Studies on the Pathogenesis and Epidemiology of Ovine Pulmonary Adenocarcinoma (OPA)

Thesis submitted for the Degree of Doctor of Philosophy

by

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Contents

Declaration

I declare that all the work presented in this thesis has been composed by myself.

Contributions to the work of this thesis by colleagues are fully acknowledged.

This work has not been submitted for candidature for any other degree.

Daniela Salvatori

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I wish to dedicate my Ph.D. Thesis

with a lot of gratitude to

Dr W.B. Martin

Abbreviations

AIDS: acquired immunodeficiency syndrome

ALV: avian leukosis virus

Amp: ampicillin

ASV: avian sarcoma virus

ATII: pneumocytes type II

ATL: adul T-cell leukemia

BAC: bronchioloalveolar carcinoma

BIV: Bovine immunodeficiency virus

BLV: bovine leukemia virus

bp: base pairs

CA: capsid protein

CAEV: caprine-arthritis-encephalitis virus

CCSP: Clara cell secretory proteins

CHV-1: caprine herpesvirus-1

CI: confidence interval

CMV: cytomegalovirus

ConA: concanavalina A

CTLs: cytotoxic T lymphocytes

DAB: 3,3'-diaminobenzidine

DC: dendritic cells

DMF: di-methylformamide,

DNA: deoxyribonucleic acid

EIAV: Equine infectious anemia virus

ELISA: Enzyme Linked ImmunoSorbent Assay

EM: electron microscopy

enJSRVs: endogenous JSRV-related sequences

ENT: enzootic nasal tumour

Env: envelope protein

ERV: endogenous retrovirus

EU: European Community

FCA: Freund's Complete Adjuvant

FeLV: feline leukemia virus

FIV: feline immunodeficiency virus

GAPDH: glyceraldehyde-3-phosphate dehydrogenase gene

GLMMs: Generalised Linear Mixed Models

GPI: glycosylphosphatidylinositol

GST: glutathione S-transferase

HBV: hepatitis B virus

H-E: hematoxylin and eosin

HERV: human endogenous retrovirus

HERV-K: human endogenous retrovirus K

HERV-W: human endogenous retrovirus W

HFV: human foamy virus

HIV: human immunodeficiency virus

HTLV: human T-cell leukemia virus

IFA: Freund's Incomplete Adjuvant

IHC: immunohistochemistry

IL-6: interleukin-6

IL-10: interleukin-10

IN: integrase

IPTG: Isopropyl-D-thiogalactopyranoside

JSRV : jaagsiekte sheep retrovirus

JSRV-SA: JSRV South African strain

LB: L-broth

LF: lung fluid

LNGV: langur virus

LOH: loss of heterozygosity

LTR: long terminal repeat

MA: matrix protein

MALDI-TOF: Matrix associated laser desorption ionisation time of flight mass spectrometry

MCF: mouse mink cell focus-forming virus

M-CSF: mononuclear phagocyte colony-stimulating factor

MDV: Marek disease virus

MLV: murine leukemia virus

MMTV: mouse mammary tumour virus

M-PMV: Mason-Pfizer monkey virus

M-tropic: macrophage tropic

MoMLV: Moloney murine leukaemia virus

MuLV: murine leukemia virus

MVV: maedi-visna virus

NC: nucleocapsid protein

NF-κB: nuclear factor κB

OD: optical density

OHV-1: Ovine herpesvirus – 1

OHV-2: Ovine herpesvirus – 2

OPA : ovine pulmonary adenocarcinoma

ORF: open reading frame

p: p-value, associated with a statistic test

PBLs: peripheral blood leukocytes

PBMC: peripheral blood mononuclear cells

PCR: polymerase chain reaction

PND: postnatal day

PHA: phytohaemagglutinin

PI: Post infection

PR: protease

PrL: protease-like

RBD: receptor binding domain

REV: reticuloendotheliosis virus

RNA: ribonucleic acid

RSV: Rous sarcoma virus

RT: reverse transcriptase

RTS: Rapid Translation System

SA-OMVV: South African ovine maedi-visna virus

SERV: sheep endogenous retrovirus

SIV: simian immunodeficiency virus

SIV-MAC: simian immunodeficiency virus-Macaques

SIV-AGM: simian immunodeficiency virus- African Green Monkeys

SMRV: squirrel monkey retrovirus

SP-A: surfactant protein A

SP-C: surfactant protein C

SRLVs: small ruminants lentiviruses

SRV-1: simian retrovirus-1

SRV-2: simian retrovirus-2

SU: surface protein

t: t statistic

TLR: toll-like receptor

TGF-β: transforming growth factor-beta

TM: transmembrane protein

TNF-alpha: tumour necrosis factor alpha

TSP-HAM: tropical spastic paresis-HTLV-1 associated mielopathy

WDSV: Walleye dermal sarcoma virus

WFB: washing fluid B

VEGF: vascular endothelial growth factor

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ABSTRACT

Ovine pulmonary adenocarcinoma (OPA) is a contagious lung cancer of sheep. OPA is found in over 20 countries on the continents of Europe, Africa, America and Asia, and in a wide variety of breeds. The tumour also has been diagnosed sporadically in goats and wild moufflon. OPA is caused by an exogenous beta-retrovirus, jaagsiekte sheep retrovirus (JSRV), which is different from the transcriptionally active endogenous retroviral sequences present in the ovine genome. A unique feature of OPA is the absence of a specific humoral immune response to JSRV, despite the highly productive infection in the lungs and the disseminated lymphoid infection. The absence of detectable antibodies has hampered the diagnosis and control of the disease before the development of clinical signs. The disease can be reproduced consistently in neonatal lambs by intra-tracheal injection of inocula containing JSRV, but this experimental model was unsuitable for various purposes, such as the assessment of potential vaccine preparations. In the present study, the clinical disease was reproduced, pathologically confirmed as OPA, in a high proportion of lambs inoculated intra-tracheally with infectious lung fluid at either one, three or six months of age. The incubation periods, however, were found to be longer in the older age groups than in one week old lambs. During the course of the experiment, viraemia was detected consistently by PCR (JSRV U3 PCR). The persistent viraemia and the delayed development of OPA in the older lambs paralleled epidemiological observations in naturally affected flocks. The optimisation of the JSRV U3 PCR represented an important breakthroughs for studies on the pathogenesis of this

infection and for the diagnosis and control of the disease. JSRV U3 PCR was employed for the first longitudinal survey for detecting JSRV infection in a flock with natural history of OPA. Ewes and offspring were followed for a period of two and half years and the JSRV U3 PCR test was repeated every 3-4 months. It was shown that the flock was heavily infected with JSRV. The lambs from both negative and positive mothers became JSRV positive as early as 20-30 days after birth. The rate of infection of the ewes and of the lambs increased significantly after the first test. JSRV incidence in the ewe population was higher in the period prior to lambing. The results suggested that Texel breedline had a higher susceptibility to JSRV infection and a higher probability of remaining positive.

New envelope and capsid recombinant proteins were produced not only for immunological assays but also for preliminary immunisation trials in sheep. The present experiments have been able to overcome previous difficulties regarding JSRV Env protein production.

In the present study IHC gave new insight about histogenesis and viral pathogenesis of OPA. In fact SU Env protein was found widespread on the surface of tumour cells. This finding proved that envelope protein is consistently produced suggesting again a role in cell proliferation. These epidemiological and experimental transmission data point to JSRV as a non-acute transforming retrovirus and raise questions about the *in vivo* role of JSRV *env* gene in transformation.

Chapter 1

Introduction

1.1 Introduction and history of ovine pulmonary adenocarcinoma (OPA)

Ovine pulmonary adenocarcinoma (OPA) is a contagious disease characterised by a tumour in the lungs of sheep (Sharp, 1987). OPA is also known as jaagsiekte, sheep pulmonary adenomatosis or ovine pulmonary carcinoma. OPA is caused by an exogenous retrovirus called jaagsiekte sheep retrovirus (JSRV).

The disease was first recognised in South Africa in the 19th century as a cause of respiratory distress in sheep. The Afrikaans name "jaagsiekte" derived from "driving" (jaag) and "sickness" (ziekte), reflected the tendency of diseased sheep to lag behind the flock during herding (Tustin, 1969).

Since then, OPA has been identified in a wide variety of breeds in over 20 countries on the continents of Europe, Africa, America and Asia, with the notable exceptions of Australia and New Zealand (Eber, 1899; Aynaud, 1926; Sigurdsson *et al.*, 1954; Shirlaw, 1959; Ceretto F. *et al.* 1967; Al-Zubaidy *et al.*, 1979; Tontis *et al.*, 1979; Stevenson *et al.* 1980; Verwoed *et al.*, 1985; Toumazos, 1989; Sharp & De las Heras, 2000). The disease does not appear to affect Australia and New Zealand, apart from two isolated reports (Dalefield & Aleey, 1988; Hooper & Ellard, 1995).

Iceland is now free from OPA but after a lot of effort and paying high economic costs for the eradication programme. OPA was introduced in Iceland in 1933 along with scrapie, maedi-visna and paratubercolosis by a small number of sheep imported from Germany (Sigurdsson *et al.* 1954). A year after the introduction of those animals, the first cases of OPA appeared in 1-3 year old sheep. During the next 2-3 years, about 60% of the breeding stock in affected flocks died of OPA. In the

following 3 years the mortality decreased to under 10% and at this time another respiratory disease called maedi-visna appeared. It was later determined that this disease was caused by a lentivirus called maedi-visna virus (MVV) (Sigurdsson, 1958).

Sigurdsson in 1954 used the adjective "slow" to describe the peculiar clinical evolution of this group of diseases. He had studied the three imported diseases of sheep peculiar to Iceland, *maedi*, *visna*, and *rida*. In 1954 he proposed that 'A number of [slow] infections exist and the following criteria could be tentatively suggested: (1) A very long period of latency lasting from several months to several years. (2) A protracted course after clinical signs have appeared usually ending in serious disease or death. (3) Limitation of the infection to a single host species and anatomical lesions in only a single organ or tissue system. These last statements may have to be modified as knowledge increases.'

OPA is a tumour. Tumours are generally uncommon in domestic livestock, but in sheep, two neoplastic diseases are recognised, OPA and enzootic intranasal adenocarcinoma (Duncan *et al.*, 1967; Charray *et al.*, 1985; De las Heras *et al.*, 1991; Gazquez *et al.*, 1992; Vitellozzi *et al.*, 1993; De las Heras *et al.*, 1995; DeMartini & York, 1997). Both types of tumour arise from secretory epithelial cells that retain their secretory function after transformation (Nisbet *et al.*, 1971). Each disease is associated exclusively with betaretroviruses that are highly related but distinct (Sharp, 1987; DeMartini & York, 1997; Ortín *et al.*, 2003).

Many scientists have noticed similarities between OPA and human

bronchioloalveolar carcinoma (Bonne, 1939; Perk & Hod, 1982; Rosenberg, 2001). Understanding the mechanisms of tumourigenesis in OPA may offer insights into some forms of human adenocarcinoma (Palmarini & Fan, 2001).

