-	-
211	+	+	+	*MSCU	+	-	-
250	+	+	+	*230909	+	-	-

* Represents the three Australian sheep

3.4 DISCUSSION

The cloning and ultimate sequencing of the full length JSRV genome proved to be a major breakthrough for JSRV research as it opened the way for further molecular studies of JSRV/OPA (York *et al*, 1992). The hybridization studies of JSRV DNA with normal sheep tissue revealed the presence in the sheep genome of 15 to 20 copies of endogenous JSRV-related retroviruses [enJSRV] (Hecht *et al*, 1994; York *et al*, 1992). This similarity between the exogenous and endogenous JSRV meant that the probes designed for the exogenous JSRV cross-reacted with that of the enJSRV and this made detection of the exJSRV amidst a high background of enJSRV difficult (Palmarini *et al*, 1996a).

With perseverance potential molecular markers unique to exJSRV were identified in *gag*, *env*, the LTR and major differences were found in the U3 region as well (Palmarini *et al*, 1996a; Palmarini *et al*, 1996b; Bai *et al*, 1996). Molecular assays based on these differences (LTR-*gag*, V1/V2 and U3/LTR region) were designed. All the parameters to ensure that these diagnostic tests had the capacity to distinguish JSRV-like virus from that of enJSRV-related forms with regard to their sensitivity and specificity in detecting JSRV were assessed.

Exogenous plasmids (JSRV21-11, 537 bp in length, JSRV 382 at position 953-3029 and JS 7 which is 7841 bp in length has both exogenous and endogenous DNA extracts) and endogenous plasmids (JS5.6A1 which is 7939 bp in length, JS5.9A1 which is 6695 bp in length and JS5F16 which is 6915 bp in length) were used to assess the sensitivities of the three molecular assays (LTR-*gag* hemi-nested PCR, V1/V2 PCR and U3/LTR hemi-nested PCR) by making 10-fold dilutions ranging from 1ng to 1fg. These dilutions were used as templates for amplification in both a one-step (V1/V2 PCR) and a hemi-nested (LTR-*gag* and U3/LTR hn PCR) assay using primers specifically designed for the regions in the LTR-*gag*, V1/V2 and U3/LTR.

The first molecular assay was based on the LTR-*gag* region where the presence of the second *ScaI* site (position 1729) identified the sample as being exogenous. Palmarini *et al* optimized this PCR for the detection of 1 to 10 copies of pJSRV 382 in 1996. This study was able to detect up to ten pg of the plasmid. The first step of the LTR-*gag* PCR used primers P1 (internal to the LTR region) and P2 (*gag* primer-see Table 3.1) that amplified a

product of 2064 bp. The hemi-nested step used P1 and a primer internal to the *gag* primer (P2), P5 that amplified a product spanning 229 bp within the *gag* gene for both the exogenous and endogenous DNA extracts. Only upon digestion of this 229 bp product with *Sca*1 enzyme into two fragments of 131 bp and 98 bp, was one able to deduce whether the extracts were from affected or unaffected sheep. The explanation being that the exogenous JSRV has two *Sca*1 sites, while the endogenous has just one. The primers were designed so that only the second *Sca*1 site was amplified (Palmarini *et al*, 1996a).

Similar dilutions of samples were also used as templates for two other molecular assays, the U3/LTR hemi-nested PCR and the V1/V2 PCR. The U3/LTR first step PCR identified a 176 bp product (data not shown), while the hemi-nested step identified a 133 bp (Fig. 3.5) exogenous JSRV product and the PCR covering the V1/V2 region a 363 bp (Fig. 3.4) product (exogenous specific).

In assessing all three assays it was found that the VR1/VR2 PCR detected only JS 21 at a low concentration of 1 μ g equivalent to 82,000 copies. According to calculations based on JS21 being 11571 bp long, 82×10^9 copies of JS21 should be detected per η g of DNA. In this study only 82×10^6 copies were detected per η g that was below that of the theoretical detection level of 82×10^9 copies/ η g. In addition, JS 7 and JS 382 were not detected with this primer set. JS 382 was not detected by primers VR1 and VR2 because the region targeted was not present in the clone.

According to Holland *et al*, 1999, the JS7 cell line has one copy of exogenous target (as measured by the U3/LTR hemi-nested PCR) per 5.98 μ g of DNA. If the weight of the genomic DNA contained in 1 cell is 6.66 μ g, then each JS 7 cell line is estimated to have 5.98 μ g/6.66 μ g copies (0.9+/-0.07 copies). This is equivalent to nearly 1 copy per cell. For the LTR-*gag* PCR in this study, the detection level for JS 7 was 100 μ g, which should then theoretically have 16.7 copies (100 μ g/5.98 μ g) and at 1 η g 167 copies. This is in line with the detection limits according to Holland *et al*, 1999. But according to calculations for JS21, there should be 82×10^9 copies/ η g of DNA detected. In other words 1 fg should have 82 copies, 10 fg 820 and 100 fg 8200 etc. The JSRV U3/LTR hemi-nested PCR (Fig. 3.5 lanes 8-14) did detect JS 21 at a concentration of 1 fg. These calculations have been based on the fact that JSRV 21 is 11571 bp in length.

Since the detection endpoint for JS 7 was the same i.e. 16.7 copies per 100 μ g for both the LTR-*gag* and U3/LTR hemi-nested PCR, one cannot state that one assay was more sensitive than the other. However, it is evident from the titration profile for JS 21 (Fig 3.2, lanes 10-14_{100fg-1 η g} and Fig 3.5, lanes 8-13_{1fg-10 μ g}), that the U3/LTR hemi-nested PCR is definitely more sensitive than the LTR-*gag* PCR in that it detected 82 copies (1fg) as opposed to that of 8200 copies at 100fg.