1.1.1 Clinical Signs and Epidemiological Features in OPA.

The incubation period in naturally infected sheep appears to be long, so that the disease is not usually seen until sheep are about 2-4 years old (Hunter & Munro, 1983). However, tumours have been seen exceptionally in lambs as young as 2 months old and sheep 11 years of age (Sharp & Angus, 1987).

Clinical signs are those of an afebrile progressive respiratory illness associated with loss of weight. The severity of the tachypnoea and dyspnoea, often associated with noticeable movement of the abdominal wall, depends on the extent of the tumour and loss of normal functional lung. A pathognomonic feature of OPA is the accumulation of lung fluid within the respiratory tract; in advanced cases "bubbling" sounds may be heard when the chest is auscultated. In addition, a very helpful test for this disease, is to lower the head of the sheep below the chest, when fluid may run from the nostrils ("wheel-barrow test") (Sharp & Angus, 1990) (Fig.1.1). Sometimes quite copious amounts of mucoid fluid, up to 300 ml, can be collected (Fig.1.2). Appetite is maintained, though loss of weight is obvious. OPA affected sheep show signs which continue over several weeks. Death is often due to inter current bacterial infection by *Mannhaemia haemolytica*. In some countries, another form of the tumour, described as atypical OPA, has been reported. This form is not

Fig. 1.1. OPA clinical signs.

Frothy seromucous seromucous fluid is discharged from the nostrils when OPA-affected sheep hindquarters are raised and the head lowered.

Fig.1.2. OPA clinical signs: fluid collection.

The amount of fluid that can be collected from an affected animal varies from 50-60ml to as much as 300 ml.

associated with excess accumulation of fluid in the lung and, therefore, generally presents as an incidental finding at necropsy or in the abattoir (Garcia-Goti *et al.*, 2000). Due to the lack of ante-mortem diagnostic tests the control of OPA is hindered severely.

Most information on the prevalence of OPA has been based on clinical observations and histopathological diagnosis. Information of the true prevalence of JSRV infection in OPA-affected flocks has been a major gap in our knowledge.

The prevalence of OPA appears to vary between countries. For example OPA is endemic in Peru, Scotland and South Africa. In these countries it can cause between 2 and 10% annual mortality in adult sheep in the affected flocks (Sharp & DeMartini, 2003).

Annual losses from OPA in affected flocks have been put from 1-2% up to 6% (Sharp & Angus, 1985; Verwoerd *et al.*, 1985; Sharp & De las Heras, 2000). Losses in infected flocks in Scotland are generally between 2% and 10% annually (Sharp, 1991). Hunter and Munro (1983) found an average annual mortality rate of 3 % in three affected flocks in Scotland. In a flock in East Anglia with concurrent maedi-visna infection and OPA, there was an increase to about 20% in the number of ewes culled prematurely for loss of condition (Pritchard & Done, 1990).

A longitudinal survey lasting five years based on histopathological studies in two OPA affected flocks in Scotland revealed that about 30% of the sheep had histologically confirmed lesions in their lungs and between 12% and 21% of the sheep had subclinical tumours (Sharp, 1981).

In contrast, OPA is infrequently diagnosed in the USA or Canada, where only few cases have been reported (Stevenson *et al.,* 1980). These differences in prevalence and incidence of OPA may reflect variations in viral strains, host susceptibility, or may reflect the result of different management systems.

The detection of JSRV in the peripheral blood of experimentally infected lambs, before the development of tumour or onset of clinical disease, therefore, was an important finding that offered a means to investigate the extent of JSRV infection in OPA-affected flocks (Holland *et al.,* 1999).

These findings have been extended in studies involving sheep from OPA-affected commercial flocks in Spain (García-Goti, 1999). Peripheral blood leukocytes (PBLs) and tissue samples from sheep were examined for JSRV by PCR. Overall, JSRV was detected in all OPA sheep, with either the classical or atypical form of OPA, as well as in 80% of OPA in-contact sheep. None of 71 samples from the control sheep was positive. Although an earlier report had indicated that as few as 1/250,000 PBLs might be positive for JSRV (Holland *et al.,* 1999), in this study, JSRV was detected in the PBLs of all classical OPA sheep, 83% of the sheep with atypical OPA, as well as 40% of lesion-free in-contact sheep. These results clearly demonstrated that JSRV could be detected in naturally infected live sheep before the onset of clinical OPA and even in the absence of discernible lung tumours. Although only a small number of in-contact sheep were examined, it seems clear that subclinical JSRV infection can reach a high prevalence in OPA-affected flocks.

To provide further information of the dynamics of JSRV infection in OPA-affected

flocks, García-Goti *et al.* in 2000 conducted a small prospective longitudinal survey in an OPA-affected flock. JSRV was detected by PCR in PBLs from 28% of the flock. Fifteen positive sheep and five negative were selected and PBLs examined by JSRV PCR at monthly intervals for the next four months. JSRV was detected in only nine of the original 15 positive sheep during this period and in four of those that were negative. These results demonstrated the fluctuation in detection of JSRV in blood and, more importantly, confirmed the high prevalence of JSRV infection in OPA-affected flocks.

1.1.2 Pathology of OPA

1.1.2.1 Gross pathology

At post-mortem examination, lungs affected with OPA are 2-4 times heavier than normal and they fail to collapse when the thorax is open (Fig. 1.3). Frequently, in the cranioventral region of the lungs there is a firm, grey-purple neoplastic mass which is often surrounded by small satellite tumour nodules (Dungal *et al.,* 1938; Cuba-Caparo *et al.,* 1961; Dualde-Pérez, 1966; Wandera, 1970; Gonzalez, 1990; Sharp & Angus, 1990), (Fig.1.4). The cut surface of the neoplastic tissue is generally moist and frothy fluid may pour from the bronchioles of the affected area. In most cases, this fluid accumulates in the airways and is discharged from the nostrils (Fig.1.5).

Lung adenocarcinoma can give metastases, mainly affecting regional lymph nodes, although some reports found metastases in extrathoracic tissue (Cuba-Caparo *et al.*, 1961; Cutlip &Young, 1982; Snyder *et al.*, 1983; DeMartini *et al.*, 1988). In the Awassi sheep of Israel, metastases have been observed in 50% of cases (Nobel *et al.*,

Fig.1.3. Classical OPA: macroscopic pathology.

Gross lesion are generally confined to the lungs. The lungs appear considerably enlarged and they do not collapse when the chest is opened. The normal weight can be increased three or more times, depending on the extent of the tumour. The neoplastic areas vary from small discrete nodules (0.5 - 2 cm) to extensive tumours involving the entire ventral half of the diaphragmatic and other lobes.

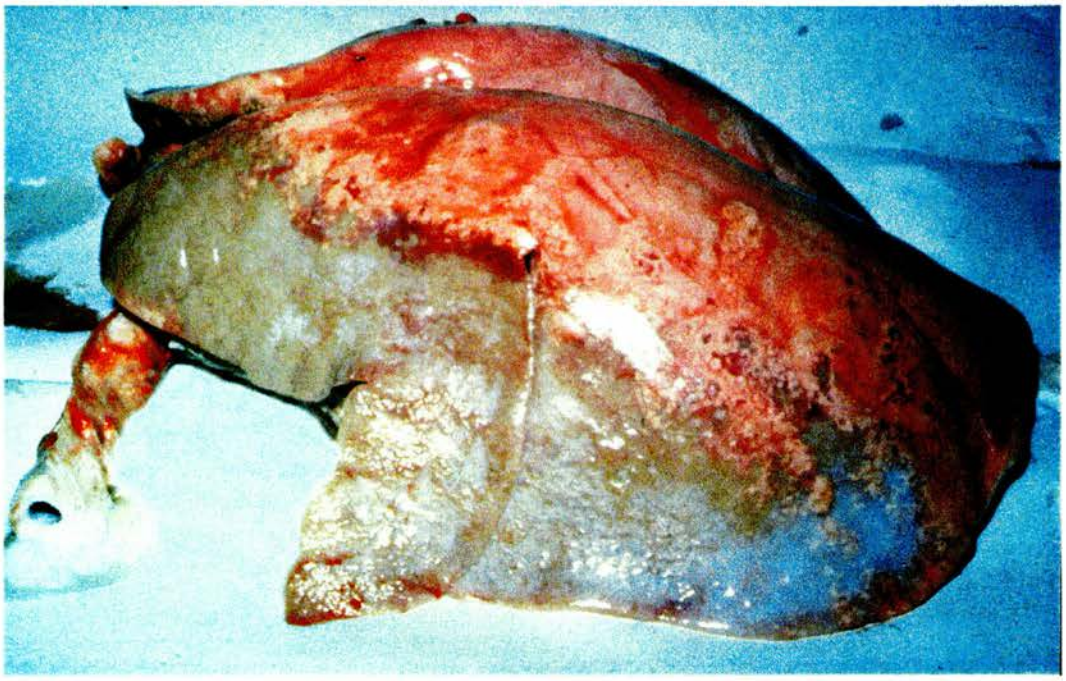


Fig. 1.4. Classical OPA: macroscopic pathology of the lung.
Tumours appear as purple coloured areas in the cranioventral part of the lung. The surface is moist and frothy fluid pours from the trachea.

Fig. 1.5. Typical OPA: section of the diaphragmatic lobe of the lung showing tumour.

Affected areas with meaty aspect and frothy fluid pouring from the bronchioles.

1969).

In some cases, an "atypical OPA" has been observed. In contrast to "classical OPA", in "atypical OPA" solitary or coalesced nodules are hard, pearly white and with a dry cut surface (Fig.1.6). The tumoral nodules are clearly demarcated from the sorrounding parenchyma, either underneath the pleura or deep in the lung tissue; fluid is absent in the bronchi (Fig. 1.7 & 1.8).

The features of atypical OPA are responsible for a subclinical course of the disease.

Infact this condition is commonly found only in abattoir studies (De la Heras *et al.*, 1992), or at necropsy of sheep dying from other unrelated causes (González, 1990).

In contrast to the classical form of OPA, there are few reports of atypical OPA in the literature. In Spain, De las Heras *et al.* (1992) and Dualde-Pérez (1966) described these lesions in the course of abattoir studies, and González (1990) did so in the course of a clinico-pathological survey of ovine chronic pneumonias. Similar lesions were also identified in Peru by Cupa-Caparo *et al.* (1961). In both Spain and Peru, classical OPA is also commonly recognised and, in some reports, specific reference is made to the fact that both forms of OPA may appear in the same flock (González, 1990). However, in other countries in which classical OPA is well recognised, such as Scotland, South Africa and Israel, there appear to be no specific references to the atypical form of the disease (Nobel, 1958; Tustin, 1969; Hunter & Munro, 1983; Sharp & Angus, 1990).