JS 382 was detected at a concentration of 10 μ g by the LTR-*gag* PCR and was detected as a contaminant by the U3/LTR hemi-nested PCR. Upon retesting with a fresh stock of JS382, the U3/LTR hn PCR was negative. Looking at the results for the exogenous plasmids, it can be stated that of the three assays used, the LTR-*gag* and U3/LTR hn PCR proved to be equally sensitive, but the U3/LTR hn PCR was 2 logs more sensitive when assessed against exogenous JSRV DNA.

The endogenous plasmids JS5.9A1, JS5.6A1 and JS5F16 were not detectable by the V1/V2PCR assay. Although the plasmids were detectable upon amplification with the LTR-*gag* PCR, the second *Sca*1 site was not in evidence upon restriction digest with the *Sca*1 enzyme proving that these were truly endogenous JSRV products. The endogenous plasmids on the other hand were completely undetectable with the U3 hemi-nested PCR, although the plasmid JS5.9A1 was detected at a concentration of 100pg. Closer examination of the nucleic acid sequences of the three endogenous plasmids with the exogenous JSRV, revealed that whereas JS5.6A1 and JS5F16 had a 5' ATTC 3' at position 7361-7364, JS5.9A1 had a 5' CTGG 3' sequence at this position and the exogenous sequence was 5' CCGG 3' at this position. The presence of this 5' C at position 7361 could be responsible for the amplification of this endogenous plasmid (see Figure 3.8). The reverse primer P3 for the first round of the PCR was therefore redesigned as can be seen in Table 3.1 (P3M). The endogenous plasmid JS5.9A1 was retested with the U3-hemi-nested PCR assay using the new reverse primer (P3M) as well as AmpliTaq Gold DNA polymerase enzyme by Applied Biosystems in a real time setting (Lightcycler by Roche Diagnostics). This enzyme is a chemically modified enzyme that requires a high temperature incubation to activate the enzyme thus reducing enzyme extensions at lower temperature and improving specific binding between primer and template. The endogenous plasmid JS5.9A1 was not detected when the hot start Taq was used. When all three assays

were compared for specificity in detecting endogenous JSRV, both the LTR-*gag* and U3 hemi-nested were found to be equally specific.

On comparing all three assays and their detection levels, all three were found to be specific. The V1/V2 PCR did not detect JS7 because this cell line has both exogenous and endogenous extracts. On the question of sensitivity, the U3-hemi-nested was found to be 2 logs more sensitive than the LTR-*gag* assay. Palmarini also confirmed this sensitivity in 1996, when the U3 hemi-nested technique was found to be 10^5 -fold more sensitive than the LTR-*gag* PCR/*Sca1* digestion method. Holland *et al*, 1999 and González *et al*, 2001, also confirmed this.

Both assays were tested against 22 sheep lung tumours. It was concluded that the U3/LTR hemi-nested assay was the most sensitive. The number of positive results obtained with the LTR-*gag*PCR/*Sca1* digestion assay was 9/22 compared to that of the U3/LTR hemi-nested technique where 100% of the positives were detected (22/22). The LTR-*gag* PCR/*Sca1* digestion did not detect 6/22 lung tumours. Of the 16 samples that were positive by this combination assay, 7 samples were detected by PCR, but their digestion products were not seen when run on a 2% agarose gel. Either the products were too faint to be seen or the material was of endogenous origin. The detection of few *Sca1* sensitive sequences at the DNA level may be due to there being a greater amount of endogenous JSRV template compared to exogenous JSRV template available (there are probably as much as 20 endogenous copies as compared to only 1 exogenous copy per cell) (Palmarini *et al*, 1996a) . Two weaknesses have been revealed in this study. The first is that an internal house keeping gene was not included for each assay. Whilst the concentration of DNA using OD 260nm and a ratio of 260nm to 280nm is useful it is not as powerful as an internal housekeeping gene. The other potential weakness is in those cases where the exogenous virus might be present in low copies. It is possible that the endogenous virus might be preferentially amplified. Such a scenario could result in a false positive result..

Preliminary investigations into the U3/LTR hemi-nested on the light cycler (Roche Diagnostics) using Taq gold and the improved primer set has proven to be excellent. It is now possible to include concentration standards to make the assay quantitative which has numerous applications including diagnosis, research and disease management.

CHAPTER 4

CHAPTER FOUR

GENERAL DISCUSSION AND CONCLUSION

The diagnosis of OPA has traditionally relied on the clinical history and physical examination of the affected animal. Laboratory confirmation at necropsy has been by the histopathological examination of infected lung tissue. For such an examination, it is a prerequisite to take specimens from several affected sites and from more than one animal. The reason for this being that secondary bacterial pneumonia which can be the immediate cause of death often masks the lesions (both macroscopic and microscopic) of the primary disease. In the absence of specific serological and molecular diagnostic tests, disease control relied on regular flock inspections and prompt culling of suspected cases and their offspring (Sharp, 2000).

The absence of any specific humoral immune response to JSRV has made JSRV unique amongst retroviruses. Even though JSRV is responsible for highly productive infections in the lungs as well as a disseminated infection of the lymphoreticular system, the absence of circulating antibodies in the sera of the affected sheep has made detection of the infection very difficult (Sharp and De Martini, 2003). There has been some controversy relating to the presence or absence of circulating antibodies in the sera of affected sheep (Ortin et al, 1998).

Many studies have failed to detect antibodies to JSRV in sera or lung fluid of affected sheep by Western blotting or ELISA (Sharp and Herring, 1983; De Martini *et al*, 1987; Kajikawa *et al*, 1990; He *et al*, 1992; Kwang *et al*, 1995; Ortin *et al*, 1998; Summers *et al*, 2002 and Sharp and De Martini, 2003 text book). It was Sharp and Herring in 1983 that were the first to describe an immunological cross-reaction by Western blotting between the 27 kDa core protein (p27) of MPMV, MMTV and a 25 kDa protein in OPA tumours and lung fluids. They failed to detect any antibodies to p25 in the sera of sheep with terminal OPA or in lambs where the tumours were induced (Sharp and Herring, 1983)

However, in 1992 the complete viral genomic sequence for JSRV was determined from a virus (constructed from a cDNA library) purified from lung wash of an OPA affected sheep from South Africa (York *et al*, 1992). This paved the way for immunoassays based on recombinant antigens expressed in the form of GST fusion proteins (Kwang *et al*, 1995; Ortin *et al*, 1998 and Palmarini *et al*, 1995). Recombinant proteins have been frequently used as diagnostic antigens in the detection of antibodies to indicate infection and disease. The sensitivity and specificity of these assays is much better than using purified whole virus preparations. The first available marker for direct or indirect detection of OPA-affected sheep was the MPMV capsid-related antigen p27. This soon became the internationally accepted OPA marker protein (Kwang *et al* 1995). A recombinant form of MPMV-p27 (expressed as a GST-p27 fusion protein) was used in the development of a simple, rapid and sensitive ELISA. This ELISA was used to detect JSRV-CA antibodies in Italian sheep flocks with a high prevalence of OPA and in North American sheep flocks with a high prevalence of chronic pneumonia (Kwang *et al*, 1995). This study detected JSRV-CA antibodies in infected and diseased sheep, but the specificity of these reactions were still to be confirmed by others.

Our studies on the expression of some JSRV genes also started soon after the JSRV genome had been identified, cloned and sequenced. At the time we also believed that purified recombinant antigens would once and for all address the question of whether there were specific antibodies being produced against the viral proteins. We felt that even the most pure preparations of JSRV from lung fluid were still contaminated with non-viral proteins.

We targeted three genes, the *orf-X* because it was unique, the transmembrane and the capsid gene, that is the JSRVp26 gene. Our attempts at expressing the *orf-X* and transmembrane were not successful. The genes were successfully cloned into the expression vectors, but all attempts at purification of these extremely hydrophobic proteins failed. We continued with the JSRVp26 expression, purification and analysis. We established that the JSRV p26 gene was identical to that present in three full-length endogenous JSRV isolates. If the endogenous viruses were expressing an identical protein to the exogenous virus, then this would support the theory that sheep do not produce antibodies to JSRVp26 because they are recognised as self-antigens. Nevertheless, we continued with our assessment of the purified protein using many assay formats such as an

indirect ELISA, Western immunoblot assay, antigen sandwich and direct conjugation. We confirmed that the purified p26 cross-reacted with the anti-MPMVp27 antibody in the Western blot immunoassay in accord with the findings of Sharp and Herring, 1983. We went on to demonstrate that an indirect ELISA using this recombinant antigen detected JSRV positive sera (6/8), however, further investigations revealed that the reaction was non-specific. Using an equivalently expressed gene we showed that the reaction was not specific to JSRV but also to the proteins associated with the expression vector. We were able to demonstrate that the “p26 specific antibodies” were competed out using an unrelated protein that was purified and expressed in the same vector. This non-specificity concurred with findings reported by Ortin *et al*, 1998.

Ortin and co-workers also expressed JSRVp26, which he called JSRV-CA, in a pGEX expression vector. Ortin showed that sheep that had been tested to have positive antisera to the JSRV-CA had no detectable tumours at necropsy and further that they came from flocks with no clinical history of OPA or ENT. Final confirmation that the assay was not specific came from a study where 7 out of 8 sera were positive from sheep that were in Australia and Falkland where neither OPA nor ENT exist. To understand these findings all sera were preabsorbed with a lysate containing GST, but with no proteins related to JSRV or ENT. This procedure removed all reactivity to the sera and confirmed the non-specificity of these reactions (Ortin *et al*, 1998).

All findings strongly support the conclusion that there are no circulating antibodies to JSRV CA (Ortin *et al*, 1998; Sharp *et al*, 1983). In light of the overwhelming evidence that that JSRVp26 does not stimulate a humoral response we believed that there might be a localized immune response in the lungs that could be detected. We therefore developed an antigen assay to investigate this. Earlier work by our group had suggested that JSRV specific IgG and IgA were being produced in the lungs of infected sheep. In this study we analysed lung lavage samples from infected and normal sheep for the presence of JS anti-p26 antibodies using an in-house JSp26 peroxidase conjugate in an antigen capture assay format (Table 2.18). Although the assay lacked sensitivity (31%), the results were encouraging and in line with our earlier findings using a much cruder preparation of viral antigens, and Palmarini *et al*, 1995, in that there was a specific localized immune response to JSRV in the lungs of OPA affected sheep. Since there were a significant number of samples that were negative we needed to improve the sensitivity of the assay but more

importantly develop an antigen capture assay in view of the absence of antibodies detected in those few JS lungs. This assay however required a specific JSRV CA antiserum. Our attempts to produce a JSRVp26 antiserum in chickens were partially successful but use was made of an alternative antiserum that became available at the time. This antiserum to the JSp26 was produced in a rabbit and was provided by Professor De Martini (University of Colorado, Fort Collins). The rabbit polyclonal viral capsid antibody facilitated the development of an antigen capture assay. This assay detected JS antigen in the lung fluid and nasal fluid of JS affected sheep and not in normal lung lavage (Table 2.22).

The conclusion from our studies were that the poor humoral response to the JSp26 protein is likely due to the fact that there are identical genes being expressed by the endogenous (normal) virus that are present in all sheep genomes. Further, that there is a predominantly localized immune response in the lungs of JS affected sheep and that the antigen capture assay is a useful method to detect the presence of virus in both the lungs and nasal fluid of affected sheep. This assay does have its limitations in that the disease in an infected animal needs to be sufficiently advanced for sufficient virus to be released before it can be detected.