Secondary bacterial infections, such as pneumonic pasteurellosis and mycoplasmosis, are common, leading to pleurisy and abscessation and they may

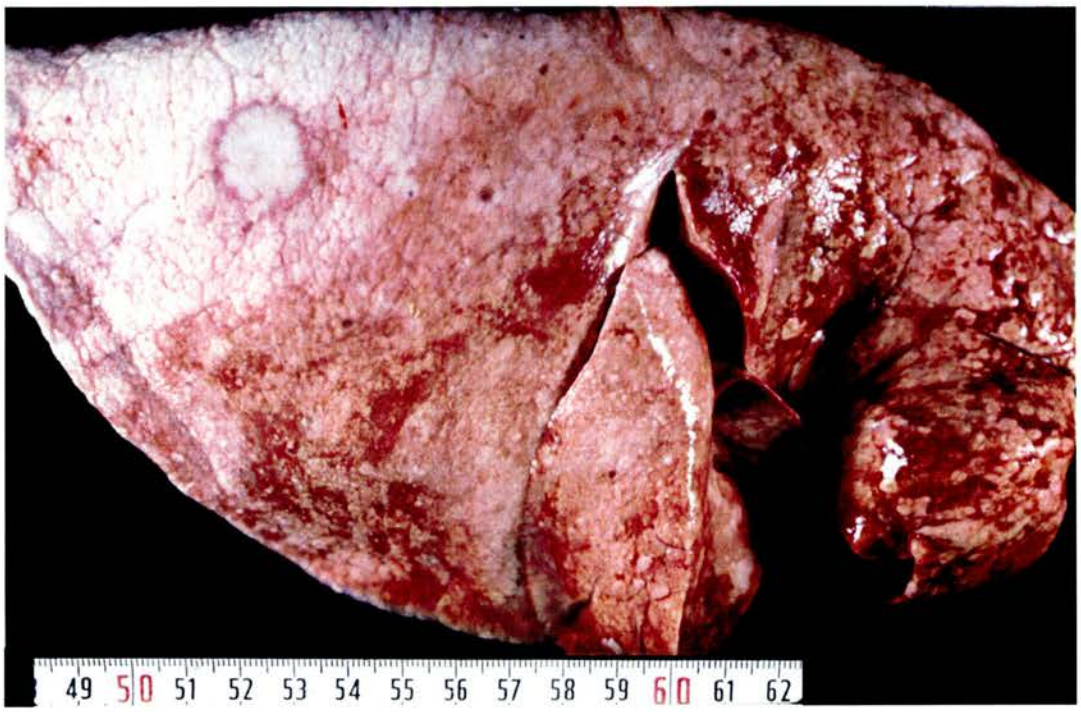


Fig.1.6. Atypical OPA.

Well demarcated white, solitary tumour nodule in the dorsocaudal area of the right lung.

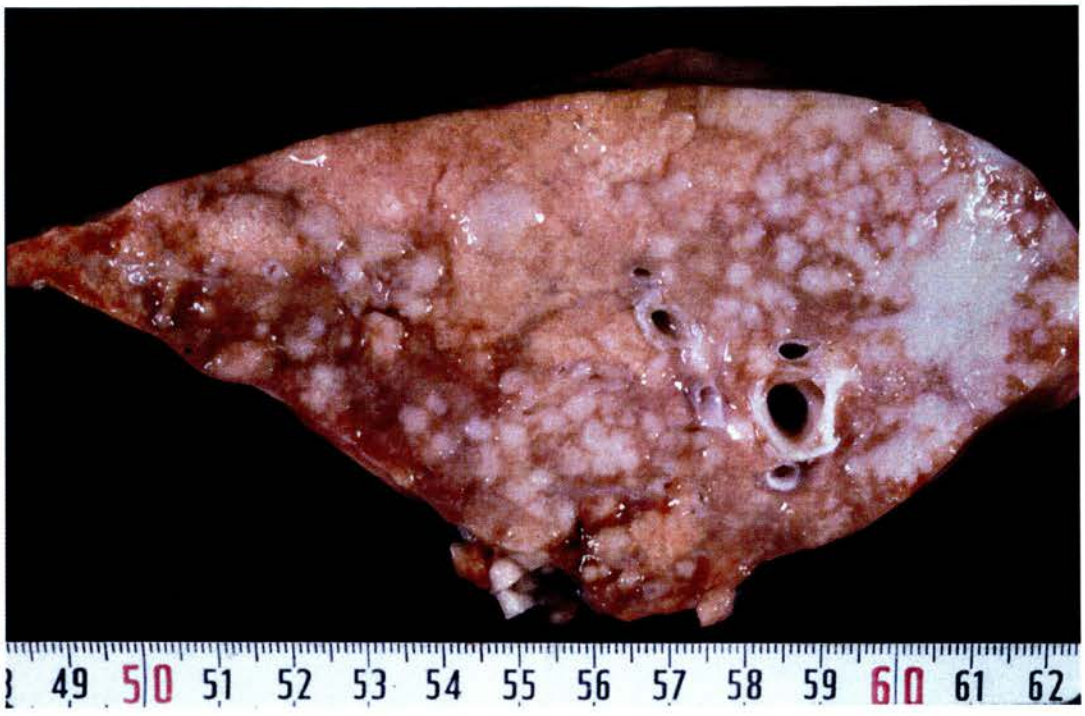


Fig.1.7. Atypical OPA: section of a lung.

Severe case of atypical OPA with disseminated, numerous, dry confluent nodules.

explain the eventual appearance of inflammatory infiltrates in the stroma of the tumour (Cutlip & Young, 1982).

1.1.2.2 Histopathology and Electron Microscopy

OPA is a tumour arising from alveolar type II pneumocytes and the non-ciliated bronchiolar (Clara) cells. Histologically neoplastic masses are composed of cuboidal to columnar epithelial cells that line the alveolar and bronchiolar air spaces exhibiting a papillary or an acinar pattern (Fig.1.9); sometimes the tumour cells form papilliform growths that project into the alveoli and bronchiolar spaces. Neoplastic areas present a thin stromal tissue in which mononuclear inflammatory cells and connective tissue fibres are usually present in small numbers. The non transformed alveoli contiguous to the neoplastic foci are usually compressed and are characteristically filled with enlarged macrophages with a foamy cytoplasm (para-adenomatous lesion) (García-Goti *et al.*, 2000).

Histological observation of lung sections from natural cases of OPA shows the presence of several foci of epithelial neoplastic proliferation in alveolar and bronchiolar regions. These proliferations can have a papillary or an acinary appearance. The alveolar neoplastic nodules compress the neighbouring alveoli causing atelactasis and/or coalescence. Cuboidal or columnar cells replace the normal flat type I pneumocytes. The nuclei of the transformed cells are located in the basal region of the cells. The cytoplasm of these cells can be homogeneously eosinophilic, clear and vacuolated and frequently it stains positively for glycogen deposits (Wandera, 1970; González, 1990; Sharp & Angus, 1990). The stroma of the

Fig 1.8. Atypical OPA: section of a solitary demarcated atypical OPA nodule.

The atypical nodules are pearly white in colour, very well demarcated from the sorrounding parenchima, very hard in consistency and sometimes look like scars.

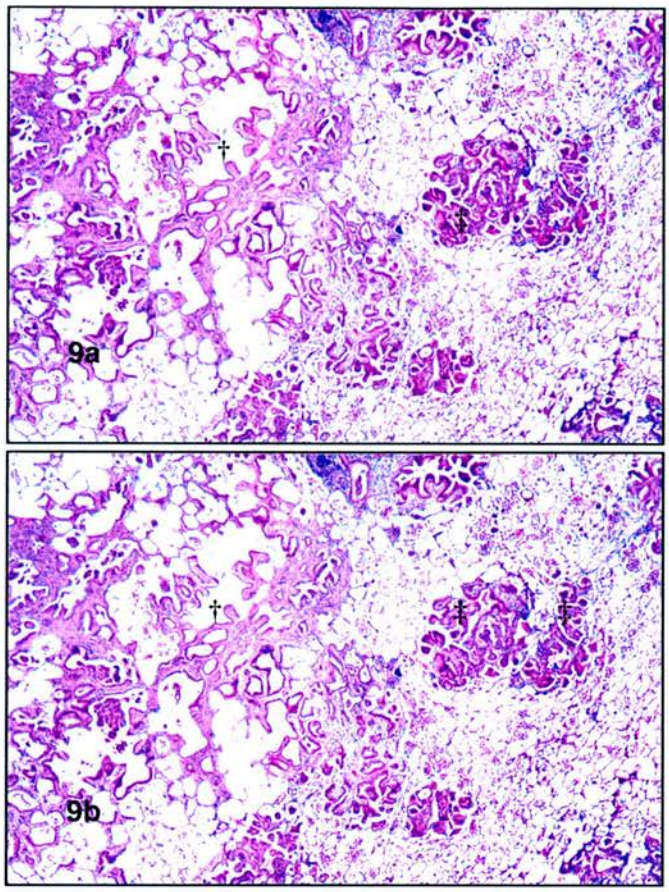


Fig.1.9. Histopathology of experimental OPA.

Neoplastic lesions in the lung of a lamb inoculated with JSRV at 1 month of age. (a) Co-existence of papillary (†) and acinary (‡) forms of the tumour and presence of desquamated alveolar macrophages. H-E, bar = 400μm. (b) Papillary neoplastic proliferations involve alveoli and bronchioles, which also show hyperplasia of associated lymphoid tissue. Para-adenomatous change is also conspicuous. H-E, bar = 200μm.

tumour is generally thin but may be infiltrated by variable amounts of lymphocytes, plasma cells and connective tissue fibres (De las Heras *et al.,* 1995). However, in more advanced tumours and particularly in their central areas, fibrosis of the stroma can be prominent (González, 1990; Sharp & Angus, 1990).

Macrophages with vacuolated cytoplasm are consistently found surrounding neoplastic alveoli and filling the lumena of the normal ones; they are called para-adenomatous areas because they look like a delimiting barrier of the tumour nodules (González, 1990; Sharp & Angus, 1990; De las Heras *et al.,* 1995). Another histopathological feature of OPA, usually seen concurrently with alveolar lesions, occurs in the terminal bronchioles, in which multiple polypoid ingrowths arise from the bronchiolar epithelium. In some cases, masses of myxomatous tissue, probably of mesodermal origin, are found in association with the epithelial neoplastic growths (Cutlip & Young, 1982; Sharp & Angus, 1990; García-Goti *et al.,* 2000).

According to De la Heras *et al.*, (1992) the histopathological expression of atypical tumours is essentially the same as that of the classical ones, but in the "atypical OPA" the stroma of the tumour is heavily infiltrated by mononuclear inflammatory cells, mostly lymphocytes and plasma cells, and connective tissue fibres; lymphoid proliferations are also consistently seen around the bronchioles of neoplastic areas (García-Goti *et al.,* 2000).

The reactive changes in the stroma of atypical OPA may be due to a host immune response. In the tumour stroma there is presence of lymphocytes, macrophages and connective tissue. The fibrocellular infiltrates are essentially the same in classical

and atypical OPA and the main difference between the two patterns is only quantitative (Dualde-Perez, 1966; Hod *et al.*, 1977; Payne & Verwoerd, 1984; De las Heras *et al.*, 1992).