It has been mentioned earlier that the complete sequencing of the JSRV genome opened many doors for Jaagsiekte research and facilitated research into the molecular aspects of JSRV (York *et al*, 1992). However, the discovery of the presence of 15 to 20 copies of endogenous JSRV-related sequences in the normal sheep genome made the obvious development of a molecular assay that much more challenging (Hecht *et al*, 1994; York *et al*, 1992). One of the immediate objectives was therefore to obtain more information on the endogenous and exogenous JSRV-related viruses. The *gag* and LTR regions of both the exogenous and endogenous JSRV were sequenced and compared (Bai *et al*, 1996). A long range PCR based on the exogenous JSRV sequences (York *et al*, 1992) was developed to characterize six endogenous retroviral loci based on restriction enzyme profiles and proviral genomic size. Restriction sites that were identified that could distinguish the exogenous JSRV from the endogenous counterparts in the same cell were *Nde*1, *Hind*111, *Sca*1, *Kpn*1 and *Eco*R1 in the *gag* region. There were also significant differences in the U3 region between the endogenous loci and the exogenous JSRV, due to deletions and many point mutations in the U3 of the LTR (Bai *et al*, 1996). The endogenous LTR's were longer (442-445 bp) than the exogenous JSRV sequence (397 bp) and there were two large

deletions of 30 bp and 16 bp at positions 198 and 261, respectively (York *et al* 1992). However, over time more endogenous sequences were added to the database. Many of the restriction enzyme sites that had been suggested as being signatures of either an endogenous or exogenous JSRV, did not withstand the test of time.

The most useful one is a unique *Sca1* restriction site (at position 1729) that was only found in the exogenous forms of JSRV. This site was found exclusively in the cDNA of OPA-affected sheep and not in unaffected control sheep (Palmarini *et al*, 1996a). A molecular based PCR assay, LTR-*gag* hemi-nested assay was designed to specifically target exogenous proviral DNA with sense primer P1 (based on an area of sequence divergence between endogenous LTR clones and the exogenous LTR sequence (JSRV) and antisense primer P2 (*gag* region) (see Table 3.1) to amplify a 2 kb product. The second round of amplification used P1 and P5 to amplify a 229 bp fragment were upon *Sca1* digestion, enabled the detection of *Sca1*-sensitive sequences in tumour samples only and not in non-tumour samples (Palmarini *et al*, 1996a). JSRV was detected in tumour and in draining lymph nodes of OPA affected sheep. Since it was not detected outside the respiratory tissues of OPA affected sheep or in any tissue of control sheep, it was felt that the detection of low copies of exogenous JSRV were masked due to the high background of sheep endogenous retroviruses. To obviate this issue, an exogenous JSRV specific U3/LTR hemi-nested PCR was designed to detect the anatomical distribution of JSRV in sheep tissues with naturally and experimentally induced OPA (Palmarini *et al*, 1996b).

This U3/LTR hemi-nested PCR primer P1 (located almost at the 5' end of the U3 region (see Table 3.1) was paired with antisense primer P3 (in the U3 region–Table 3.1) to amplify a 176 bp product. The sensitivity of this reaction was improved by including a primer P4 (see Table 3.1) internal to the PCR product. This primer was paired with primer P1 in a second round hemi-nested PCR so that a product of 133 bp was amplified from all lung fluid and lung tumour PCR products. This assay detected a single molecule of template in 500ng of normal sheep genomic DNA, whereas the LTR/*gag* hemi-nested PCR followed by *Sca1* digestion was 10^5 -fold less sensitive, because it was not able to detect 10^5 copies of the exogenous clone, pGem-T LTR-*gag* in a background of 500ng of normal sheep kidney DNA (Palmarini *et al*, 1996b). Moreover, exogenous sequences were detected only when the ratio of exogenous/endogenous sequences were 1:10 and not 1:100 (Palmarini *et al*, 1996b)

This study, besides assessing the sensitivity and specificity of the LTR-*gag* PCR and the U3/LTR hemi-nested PCR, also included a further assay that covered the V1/V2 region, which was a single step PCR. This was possible when our group sequenced three full-length endogenous JSRV genomes. The outcome of the present molecular study is in line with those findings of Palmarini *et al*, 1996b, Holland *et al*, 1999 and González *et al*, 2001 where the U3/LTR hemi-nested assay was found to be a more sensitive assay than the LTR-*gag* PCR. In this study the U3/LTR hemi-nested PCR detected the JS 21 infectious clone at a concentration of 1fg compared to 100fg for the LTR-*gag* PCR being 2 logs more sensitive. The extreme sensitivity of the U3/LTR hemi-nested assay detected a minor contamination of the JS 382 plasmid that contains a portion of the *gag* gene. The experiment was repeated with dilutions from fresh stocks of JS 382 not previously used and turned out to be negative. Further assessment of the assay revealed that this assay also detected the endogenous JSRV5.9A1 loci. This was overcome by redesigning a more specific primer P3M (see Table 3.1 and Figure 3.8) for the first step of the U3/LTR hemi-nested PCR. This primer together with the use of AmpliTaq Gold DNA polymerase improved the specificity of the assay. We concluded that this assay was both the most sensitive and specific.

The U3/LTR hemi-nested PCR has been the molecular assay of choice for many of the recent studies (Holland *et al*, 1999; González *et al*, 2001 and Summers *et al*, 2002) to determine in which organs JSRV could be detected. This assay is becoming the internationally accepted molecular method to identify sheep infected with the exogenous infectious form of JSRV. Recently the assay was used to confirm an outbreak of OPA in Patagonia, Argentina, which would normally have been diagnosed using gross and histological changes (Uzal *et al*, 2004).