Regional lymph nodes of OPA affected lungs may show either depletion of cortical follicles and moderate plamacytosis in the medullary area (De las Heras *et al.,* 1995), hyperplastic reactive cortices, or no specific changes (Sharp & Angus, 1990). Lymph node metastases when present, consist of epithelial neoplastic cells arranged in a similar acinar or papillary pattern as in the lung tumour, but scirrous and mixed epithelial/scirrous proliferations have also been described (Nobel *et al.,* 1969). The scirrous proliferation in the metastases very much resembles the myxomatous proliferation in the lung (Nobel *et al.,* 1969). Metastases in regional lymph nodes seem to be less frequent in atypical OPA than in classical OPA (García-Goti *et al.,* 2000).

The major capsid protein of JSRV (JSRV CA) can be detected in OPA tumours by immunohistochemical (IHC) techniques (Palmarini *et al.,* 1995). This viral protein is located in the cytoplasm of transformed alveolar and bronchioalveolar cells, and can be shown both in classical and atypical OPA cases (Fig.1.10). Positive labelling can also be found in a few mononuclear cells with lymphocyte morphology in the paracortical areas of the regional lymph nodes. Within a tumour nodule individual cells or group of cells may stain intensely for JSRV CA while other cells within the same tumour nodule may not stain at all (Palmarini *et al.,* 1995; Platt *et al.,* 2002).

Immunohistochemical studies carried out by García-Goti *et al.*, 2000, revealed fewer

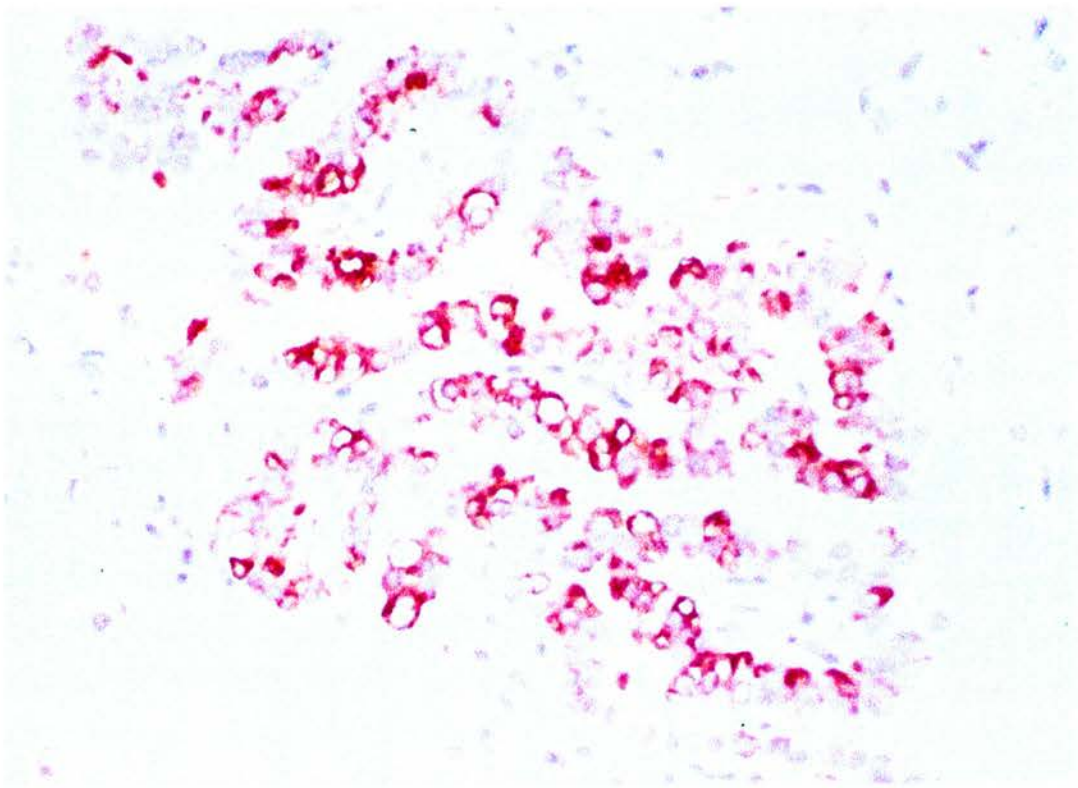


Fig. 1.10. Immunohistochemistry for JSRV-CA protein.

Immunolabelling is associated with the cytoplasm of neoplastic alveolar cells. Immuno-histochemistry and counterstaining, x25.

JSRV-positive cells in the atypical tumours than in the classical ones. This finding agrees with the results obtained from the detection of JSRV proviral DNA when a smaller number of positive replicates was obtained from atypical tumours, which would indicate a lower number of target proviral DNA copies than in classical tumours. In addition, viral dissemination in the host appeared to be less pronounced in atypical OPA, as demonstrated by the lower numbers of positive samples and positive replicates in PCR on blood than in the cases of classical OPA.

Thus, JSRV-exogenous specific U3 long terminal repeat (LTR) sequence and JSRV capsid protein (CA) were detected in lungs from animals with classical OPA and from those with atypical OPA. The LTR is the region that presents most variations in different retroviruses, such as JSRV, sheep endogenous retroviruses and ovine enzootic nasal tumour virus; even sequencing did not make possible to distinguish atypical from classical samples (García-Goti *et al.*, 2000).

More extensive studies are needed to confirm this apparent common aetiology, as differences in other regions of the JSRV genome may exist and account for the different lesions in classical and atypical OPA.

Based on electron microscopy (EM), the alveolar type II cells and the non ciliated bronchiolar Clara cells are the predominant neoplastic cell phenotypes in naturally occurring OPA and in experimentally induced OPA (Nisbet *et al.*, 1971; Perk *et al.*, 1971; Hod et al., 1977; Sharp, 1987; Platt *et al.*, 2002). Transformed cells maintain the appearance of the cells from which they originated. Type II pneumocytes and Clara cells are both secretory epithelial cells. In type II pneumocytes there is

synthesis, storage and secretion of the alveolar surfactant (Voelker & Manson, 1989; Mason & Shannon, 1997). Clara cells are involved in the synthesis, storage and secretion of protein components of the extracellular lining layer of bronchioles (Massaro, 1989; Plopper *et al.*, 1997). Ultrastructurally, tumour cells of type -II cell origin are usually well differentiated, with microvilli, desmosomes, and intracellular lamellar bodies; tumour cells of Clara-cell origin contain cytoplasmic dense bodies instead of lamellar bodies (Nisbet *et al.*,1971; Perk *et al.*,1971; Payne & Verwoerd, 1984; Cutlip & Young, 1982; Herring *et al.*, 1983; DeMartini *et al.*, 1987). By IHC studies, both cell types are occasionally found in the same tumour but they are involved in different percentages. To further characterise the neoplastic cell population, Platt *et al.*, 2002 immunostained sections of tumours with antibodies to surfactant protein A (a phenotypic marker for ATII pneumocytes and Clara cells), surfactant protein C (specific marker for ATII cells) and Clara cell 10-Kd protein. Overall surfactant Proteins A and C were expressed in 70% and 80% of tumour cell, respectively, whereas Clara cell 10-Kd protein was expressed in 17% of tumour cells.

JSRV CA was detected in 71% of tumour cells and macrophages (5/12 tumours examined) and in non neoplastic and bronchiolar cells (6/14 tumours) (Platt *et al.*, 2002).

1.1.2.3 OPA in other species

There are few descriptions of natural OPA in goats. In the pathology reports from India the features of the tumours are very similar to those for sheep (Rajya *et al.*,

1964; Sharma *et al.*, 1975). OPA has been experimentally reproduced in goats after intracheal inoculation of very young goat kids with concentrated lung fluid of sheep origin (Sharma *et al.*, 1975; Tustin *et al.,* 1977; Sharp *et al.,* 1986).

In the mid-1980s, a severe outbreak of a disease resembling OPA was described in captive Sardinian moufflon (*Ovis musimon*). Moufflon are wild sheep phylogenetically related to the domestic sheep, *Ovis aries (*Hiendleder *et al.,* 1998). A flock of over 60 mufflon was kept on a game farm in Sassari (Sardinia, Italy) with the aim of producing animals to be released later into their natural habitats. The moufflon and a flock of sheep in which OPA was endemic had repeated contacts (Ceretto & Deiana, 1967). Studies on DNA from archival materials concluded that JSRV was the cause of OPA in the moufflon (Sanna *et al.*, 2001).

Bronchiolo-alveolar carcinoma has been described in human beings and in several animal species such as, cattle, horses, pigs, cats, dogs, chinchilla, Virginia opossum (*Didelphis Virginiana*)and mice but the investigations on the moufflon resulted in the first report that positively associated JSRV with bronchiolo-alveolar carcinoma in an animal species other than the domestic sheep and goats (Tyzzer, 1907; Theiler, 1918; Grumbach, 1926; Monlux *et al.*, 1953, 1955; Liebow, 1960; Sherwood *et al.*, 1969; Perk & Hod, 1982; Sriraman *et al.,* 1982; Maruo *et al.,* 1995; Hahn *et al.*, 1996; Okada *et al.*, 1998; Kim *et al.,* 2002; Grossman *et al.,* 2002 a & b).

1.1.3 Viruses associated with OPA

From the beginning of the century OPA has been associated with different organisms

such as the sheep lung worm Muellerius capillaris, Mycoplasma and Chlamydia but none of them was demonstrated to be the etiological agent of OPA (McFadyean, 1894 and 1920; Mackay, 1969a and b; Tustin, 1969).

An interesting finding was the isolation of herpesviruses from lungs of OPA sheep in geographically distinct locations such as Scotland, ex-Yugoslavia, Kenya and South Africa (Mackay 1969a and 1969b; Nevjestic *et al.,* 1971; Malmquist *et al.,* 1972; De Villiers *et al.,* 1975; Martin *et al.,* 1976 and 1979). The different herpesvirus isolates were all antigenically related and could be differentiated by restriction endonuclease analysis (Scott *et al.*, 1984). This herpesvirus was initially classified as caprine herpesvirus-1 (CHV-1) and later as ovine herpesvirus-1 (OHV-1) (Roizman *et al.,* 1992). Although ovine herpesvirus 1 (OHV-1) had been isolated exclusively from OPA tumours, epidemiological studies and experimental infections provided no evidence for a role in the aetiology of OPA (De Villiers *et al.,* 1975, Martin *et al.,* 1976 & 1979; Scott *et al.,* 1984). Ovine herpesvirus 2 (OHV-2) is the sheep-associated malignant catarrhal fever herpesvirus and has never been linked to OPA (Baxter *et al.*, 1993; Bridgen & Reid, 1991).

It is an interesting possibility that retroviral information may be transmitted by herpesvirus and that herpesvirus expression can be modulated by retroviral elements. Retroviruses and herpesviruses are naturally occurring pathogens of humans and animals. Co-infection of the same host with both these viruses is common. It has been reported that a retrovirus can integrate directly into a herpesvirus genome. Specifically, insertion of a non acute retrovirus, reticuloendotheliosis virus (REV), into a herpesvirus, Marek disease virus (MDV) has been demonstrated. Both viruses

are capable of inducing T lymphomas in chickens and often coexist in the same animal. REV DNA integration into MDV occurred in a recently attenuated strain of MDV and in a short-term coinfection experiment in vitro. There is also provide suggestive evidence that REV has inserted into pathogenic strains of MDV in the past. Sequences homologous to the REV long terminal repeat are found in oncogenic MDV but not in non oncogenic strains (Isfort *et al.,* 1992). Recombination after integration of retrotransposons or retroviruses into the genome of another large dsDNA virus i.e. baculovirus has been documented (Malik *et al.,* 2000; Pearson & Rohrmann, 2002).

The association of retroviruses with OPA has been recognised for several years.

The earliest electron microscopy studies in OPA tumour cells were consistent with findings of A and B type retroviruses particles. However there were also reports of isolation from OPA tumours of type C retroviral particles (Malmquist *et al.,* 1972; Irving *et al.,* 1984; Perk *et al.,* 1985; Payne *et al.*, 1986). The nucleotide sequence of one of the South African isolates [South African ovine maedi-visna virus (SA-OMVV)] confirmed that the virus was related to the icelandic maedi-visna virus strain 1514 (Querat *et al.,* 1987). Experimental infection of sheep with this lentivirus caused mild immunosuppression, but no tumours were ever produced (Myer *et al.,* 1988). This situation could be explained by the fact that concurrent "maedi" and OPA infection have been recognised frequently in several countries. Many authors have proposed a synergism between these two retroviruses (Al-Zubaidy & Sokkar, 1979; Cutilip & Young, 1982; Markson *et al.*, 1983; Snyder *et al.,* 1983; Rosadio *et*

al., 1988; González *et al.*, 1993).

Different hypothesis could be formulated about a possible pathogenetic mechanism arising from a double infection maedi-visna/OPA or OHV-1/OPA.

Synergism between maedi-visna and OPA could arise from the presence of an abundant population of macrophages in OPA lesion representing an abundant substrate of target cells for MVV (Payne *et al.*,1986; Demartini *et al.*,1987; Dawson *et al.*, 1990). Nevertheless, recent investigation on atypical OPA cases indicated that the inflammatory changes in the tumour stroma were not connected with concurrent maedi-visna infection in the non neoplastic areas of the lung (Garcia-Goti *et al.*, 2000). A further possibility is that OHV-1 plays a role in the reactive changes in atypical OPA. This theory is supported by the fact that infection of young lambs with OHV-1 results in interstitial pneumonia (Scott *et al.*, 1984) although OHV-1 has been isolated on a number of occasions from lungs with lesions of classical OPA, without significant interstitial inflammation (Sharp & Angus, 1990).

1.1.4 General features of the Family Retroviridae

Retroviruses comprise a large and diverse family of enveloped, RNA viruses (Coffin, 1992a and 1992b; Coffin *et al.*, 1997). The virions are 80-100 nm in diameter (Fig.1.11). The virion RNA is 7-12 kb in size, and it is linear, single-stranded, diploid, non-segmented, and of positive polarity. The retroviral replicative strategy includes as essential steps reverse transcription of the virion RNA into linear double-stranded DNA by a virus-coded polymerase called reverse transcriptase (RT), and then subsequent integration of this DNA into the genome of the cell.

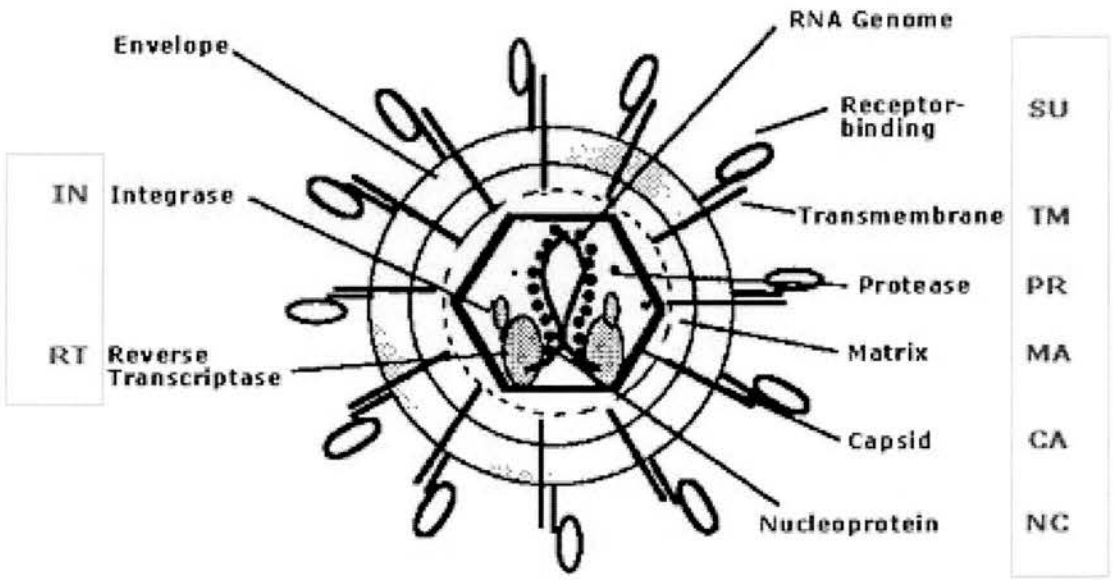


Fig. 1.11. Schematic representation of a retroviral particle.

The viral envelope is formed by a cell-derived lipid bilayer into which proteins encoded by the *env* region of the viral genome are inserted. These consist of the transmembrane (TM) and the surface (SU) components linked together by disulfide bonds. Internal nonglycosylated structural proteins are encoded by the *gag* region of the viral genome. They are the matrix (MA) protein, capsid (CA) protein, and nucleocapsid (NC) protein. Major products of the *pol*-coding region are reverse transcriptase (RT) and integrase (IN). The protease (PR) is derived from the *pro* gene between *gag* and *pol (*Coffin *et al.,* 1997*)*.

From this reverse flow of genetic information originates the terms "retroviruses" and "reverse transcriptase."

The family *Retroviridae* is divided into seven genuses (Van Regenmortel, 2000) and the nucleocapsid is concentric for *Alpharetrovirus*, *Gammaretrovirus*, *Deltaretrovirus* and *Spumavirus*, and rod or truncated coneshaped, for *Lentivirus* and *Betaretrovirus* (Table 1.1). The *Epsilonretrovirus* is a recently discovered group that is not well understood, although it appears to group with the mammalian type C retroviruses. The form of assembly was used as means of classification, where *Betavretrovirus* assembled A-type particles (immature capsid) in the cytoplasm and budded as a B-type (MMTV, Mouse mammary tumour virus) or as a D-type (M-PMV). *Alpharetrovirus* and *Gammaretrovirus* that assemble their capsids on the plasmamembrane, were classified morphologically as C-type viruses.

The early classification had three subfamilies, *Oncovirinae*, *Lentivirinae* and *Spumavirinae*, having three different outcomes of infection; leukaemia and sarcomas from the oncoviruses, immunodeficiencies and neurological disorders from lentiviruses and spumaviruses that are not presently associated with any known disease *in vivo*. Retroviruses are widely distributed as exogenous infectious agents of vertebra above or as endogenous proviruses that have resulted at some time from infection of germline cells and that are inherited as Mendelian genes. Endogenous retrovirus (ERV) families are derived from their exogenous counterparts by means of a process of germ-line infection and proliferation within the host genome. Several families in the human and mouse genomes now consist of many hundreds of

Table 1.1. Genera of the family *Retroviridae.*

Genus	Example
Alpharetrovirus	**ALV (Avian leukosis virus)**
Betaretrovirus	**MMTV (Mouse mammary tumor virus)**
Gammaretrovirus	**MLV(Murine leukemia virus)**
Deltaretrovirus	**BLV (Bovine leukemia virus)**
	HTLV (Human T-cell leukemia virus)
Epsilonretrovirus	**WDSV (Walleye dermal sarcoma virus)**
Lentivirus	**HIV (Human immunodeficiency virus)**
	MVV (Maedi visna virus)
Spumavirus	**HFV (Human foamy virus)**

elements (Belshaw *et al.,* 2004).

Retroviruses are associated with a variety of diseases. These comprise malignancies, including certain leukemias, lymphomas, sarcomas; mammary carcinomas and carcinomas of lung, liver and kidney; immunodeficiencies (such as AIDS); autoimmune diseases; lower moton neuron diseases; and several acute diseases involving tissue damage. Some retroviruses are non pathogenetic. In general transmission of retroviruses is horizontal via a number of routes, including blood, saliva, sexual contact, etc., and vertical via direct infection of the developing embryo, or via milk or perinatal routes. Endogenous retroviruses are transmitted by inheritance of proviruses (van Regenmortel *et al.,* 2000).

1.1.4.1 Genome organization

All retroviruses contain three major genes with information for virion proteins: *gag*, synthesis internal virion proteins that form the matrix, the capsid, and the nucleoprotein structures; *pol* contains the information for the reverse transcriptase and integrase enzymes; *env* contains the information for the surface and transmembrane components of the viral envelope protein. An additional, smaller, coding gene present in all retroviruses is *pro*, which encodes the virion protease. Simple retroviruses usually carry only these four genes, whereas complex retroviruses have additional genes (accessory genes) that code for additional regulatory non virion proteins. Retroviruses can be divided into two categories: simple and complex distinguishable by the organisation of their genomes (Fig. 1.12) (Coffin, 1992a; Murphy *et al.,* 1994). Five of the seven genera include retroviruses

with oncogenic potential (also referred to as oncoviruses), and the other two groups are the lentiviruses and the spumaviruses. All oncogenic members except the human T-cell leukemia virus, bovine leukemia virus (HTLV-BLV) genus are simple retroviruses. HTLV-BLV and the lentiviruses and spumaviruses are complex . A fish virus genus (Epsilonretrovirus) also contains "accessory" genes. Accessory genes regulate and coordinate viral gene expression. These genes are located between *pol* and *env*, just downstream from *env* including the U3 region of the LTR, or overlapping portions of *env* and each other. Lentiviruses demonstrate a genomic complexity far greater than that of a prototypic retrovirus, possessing as many as six additional genes, which are involved in regulatory processes.

All known lentiviruses encode an apparent transactivating protein (herein referred to as Tat) and a Rev protein that are both essential for virus replication (Cullen, 1998; Emerman & Malim, 1998). In addition to *tat* and *rev*, HIV-1 contains four additional regulatory genes: *nef*, *vif*, *vpr*, and *vpu*, encoding the so-called accessory proteins. It is noted that whereas HIV-2 and SIV genomes lack the *vpu* gene, they contain another gene, *vpx*. Some of the accessory proteins are not absolutely required for viral replication in all *in vitro* systems but represent critical virulence factors *in vivo*. Nef is expressed from a multiply spliced mRNA and is therefore Rev independent. In contrast, Vif, Vpr, and Vpu are the products of incompletely spliced mRNA and thus are expressed only during the late, Rev-dependent phase of infection from singly spliced mRNAs.