In our study, JSRV antigens were detected using an antigen capture assay in nasal fluids and lung washes, but not in the sera of affected sheep. The U3/LTR hemi-nested PCR detected JSRV in the tumours of lungs obtained from affected sheep. These lung tumours were the only sheep organs available for the purposes of this molecular study. Early studies identified the epithelial tumour cells of the lungs as sites of JSRV replication (Palmarini *et al*, 1995). Viral RNA has been found in lung tumours, lung fluid and draining lymph nodes of OPA affected sheep (Palmarini *et al*, 1996a). With the introduction of a much more sensitive assay, the U3/LTR hemi-nested assay has been extended to other anatomical sites

where JSRV DNA has been detected in the mediastinal lymph nodes, spleen, and bone marrow. Viral RNA was found in these sites as well as the thymus and peripheral blood mononuclear cells (PBMC). Proviral DNA was not detected at these sites (Palmarini *et al*, 1996b). The adherent cell population consisting of macrophages/monocytes of the lymphoid cells was identified as having the greatest proviral burden. In OPA affected animals these are the cells that are most abundantly found in the lungs. In the non-adherent cell population, the surface immunoglobulin positive B cells contained the greatest proviral burden, whilst the CD4⁺ and CD8⁺ T cells the lowest levels of JSRV proviral DNA. JSRV provirus was also found in the PBMC population (Holland *et al*, 1999). The most interesting information from this study of Holland *et al*, 1999, was the detection of JSRV as early as 7 days after experimental inoculation of new born lambs with JSRV before the onset of OPA or any histological signs.

In a recent study sheep peripheral blood leukocytes (PBL's) and tissue samples were examined from 36 sheep, of which 10 were non-affected sheep from OPA-affected flocks (in contact sheep) and 10 from non-affected OPA-free flocks. The other 16 sheep were from classical OPA cases and sheep with atypical OPA. This study was the first to detect JSRV in naturally infected sheep (4/10 PBL's) from the in-contact group and in a lower number from the mediastinal lymph node, spleen, mammary lymph node and neo-plastic lung samples using the U3/LTR hemi-nested assay before the onset of any clinical disease or development of tumours (González *et al*, 2001).

Overall the lung tissue contained the highest number of PCR positive results, in agreement with previous findings on JSRV detection by immunohistochemistry or blocking ELISA techniques (Palmarini *et al*, 1996a and González *et al*, 2001).

The present study confirmed the sensitivity of the U3/LTRhemi-nested PCR and improved its specificity by redesigning one of the primers used in the first step (P3M-Figure 3.1) and the utilization of a thermally activated AmpliTaq Gold DNA polymerase (*PE* Biosystems, Applied Biosystems, USA) that enhances the specificity of the primers. We also developed a real-time U3/LTR hemi-nested PCR assay using sybr green and the Light cycler (RocheDiagnostics) real time machine. This technology has a number of advantages in that there is less handling of the samples and it has the capability of being quantitative. Collaborative work will continue and the assay will be modified to include a house keeping

gene as an internal control and primers will be modified to improve the amplification of the exogenous virus at a low copy number in the presence of high copies of the endogenous viruses.

In concluding, this study has spanned nearly a decade of research that has included many different techniques and experiments, many of which have not been presented or discussed in this thesis. Only aspects that were associated with the development of a JSRV diagnostic assay have been included. The serological study confirmed the work of others in that there are no circulating antibodies to the JSRV p26 capsid gene but it is possible to detect viral antigens using an optimal p26 antigen assay. However sufficient virus needs to be present before it is detected. Future studies looking for circulating viral antibodies will involve the expression of other JSRV proteins or fragments, especially those peptides that differ between the endogenous and exogenous isolates. The development of a molecular assay had to initially overcome the problem of the endogenous JSRV sequences. However, as more sequences became available it became possible to target regions that were uniquely exogenous. Improving the sensitivity of the assay using a hemi-nested format has finally resulted in a molecular diagnostic assay that can now be used to detect the presence of the infectious exogenous virus even in sheep that are asymptomatic. It is therefore possible to use this assay to screen infected sheep populations to identify infected sheep and in this way eradicate the disease through a selective culling program. Evidence that this is possible is the Icelandic story where that country successfully eradicated JSRV following an extensive slaughtering out campaign where all sheep on the Island were slaughtered except for a small population on the Western peninsular that were not exposed to the virus. The Island has been free from JSRV for nearly half a century.

Future research will focus on understanding the mechanisms of transformation and the immune response. The molecular tools are now available to facilitate research into this most interesting disease, which remains the best natural outbred animal model of a contagious cancer that is caused by a virus. This model will have important implications for research into human breast and lung cancer.

APPENDIX A

DNA Sequence of the 5' end of GST-JSp26 in pGEX-1 expression vector with p26 inserted into the correct reading frame.

Shown in bold print and underlined is the sequence of the 5' pGEX sequencing primer and only underlined is the 5' JSp26 *Bam*HI primer. This sequence showed 100% homology with the Genbank sequence. Also shown is the amino acid translation of the sequence of JSp26.

1 5' GGACCCAATG TGCCTGGATG CGTTCCCAAA ATTAGTTTGT TTAAAAAAC
51 ATTGAAGCTA TCCCACAAAT TGATAAGTAC TTGAAATCCA GCAAGTATAT
101 TGGCCTTTGC AGGGCTGGCA AGCCACGTTT GGTGGTGGCG ACCATCCTCC
151 AAAATCGGAT CCTGTCTTCG AAAATAACAA CCAGCGTTAC TATGAATCAC
201 TGCCGTTTAA ACAACTAAAA GAGTTAAAGA TTGCTTGTTT GCAATATGGT
251 CCTACCGCTC CATTTACTAT TGCTATGATA GAAAGTTTGG GTACTCAAGC
301 TTTGCCCCCG AATGACTGGA AACAGACCGC TAGGGCATGT CTCTCAGGGG
351 GAGATTATTT ATTATGGAAA TCTGAATTTT TTGAACAATG TGCTCGTATA
401 GCTGATGTTA ACCGACAGCA AGGTATACAG ACCTCCTATG AAATGTTGAT
451 TGGCGAAGGC CCTTATCAGG CTAC 3'

Translating from BamHI: GGATCCT (-1)

157 DPVFENNNQR YYESLPFKQL KELKIACSQY GPTAPFTIAM IESLGTQALP
207 PNDWKQTARA CLSGGDYLLW KSEFFEQCAR IADVNRQQGI QTSYEMLIGE
257 GPYQAXXTX

APPENDIX B

Alignment of exogenous and endogenous JSRV gag sequences and positions of primers used for the V1/V2 region one step PCR

Nucleotide sequences obtained from GENBANK using accession numbers for JSRV21 (AF105220); JSRV-SA (M80216); enJS5.6A1 (AF153615); enJS5F16 (AF136224) and enJS5.9A1 (AF136225)

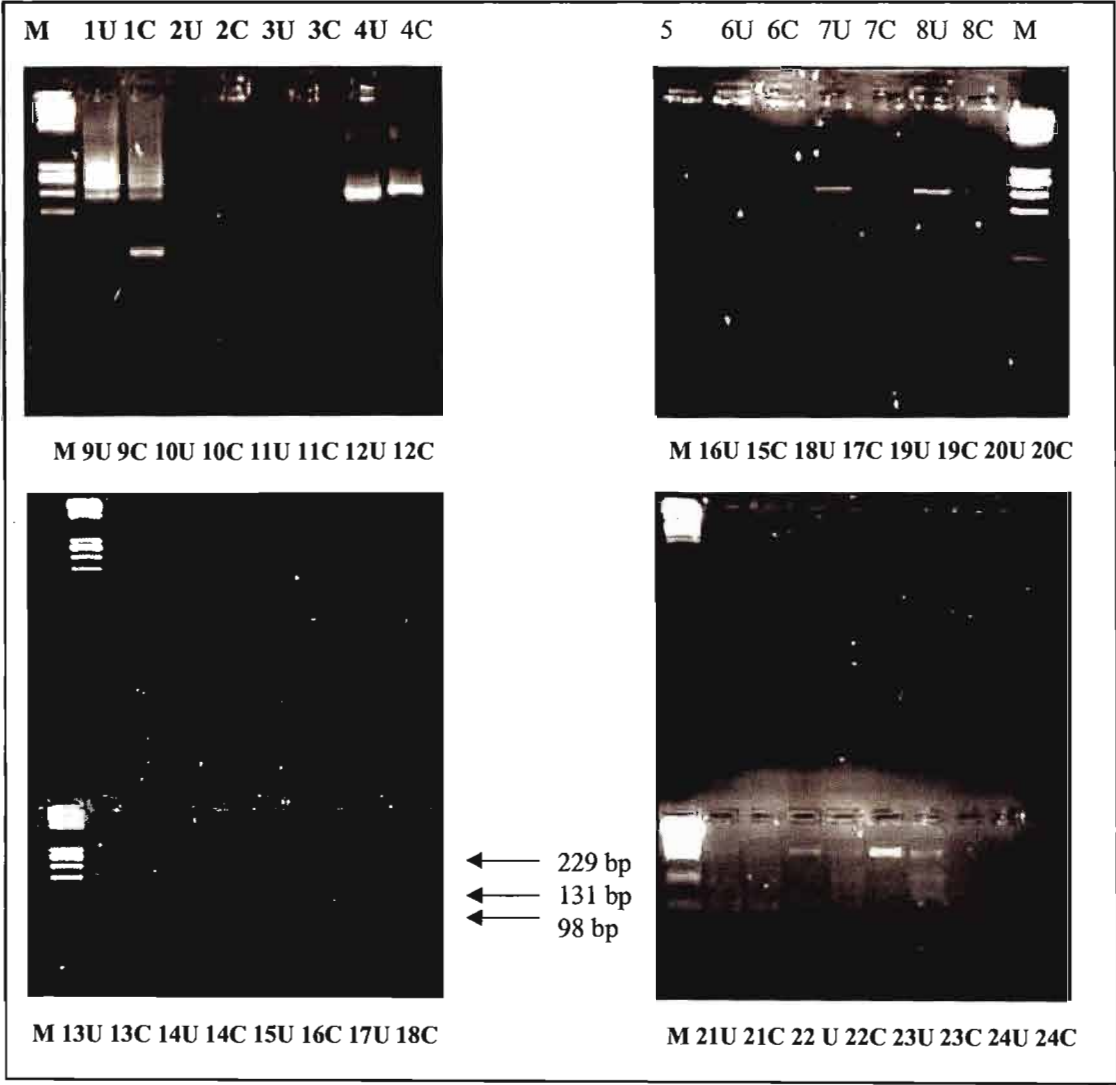
	610	620	630	640	650	660
					
JSRVCG	CTGATT	CGGTACCTAGTTATGATCCTCCTCCTCCTCCT	-----	CCG-CCATCTCTGA		
en56A1GAG	CTGATT	CGGAACCCAGATATGCTGTTCCCGAGGGGGTTAAAAGCGACCTCCGTTTTCTA				
en5F16GAG	CTGATT	CGGAACCCAGATATGCTGTTCCCGAGGGGGTTAAAAGCGACCTCCGTTTTCTA				
en59A1GAG	CCGAT	CCGGAACCCAGGTACGCTGTTCCCGAGGGGAGCTGAAGGCGACCTCAGTTTTTTTA				
JSRVSAAGAG	CTGATT	CGGTACCTAGTTATGATCCTCCTCCTCCTCCT	-----	CCG-CCATCTCTGA		
JSRV21GAG	CCGATT	CGGGACCTAGTTATGATCCTCCTCCTCCTCCT	-----	CCC-CCATCTCTGA		
JS613F	-----	CTAGTTATGATCCTCCTCCTCCTCCT	-----			
JS976R	-----		-----			