Most of the small accessory proteins of HIV have multiple functions manifested at

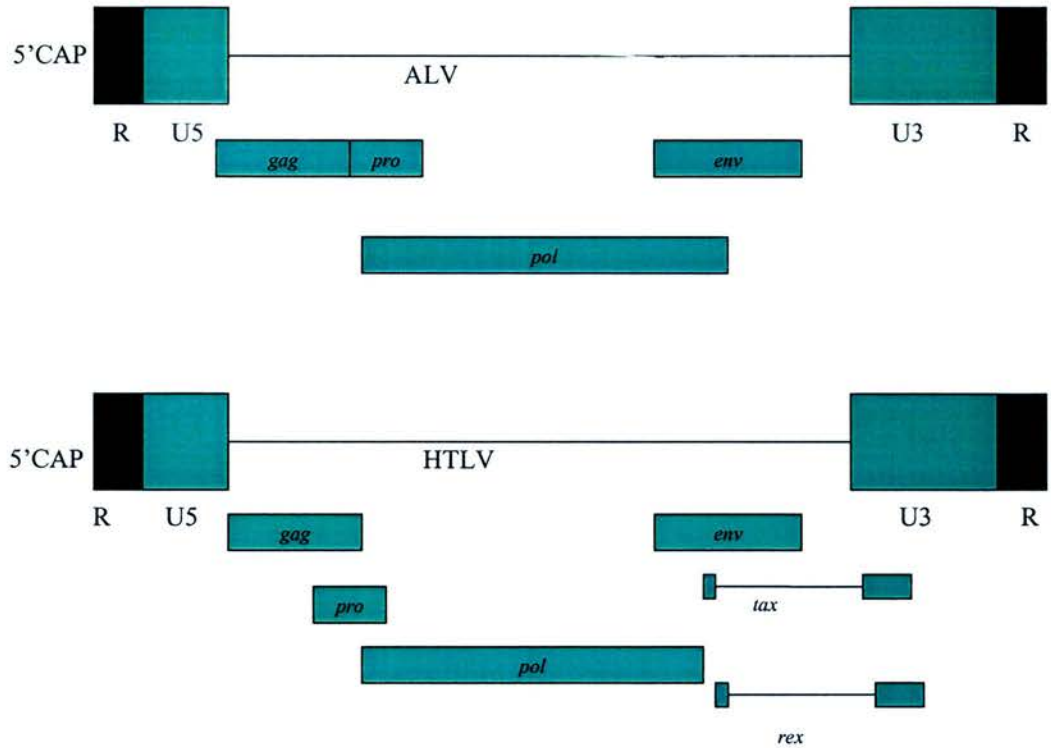


Fig. 1.12. Retroviral genome.

(*A*) A simple retroviral genome. The genetic map of an ALV contains four major coding regions, *gag*, *pro*, *pol*, and *env* and *pro* gene.The terminal noncoding sequences include two direct repeats (R), a U5 (5' unique), and a U3 (3' unique) sequence. (*B*) A complex retroviral genome. The genetic map of HTLV contains, besides the major coding domains, information for two regulatory proteins, Tax and Rex, encoded in regions (boxes) joined by RNA splicing.

different stages of virus replication (Miller *et al.*, 2000).

1.1.4.2 Replication

Retroviruses enter the host cell through the attachment of their surface glycoproteins to specific plasma membrane receptors, which leads to fusion of virus and cell membranes. The interaction of virus and cell surfaces is highly specific; it constitutes the main determinant of viral host range, defining susceptible animal species and target cells within the host. After penetration into the cell, the RNA genome, still contained in a core complex of non-glycosylated proteins and associated with the virion reverse transcriptase (RT), is transcribed into a double-stranded DNA. Transcription into DNA involves two jumps of the reverse transcriptase from the 5' terminus to the 3' terminus of the template molecule. These sequences then occur fused in tandem on both ends of the viral DNA, forming the long terminal repeats (LTRs) (Fig.1.13). The LTR regions regulate viral gene expression and therefore replication and pathogenesis.

Reverse transcription takes place in the cytoplasm; the viral DNA is translocated into the nucleus where the linear copy of the retroviral genome is inserted into chromosomal DNA with the aid of the virion integrase to form a stable provirus. Integration does not change the linear order of the proviral sequences, LTR-*gag-pol-env*-LTR. The number of possible sites of integration into the cellular genome is very large and very widely distributed. With integration, the provirus achieves the status of a cellular gene and is expressed by the cellular RNA polymerase II and replicated by cellular enzymes. Control of proviral transcription remains largely with

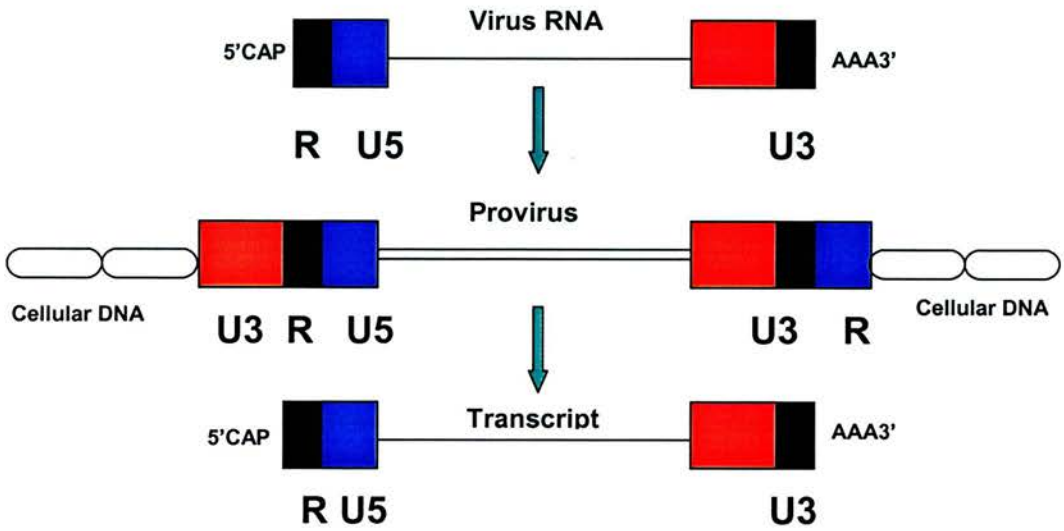


Fig. 1.13. Comparison of the RNA and the DNA forms of the viral genome.

Reverse transcription of the RNA genome generates identical structures referred to as long terminal repeats (LTRs) found at both ends of the DNA provirus. Transcription of the provirus between the upstream U3 and downstream U5 regions generates RNA with the same terminal organization as in the parental virus. (R) Terminal direct repeat RNA; (U5) unique regulatory sequences at the 5' end; (U3) unique regulatory sequences at the 3' end.

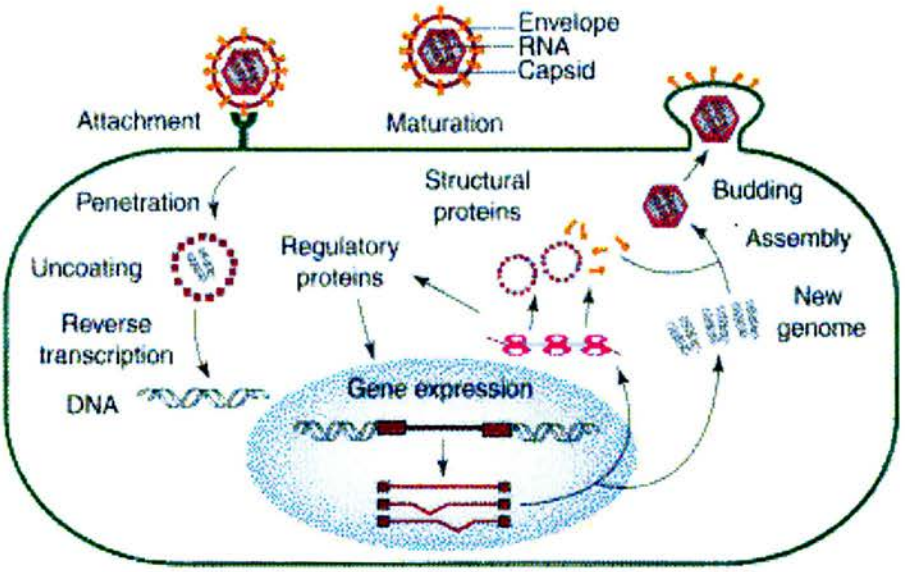


Fig. 1.14. Retroviral life cycle.

The life cycle of retroviruses is defined by the binding of virion to the host cell receptor, cell entry, followed by reverse transcription and subsequent integration into the host genome. Full length viral transcripts and individual viral mRNAs are synthesized in the nucleus, which subsequently encode both structural and regulatory proteins, permitting viral assembly and eventual budding of new virions.

the non coding sequences of the viral LTR. Transcription of the provirus generates spliced and unspliced mRNAs and full-length progeny RNA genomes. In infection with simple retroviruses, control of transcription is mediated by interaction of cellular factors with the DNA of the LTR. Complex viruses, in contrast, take a more active role, encoding *trans*-activating factors that affect the levels of transcripts and the relative amounts of the products of the various genes.

Viral messages are translated on cellular ribosomes. The translation products, together with progeny RNA, are assembled at the cell periphery into viral particles that are released from the cell by budding of the plasma membrane. Budding of viruses is followed by proteolytic cleavage of virion polyproteins by a viral protease and by cellular proteases (Fig.1.14). Productive retroviral infection is not of necessity cytopathic; infected cell cultures often show no visible effects of viral production. In some congenital infections in animals, virus can be produced by most cells in most tissues without deleterious effect on the development and function of the organism (Rubin *et al.,* 1961 and 1962).

1.1.4.3 Provirus and interaction with cellular genome

A provirus, once integrated into the cellular genome, has the genetic stability of a cellular gene. Reverse transcription and integration make retroviral infection permanent, as integrated proviruses are only rarely lost from the cellular genome: "A retrovirus is forever."

Retroviruses have developed mechanisms of pathogenicity involving the transfer or

transcriptional activation of specific cellular genes. These mechanisms are based on genetic recombination between virus and cell and between viral genomes. Retroviral particles contain two copies of their genome linked by regions near the 5' termini (Billiter *et al.,* 1974; Kung *et al.,* 1975; Beemon *et al.,* 1976). A direct consequence of diploidy is the formation of heterozygote virions in cells that are infected with two or more genetically distinct but related retroviruses. Such heterozygotes give rise, in the next cycle of infection, to stable genetic recombinants that are formed during the process of reverse transcription of the two parental genomes from the same viral particle. Rates of recombination between related retroviruses are high (Temin, 1964; Coffin, 1979; Linial & Blair, 1982).