	970	980	990	1000	1010	1020
					
JSRCG Jaagsiekte sheep retrovi	CTAAACATATGTCTCCATTGCAAAAGGCATTAAGACAGGCACAACGACTCGGTGAAGTTG					
en56A1GAG	CTGAGAACATGTCTCCGCTGCAAAAGGCATTGAGACAGGCACAGCGACTTGGTGAGGTTG					
en5F16GAG	CTGAGAACATGTCTCCGCTGCAAAAGGCATTGAGACAGGCACAGCGACTTGGTGAGGTTG					
en59A1GAG	CTGAGGTATGTCTCCGCTACAGAAGGCATTGAGGCAAGCACAACGACTCGGTGAAGTTG					
JSRVSAAGAG	CTAAACATATGTCTCCATTGCAAAAGGCATTAAGACAGGCACAACGACTCGGTGAAGTTG					
JSRV21GAG	CTAAACATATGTCTCCCTTACAAAGGGCCTTAAGACAGGCACAGCGACTTGGTGAGGTTG					
JS613F	-----					
JS976R	-----	ATGTCTCCCTTACAAAGGGCCTT	-----			

APPENDIX C

Ethidium bromide stained agarose gel (2%) analysis of LTR-*gag* PCR products of amplification from lung tumour tissue that have been digested with *Sca*1 restriction enzyme

Lanes 1, 2, 3, 4, 6 to 17, and 19 to 24 represent amplified products that have been digested with *Sca*1 restriction enzyme. U represents undigested PCR products and C digested PCR products. Lane M represents DNA Marker 1X (Roche Diagnostics, Mannheim, Germany)



APPENDIX D

1. Nuclei lysis buffer

10mM Tris
100mM NaCl
10mM EDTA
pH 8.2

2. 10% SDS (w/v)

10g SDS
Dissolve in d.H₂O with gentle stirring
Make up to 100ml. Store at RT.

3. Chloroform isoamylalcohol (24:1)

To 24 volumes of chloroform add 1 volume of isoamylalcohol.
The mixture can be stored at room temperature in a dark bottle.

4. 70% Ethanol

70ml 100% ethanol (AR Merck)
30ml distilled water

5. TE buffer

10mM Tris/HCl
1mM EDTA
pH 8.2

6. Deoxyribonucleic triphosphates (dNTP's) 200 μ M

Pharmacia Biotech supplies 100mM stock each of dATP, dCTP, dGTP and dTTP.

On arrival stored at -20°C . Working solution is 2.5mM. dNTP's to be diluted at 1:40.

In a 15ml tube add 100 μ l of each dNTP to 3.6ml d.H₂O.

Mix well and aliquot 200 μ l per 0.5ml eppendorf tube. Store at -20°C .

7. Ethidium Bromide (10mg/ml)

Add 0.1g of ethidium bromide to 10ml of H₂O.

Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved.

Transfer solution to a dark bottle. Store at room temperature.

8. 5x stock Tris-borate buffer (TBE)

108g Tris base

55g Boric acid

20ml of a 0.5M EDTA (pH 8.0)

Buffer was made up to 2litres with d. H₂O

To be diluted 1/10 for use.

9. 6x loading buffer

0.25% bromophenol blue

40% (w/v) sucrose in water

Stored at 4°C

10. LB Agar

10g Bacto-tryptone

5g Bacto-yeast extract

10g NaCl

Add 900ml dH₂O. Adjust to pH 7.

Add 15g Bacto-agar and make up to 1L with dH₂O. Autoclave to sterilize.

11. Stock Ampicillin (100µg/ml)

Dissolve 500mg Ampicillin in 5ml d.H₂O.

Aliquot in volumes of 500µl using sterile 1.5ml tubes covered with foil.

Store at -20°C. Use at 100µg/µl.

12. Tetracycline (5mg/ml)

Dissolve 5mg tetracycline in 1ml ethanol.

Use at 15ug/ml (1/333)

13. X-Gal solution (20mg/ml)

To 50mg of X-Gal add 2.5ml of dimethyl formamide.

Store in the dark (foil- covered tubes) as X-Gal is light sensitive at -20°C.

Use at 35µl/plate.

14. IPTG solution (200 mg/ml)

Dissolve 2.0g IPTG in 8ml distilled water.

Make up to 10ml. Sterilize by filtration through a 0.22µm disposable filter.

Aliquot into volumes of 500µl using sterile 1.5ml tubes.

Store at -20°C. Use at 20µl/plate.

15. SOC Medium

2g bacto-tryptone

0.5g bacto-yeast extract

0.059g NaCl

0.019g KCl in d.H₂O

Adjust pH to 7 with HCl, fill up to a volume of 97ml and autoclave.

Allow cooling to 60°C.

Add 2ml of 1M MgSO₄ and 1ml of 2M Glucose (glucose has been filter sterilised).

16. LB Agar with 100µg/ml ampicillin

Prepare LB agar. After autoclaving, cool to 60°C. Add 2ml/litre of 50µg/ml ampicillin stock and mix. Pour the medium into the petri dish until the bottom of the plate is fully covered. Allow setting, covering with aluminium foil. Store at 4°C

17. Solution I

50mM glucose

25mM Tris.Cl (pH 8)

10mM EDTA (pH 8.0)

Solution was autoclaved. Stored at 4°C.

18. Solution II

0.2N NaOH

1% (w/v) SDS

19. Solution III

5M Potassium acetate 60ml

Glacial acetic acid 11.5ml

dH₂O. 28.5ml

The resultant solution is then 3M with respect to potassium and 5M with respect to acetate.

20. 10X One-Phor-All-Buffer plus (OPA)

100mM Tris acetate

100mM Magnesium acetate

500mM Potassium acetate (pH 7. 5)

21. 3M Sodium Acetate (pH 5.2)

Dissolve 102.25g of sodium acetate.3H₂O in 60ml H₂O. Adjust the pH to 5.2 with glacial acetic acid. Adjust volume to 100ml with H₂O.
Dispense into 10ml aliquots and sterilize by autoclaving.

22. SOB Agar plates

- 10g Bacto-tryptone
- 2.5g Bacto- yeast extract
- 0.295g NaCl
- 0.09g KCl in d.H₂O

Make up to 475ml with d.H₂O.
Adjust to pH 7 with HCl. Add 7.5g bacto agar to medium and adjust volume to 490ml.
Autoclave to sterilize. Allow cooling to 60°C. Shortly before pouring into petri dishes; add 10ml of 1M MgSO (solution autoclaved separately).

23. Transformation Buffer (TFB)

- 0.74g KCl
 - 0.89g MnCl2.4H₂O
 - 0.15g CaCl₂.2H₂O
 - 0.08g HAcOCl₃
 - 10ml 0.5M KMES (Equilibrate a 0.5M solution of MES (2[morpholino]ethonesulphonicacid) to pH 6.3 using concentrated KOH)
- Make up to 100ml with d.H₂O.
Sterilize by filtration through a 0.22μ disposable filter.
Store in aliquots at -20°C.

24. Luria Bertani (LB) Broth

10g Bacto-tryptone
5g Bacto-yeast Extract
10g NaCl

Add 900ml d.H₂O. Adjust to pH 7. Make up to 1litre. Dispense in aliquots of 20mls using sterile 50ml Schott bottles. Sterilize by autoclaving.

25. 10% Ammonium Persulfite (APS)

1g ammonium persulfite

Dissolve in 10ml d.H₂O.

Dispense in aliquots of 100 μ l using sterile 0.5ml tubes.

Store at -20°C.

Do not refreeze APS.

26. 10X TBE Buffer

108g Tris base
55g Boric acid
9.3g EDTA

Make up to 1 litre with d. H₂O.

27. 1M IPTG

2.38g IPTG

Make a solution of IPTG by dissolving in 8ml d.H₂O.

Adjust the volume to 10ml with dH₂O

Sterilize by filtration through a 0.22 μ disposable filter.

Dispense in 1ml aliquots.

Store at -20°C.

28. Phosphate Buffered Saline (PBS)

8g	NaCl
0.2g	KCl
0.12g	KH ₂ PO ₄
0.91g	Na ₂ HPO ₄

The buffer was made up to 1 litre with d.H₂O, pH 7.5, then autoclaved.

29. 2X Denaturing Sample Buffer (10 ml)

2.5ml	0.5M Tris-HCl pH 6.8
2.0ml	Glycerol
4.0ml	10% SDS
0.25ml	1% Bromophenol Blue (w/v)
0.5ml	2-mercapto-ethanol
0.75ml	deionised water

Store at -20°C.

30. 30% Acrylamide:Bis stock (29:1)

29.2g	Acrylamide
0.8g	bis-acrylamide

Dissolve in 50ml d.H₂O by heating at 37°C.

Make up to 100ml with d.H₂O.

Store at 4°C for up to 6 mths.

31. 12% Separating Gel 0.375 M Tris pH 8.8 (20 ml)

6.7ml	Water
8.0ml	30% Acrylamide bis acrylamide
5.0ml	1.5 M Tris HCl pH 8.8
0.2ml	10% SDS
0.008ml	Temed
0.2ml	10% APS (0.1 g/ml)

32. Water Saturated n-Butanol

Add water to n-Butanol until Butanol is saturated. Two phases appear: n-Butanol at the top, and water at the bottom

33. 1.5M Tris (pH 8.8)

27.23g Tris base

Dissolve in 80ml d.H₂O. Adjust to pH 8.8 with 1N HCl. Make up to 150ml with d.H₂O

34 1M Tris (pH 6.8)

12.1g Tris base

Dissolve in 60ml d.H₂O. Adjust pH to 6.8 with 1N HCl. Make up to 100ml with d.H₂O.

Store at 4°C.

35. 5% Stacking Gel 0.125M Tris pH 6.8 (5.0 ml)

3.4ml	Water
0.83ml	30% Acrylamide bis acrylamide
0.63ml	1M Tris HCl pH6.8
0.05ml	10% SDS
0.05ml	10% APS
0.005ml	Temed

36. Staining Solution (100ml)

40%	Methanol
10%	Acetic acid
0.1%	Coommassie Brilliant Blue G250
49.9ml	Deionised water

37. Destaining Solution (100ml)

40% Methanol
10% Acetic acid
50ml Deionised water

38. 10X Tris/Tricine/SDS Electrode Buffer

121g Tris base
179g Tricine
100ml 10% SDS

Make up to 1L with deionised water. Store at RT.

Dilute 1:10 for use.

39. Pefabloc (500mM)

Add 835ml d.H₂O to 100mg of Pefabloc.

Use at 1:1000 dilution ie 0.5mM.

40. 50mM Tris HCl pH 8

Dissolve 0.61g Tris base in 80ml d.H₂O.

Adjust to pH 8 with HCl.

41. 10mM Reduced Glutathione

10mM reduced glutathione in 50mM Tris HCl pH 8,

42. 10X Transfer Buffer

30.3g Tris Base
144.2g Glycine

Dissolve in d.H₂O up to 1000ml.

Dilute 1:10 for use

43. Blocking/Incubation Buffer for Immunoblot

5g fat free milk powder

1:5 dilution of 10X Wash buffer

Dissolve fat free milk powder in 1X wash buffer.

44. 10X PBS/0.5% Tween 20 Wash Buffer for Immunoblot

99.5ml PBS

0.5ml Tween 20

Dilute 1:10 for use as wash buffer

45. Peroxidase Substrate

Solution 1: 10ml ice cold methanol plus 1 tablet 4-Chloro-1-Naphthol 4CIN.

Solution 2: Add 30 μ l Hydrogen peroxide to 50ml PBS

Mix together, solution 1 and solution 2, and use immediately.

46. 3M Sodium chloride

Dissolve 17.4g in 100ml d.H₂O. Autoclave dissolved solution and store at 4°C.

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