A plausible hypothesis for this acquisition of cellular genetic material postulates that a provirus integrates upstream of a cellular gene and leads to the production of chimeric virus-cell transcripts. In the next round of replication, non homologous recombination between virus and cell sequences leads to the incorporation of the cellular gene into the retroviral genome, so that it is now transported by the virus from cell to cell and expressed under control of the viral LTR (Goldfarb & Weinberg, 1981; Swannstrom *et al.,* 1983; Raines *et al.,* 1988; Felder *et al.,* 1991, 1993; Coffin, 1992b; Swain & Coffin, 1992). The usual product of this transduction process has acquired the host sequence at the cost of one or more viral genes. Such viruses are therefore generally defective for replication, requiring the presence of a replication-competent provirus in the same cell to provide viral proteins for replication. The transduction of cellular genes has been found only with simple retroviruses and not with complex retroviruses. The reasons for this difference are

not clear, but they may have to do with the mechanism by which retroviruses acquire cellular sequences or with viral genome organisation that must incorporate foreign inserts (Coffin *et al.*, 1997).

The modified cellular genes carried by retroviruses convey a high degree of tumorigenicity to the virus. These viral or v-*onc* genes are usually mutated growth-regulatory genes. Their cellular progenitors are referred to as protooncogenes or c-*onc* genes (Bishop, 1983; Varmus, 1984). Overexpression or inappropriate expression, often combined with mutation of an oncogene that has become part of a viral genome, results in a gain of function of a positive growth signal and in tumourogenesis.

Retroviruses with oncogenes in their genomes are particularly fast-acting carcinogens and in most cases also transform cells in culture.

Retroviruses lacking an oncogene do not transform cultured cells, but some can induce tumours in animals. This process is characterized by a long latent period. In this case, it is a cellular homolog that is activated through the insertion of a provirus. The viral LTR, through its promoter and enhancer sequences, alters expression of the neighbouring cellular oncogene (Lazo & Tsichlis, 1990; van Lohuizen & Berns, 1990; Kung & Vogt, 1991). Transduction and insertional activation of cellular oncogenes are the two main mechanisms by which most retroviruses induce tumours.

For other retroviruses the mechanisms of oncogenesis are not clear. One example is by Human T-cell leukemia virus type-I (HTLV-1) is the first discovered human

retroviral pathogen (Poiesz *et al.,* 1980). It has been implicated with the etiology of an aggressive malignancy known as adult T-cell leukemia (ATL) and of a neurological progressive inflammatory syndrome called tropical spastic paresis or HTLV-1 associated myelopathy (TSP-HAM) (Barmak *et al.,* 2003; Jeang *et al.,* 2004) HTLV-1 is a complex retrovirus that, in addition to the two long terminal repeats (LTRs) and the *gag, protease*, *pol* and *env* genes, which are typical to most other retroviruses, its genome contains an additional region called pX, which resides between the env gene and the 3'-LTR. This region includes four partially overlapping reading frames (ORFs), of which the most investigated ones are ORFs III (Silverman *et al.,* 2004) and IV that encode for the viral regulatory Rex and Tax proteins respectively. This virus neither carries a transduced oncogene in its genome nor activates a resident oncogene by insertion; it may cause tumours through one of its regulatory proteins, possibly by changing the expression levels of cellular genes (Feuer & Chen, 1992; Azran *et al.,* 2004).

A unique model of tumor induction is also seen with a form of the Friend murine leukemia virus. In the past two decades, several oncogenes and tumour suppressor genes, which play critical roles in the induction and progression of Friend erythroleukemia, have been identified. Retroviral insertional activation of Fli-1 and Spi-1/PU.1, as well as loss of tumour suppressor genes such as p53 or p45 NFE2 have been shown to be critical for the induction and progression of Friend virus-induced erythroleukemias (Lee *et al.,* 2003). It produces a modified Env protein that appears to mimic a component of the erythropoietin signalling pathway (Li *et al.,* 1990; D'Andrea *et al.,* 1992; Johnson & Benchimol, 1992). In Table 1.2 genomic

Table 1.2. Genomic organization and pathogenetic features of the betaretrovirus MLV and some primate and non –primate lentiviruses (ID=mmunodeficiency; int. = intermittent).

	Genomic organization												
Virus	Host	Disease	ID	Lymphtropic	Viraemia	rev	tat	vif	vpr	vpr/vpx	vpu	nef	Other ORF
MLV	Rodents	Yes	Yes	Yes	Yes								
HIV-1	Humans	Yes	Yes	Yes	Yes	V	V	V	V		V	V	
HIV-2	Humans	Mild	Mild	Yes	Yes	V	V	V		V		V	
SIV(MAC)	Monkeys	Yes	Yes	Yes	Yes	V	V	V		V		V	
SIV(AGM)	Monkeys	Yes	Yes	Yes	Yes	V	V	V	V			V	
FIV	Cats	Yes	Yes	Yes	Yes	V		V					A
EIAV	Horses	Yes	No	No	Yes int.	V	V						S2
CAEV	Sheep&Goats	Yes	No	No	Yes	V	V	V					
Visna	Sheep&Goats	Yes	No	No	Yes	V	V	V					
BIV	Cattle	No	No	Yes	?	V	V	V					

organization of some retroviruses and some primate and non –primate lentiviruses are correlated with their pathogenetic features.

1.1.5 Retroviruses and cancer

Oncogenic retroviruses occur in all classes of vertebrates. Tumour-inducing retroviruses have been isolated from rodents, cats, cows, primates and other animals (Gross, 1970). Some of the more studied are Rous sarcoma virus (RSV), a highly pathogenic agent inducing connective tissue tumors in chickens (Rous, 1911); mouse mammary tumor and murine leukemia viruses (MMTV and MLV); and more recently HTLV. A representative example is human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS).

With regard to the incubation time of the tumour to appear and from a clinical point of view retroviruses can be divided in two classes (Rosenberg & Jolicoeur, 1997):

1) acutely transforming retroviruses;

2) non- acutely transforming retroviruses.

The acutely transforming retroviruses cause tumours rapidly (1 week to a few weeks) and are able to transform cells in culture, whereas the non acute retroviruses induce disease slowly (months to years) and cannot transform target cell *in vitro*.

1.1.5.1 Acutely transforming retroviruses

Retroviruses that carry v-*onc* genes (sometimes called transforming viruses) induce a wide range of malignancies including sarcomas and hematopoietic cell tumours.

Their ability to induce tumours rapidly and to transform cells in vitro has made them powerful tools for studies addressing the ways in which oncogenes alter cell growth

The acutely transforming retroviruses possess a cellular oncogene (known as proto-oncogene or c-onc) in their genome often altering its structure (deletions, point mutations, etc.) and (Duesberg & Vogt, 1970; Stehelin *et al.,* 1976; Bishop *et al.,* 1984; Rasheed, 1995). Cellular proto-oncogenes are usually conserved over long evolutionary distances, suggesting that they are indispensable for fundamental cellular functions (Benjamin & Vogt, 1990). Viral oncogenes work through the synthesis of proteins (oncoproteins) that have the potential to transform a normal cell into a neoplastic one. Most retroviral oncogenes have homology with cellular genes (proto-oncogenes), a hallmark of an oncogene. For example, the avian erythroblastosis virus strain H carries the *erbB* oncogene that has close homology to the chicken epidermal growth factor receptor (Downward *et al.,* 1984). *v-onc* are usually classified in families based on the function of the protein encoded by their protooncogene parent. Most classes of proteins involved in growth stimulation are represented by growth factors, growth factor receptors, non receptor protein tyrosine kinases, G proteins, serine/threonine kinases, and transcription factors (Blackwood *et al.,* 1992a and 1992b).

As a result of the transduction of the oncogene, the acutely transforming retroviruses typically lack portions of their structural genes and are replication defective. Acutely transforming retroviruses are thus propagated in conjunction with a replication-competent virus called "helper" that encodes all of the viral structural and enzymatic proteins (*Fung et al.,* 1983; Green *et al.,* 1987). Viruses containing oncogenes are

relatively rare in naturally occurring retrovirus-induced tumors for several reasons: 1) Oncogene capture is an unlikely event, 2) the rapid and lethal course of the induced disease could limit the spread of the infection, and 3) there is probably no evolutionary-positive selection of replication-defective oncogene-containing retroviruses (Palmarini & Fan, 2001).

1.1.5.2 Non- Acute transforming retroviruses

The majority of oncogenic retroviruses do not carry an oncogene in their genome (Teich *et al.*, 1984; Benjamin & Vogt, 1990; Rosenberg & Jolicoeur, 1997). Such viruses are called "non acute retroviruses" because the incubation period of the resulting neoplasia is usually several months or more. Non acute retroviruses are replication competent. Tumour induction appears to occur through multiple steps, many with similarities to non-virus-induced cancer. The best understood mechanism of nonacute retroviral carcinogenesis is LTR activation of proto-oncogenes, also known as insertional activation, *cis*-activation, or insertional mutagenesis. In these tumors, a provirus is integrated in the vicinity of a cellular proto-oncogene, resulting in overexpression of the proto-oncogene by one of several mechanisms. Retroviruses that act by insertional activation generally induce tumors with a long incubation period because retroviral insertion near oncogenes occur by chance (Steffen & Weinberg, 1978; Brown, 1997). Thus, multiple rounds of infection are typically necessary before integration near (e.g., few to a hundred kilobases) a proto-oncogene occurs in one cell. Under the influence of the viral LTR, the transcription of the proto-oncogene is elevated in that cell and triggers transformation (Hayward *et al.*,

1981; Nusse & Varmus, 1982; Fung *et al.,* 1983; Cuypers *et al.,* 1984; Dickson *et al.,* 1984).

Insertional inactivation of tumor suppressor genes in retrovirus-induced tumors is very rare because, unlike proto-oncogenes, the loss of function of a tumor suppressor gene requires that both copies of the gene be damaged or lost. However, the insertional inactivation of one allele could potentiate spontaneous mutations occurring in the other allele, leading to transformation. For instance, the insertional inactivation of p53 has been observed in a proportion of non-B-, non-T-cell lymphomas induced by the Cas-Br-E virus (Bergeron *et al.,* 1993; Palmarini & Fan, 2001).

1.1.6 Endogenous retroviruses (ERVs)

The genomes of all vertebrates, humans included, have been colonized during evolution processes by retroviruses *via* integration of their genomes into the germ line and subsequent fixation in the gene pool of the host population (Vogt, 1997). These viruses are called endogenous retroviruses (ERV), are inherited by the host vertically, according to the mendelian principles. In contrast, exogenous retroviruses are horizontally transmitted and they do not efficiently infect the germ line (Boeke & Stoye, 1997; Patience *et al.,* 1997; Löwer, 1999). Like other transposable elements, ERV are thought to have played an important role in the evolution of mammalian genomes and the human genome sequence has already been used in phylogenetic studies.

Most ERVs have retained some similarity to their exogenous counterparts but they

have lost replication competence as a result of the accumulation of deletions and mutations acquired over the course of evolutionary time so that most of them are now defective and incapable of producing protein. ERVs have been suggested to play important roles in a number of physiological and pathological processes.

ERVs have the potential to interfere with the exogenous counterparts and with the host immune system. For example *Human endogenous retrovirus W (*HERV-W) is specifically expressed in the syncytiotrophoblast of the human placenta. It is possible that HERV-W envelope protein induces formation of syncytia, thereby sustaining the hypothesis that HERV-W is involved in human placental morphogenesis (Mi *et al.,* 2000; Frendo *et al.,* 2003). Another human endogenous retrovirus, ERV-3, is conserved throughout primate evolution and ERV-3 is highly expressed in the trophoblast of the normal placenta; the similarity between a portion of the transmembrane (TM) glycoprotein of ERV and a putatitve immunosuppressive region of gammaretroviruses, p15E, led to the speculation that ERV-3 may protect the implantation of the foetus protecting it from a maternal adverse immunological reaction (Boyd *et al.,* 1993; Venables *et al.,* 1995; Medzhitov & Janeway Jr., 2002). ERVs may also protect the host against infection by related exogenous retroviruses. For example, ERV loci of chickens express envelope proteins that confer resistance to Rous sarcoma virus subgroup E infection through receptor interference (Payne & Pani, 1971). A similar situation has been described in mice in which the receptor for Murine Leukaemia virus (MLV) is blocked by an endogenous synthesis of Gag gp 70 (Kozak *et al.,*1984).

Endogenous interference through the immune system is well established in *Mouse*

mammary tumour virus (MMTV) model through the expression of a superantigen (sag). Expression during ontogeny of of the endogenous sag sequences in the developing immune system of mouse strains induces clonal deletion of the reactive T cell required, thus preventing infection of activated leukocytes (the intermediate carriers of MMTV) with related exogenous strains (Golovkina *et al.,* 1992; Held *et al.,* 1993; Karapetian *et al.,* 1994).

In terms of negative effects, the generation of pathogenic retroviruses can result from reactivation and/or recombination among different endogenous loci or by recombination with related exogenous viruses (van Nie & Verstraeten, 1975; Hartley *et al.*, 1977; van Nie *et al.,*1977; Verstraeten & van Nie, 1978 Imai *et al.,* 1983; Stoye & Coffin, 1987; Neil *et al.*, 1991; Stoye *et al.*, 1991; Golovkina *et al.*, 1994; Golovkina *et al.,* 1996; Benson *et al.,* 1998; Golovkina *et al.*, 1997; Ruis *et al.,* 1999).

In mice, chickens and cats, endogenous retroviruses have been demonstrated to be involved in recombination events leading to the generation of oncogenic retroviruses or virus with a broader host range (Stoye & Coffin, 1987; Fan, 1994; Golovkina *et al.*, 1994; McDougall *et al.*, 1994; Bai *et al.*, 1995).

Infection of both mice and cats may lead to the emergence of new retroviruses through recombination between the incoming exogenous viruses and endogenous viral sequences (Kim *et al.,* 2004). The mouse mink cell focus-forming (MCF) viruses, also designated polytropic MLV, were the first retroviruses identified as resulting from such *de novo* recombinations following MLV infection (Fischinger *et*

al., 1975; Hartley & Rowe, 1976). Subsequent to infection by an ecotropic MLV, acquired *mcf env* sequences broaden the cellular tropism of the parental ecotropic MLV through the acquisition of heterologous receptor-binding determinants in the *env* SU (Fan, 1997). Generation of new *env*-recombinant viruses also occurs upon infection of cats with feline leukemia viruses (FeLV). Thus, infection with the FeLV-A subtype can result in the generation of FeLV-B subtypes with markedly modified tropism (Stewart *et al.*, 1986).

Infection of T lymphocytes by the cytopathic retrovirus feline leukemia virus subgroup T (FeLV-T) requires FeLIX, a cellular coreceptor that is encoded by an endogenous provirus and closely resembles the receptor-binding domain (RBD) of feline leukemia virus subgroup B (FeLV-B) (Barnett *et al.* 2003).

The acquisition of heterologous viral sequences, however, is not limited to *env*, and it is important to note that recombination among or with *mcf* sequences involves other regions of the MLV genome as well (Evans & Cloyd, 1985). Irrespective of the region of the viral genome, it is generally accepted that recombination between endogenous *mcf* and exogenous MLV sequences occurs after coencapsidation of heterologous genomic RNA into virus particles (Katz & Skalka, 1990). The production and characteristics of the resulting recombinant viruses depend upon the subspecies or strain of mouse and the type of exogenous infectious virus inoculated (Chesebro *et al.,* 1983; Lavignon *et al.,* 1997). It is also noteworthy that the initial dissemination of these recombinant viruses depends largely on pseudotyping with *env* from the infecting, exogenous MLV (Sitbon *et al.,* 1985).

HERVs have frequently been proposed as etiological cofactors in chronic diseases such as cancer, autoimmunity and neurological diseases (Löwer, 1999).

In human, for example, HERV-W has been widely investigated following the isolation of an HERV-W- related retrovirus (multiple sclerosis-associated retrovirus, MSRV) from retroviral particles produced in cell cultures from patients with multiple sclerosis (Perron *et al,* 1997). HERV-W are under investigation as potential causative agents of various human diseases including cancers following several findings suggesting that HERV-W elements are actively expressed in human tissues and cancer cells (Yi *et al.,* 2004; Yi *et al.,* 2002).

Another human endogenous retrovirus family (HTDV/HERV-K family) has links with testicular tumours. This family is interesting because it has retained coding capacity in all viral genes. In patients with testicular tumours, an immune response is induced against the Gag and Env proteins and it declines after tumour removal (Gotzinger *et al.,* 1996; Tonjes *et al.,*1996).

Most studies on the pathological potential of HERVs have looked for expression of HERV RNA or protein, on the assumption that disease symptoms result from inflammatory or autoimmune reactions to HERV proteins. The effect of HERVs in disease may be at the level of cellular gene transcription, however, since it is well known that enhancer and promoter elements in retroviral LTRs can influence the transcription of neighbouring genes. This can result in transcriptional activation or gene silencing and in changes in tissue specificity of expression (Ting *et al.,* 1992; Schulte *et al.,* 1996). Data mining of the human genome sequence has already been

used to identify two HERV-E LTRs that act as alternative promoters for cellular genes (Kowalski *et al.,* 1999).

1.1.7 JSRV

The genome of JSRV is 7,462 nucleotides with the typical organization of type B and D retroviruses with the overlapping *gag*, *pro* and *pol* reading frames except for an additional open reading frame (*orf-x*) of 683 bp overlaps *pol*. Orf-x has a codon usage different from that of other genes within JSRV, and a very hydrophobic predicted amino-acid sequence (Bai *et al.,* 1999; Rosati *et al.* 2000). The role of this open reading frame is unknown but orf-x region of endogenous and exogenous JSRV isolates collected from UK, Italy, Spain and South Africa are highly conserved (Rosati *et al.,* 2000). *Orf-x* does not seem to be required for JSRV replication *in vitro* (Palmarini, M. & Fan, H., unpublished results) or for cell transformation *in vitro* (Maeda *et al.,* 2001). The long terminal repeat of JSRV provirus is 397 bases in length. The U3 region contains regulatory signals for viral transcription (York *et al.,* 1992) (Fig. 1.15).

So far, three strains of JSRV have been completely sequenced: the original South-African strain (JSRV-SA) (York *et al.,* 1991, 1992), JSRV21 (Palmarini *et al.,* 1999a) and JSRVJS7 (DeMartini *et al.,* 2001). Partial sequences of JSRV isolates have been obtained from different strains from various geographical locations (Bai *et al.,* 1996; 1999; Hecht *et al.,* 1994; Palmarini *et al.,* 1996a and 1996b; Rosati *et al.,* 2000). The sequence of JSRV21 confirmed the genomic structure published for JSRV-SA by York *et al.,* 1991 and 1992.

1.1.7.1 JSRV mRNA Splicing Pattern

Retroviral proviruses contain a single transcriptional unit that is transcribed in the nucleus in full-length genomic RNA. The viral RNA is then transported to the cytoplasm with and without RNA splicing. All retroviruses use full-length genomic RNA as mRNA for the Gag, Pro and Pol products. A single spliced mRNA consisting of the 3' end of the genome of the *env* gene is used for translation of the envelope proteins (Coffin *et al.,* 1997). Simple retroviruses usually use only this two form of RNAs. In contrast complex retroviruses encode additional subgenomic single or double spliced mRNAs for the synthesis of regulatory proteins (Rabson & Graves, 1997). The full-length viral mRNA is translated on polyribosomes free in the cytoplasm; the subgenomic mRNAs and env mRNA are translated on polyribosomes associated with the rough endoplasmic reticulum. The cleavage of Env polyprotein happens in the Golgi apparatus producing the SU and TM proteins; SU and TM remain associated with each other on the surface of virions.

Palmarini *et al.,* (2002) characterized the virus-specific RNAs in 293T cells transiently transfected with a human cytomegalovirus promoter-driven JSRV expression plasmid, in productively infected OHH1.LU deer lung cells, and in OPA tumors from field isolates. Typical unspliced (7.5kb) (for *gag, pro,* and *pol*) and singly spliced (2.4kb) *env* mRNAs were detected. In addition, six other virus-specific RNAs were detected that resulted from the use of alternate splice acceptor sites and two premature polyadenylation sites (located in *gag* and in *env*). The *orf-x* gene of the virus appeared to be expressed from two singly spliced subgenomic mRNAs of 3.2 kb that could encode an independent orf-x protein of 179 amino

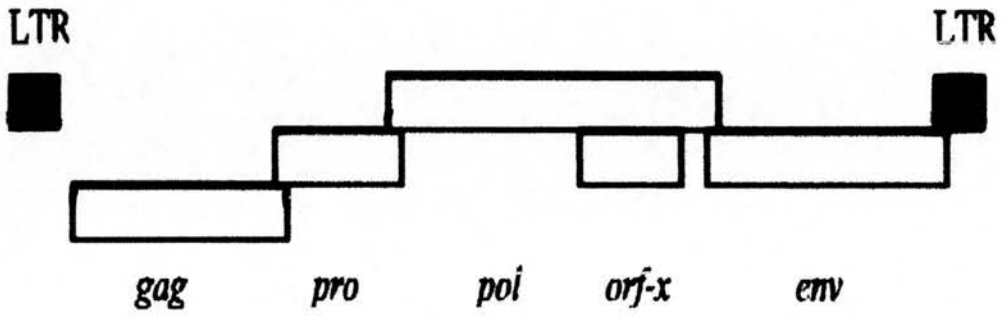


Fig. 1.15. Schematic representation of the genome of jaagsiekte sheep retrovirus (JSRV).

The JSRV genome contains the retroviral *gag*, *pro*, *pol*, and *env* genes as well as an additional open reading frame (*orf-x*) that overlaps *pol* and whose function is unknown. LTR = long terminal repeat.

acids.

1.1.7.2 JSRV proteins

The Gag precursor polyprotein is a 612-amino-acid polypeptide with a calculated molecular weight of 68 kDa. Alignment of the predicted JSRV Gag polyprotein with those of MPMV, squirrel monkey retrovirus (SMRV), and MMTV shows that there is a strong sequence conservation corresponding to the capsid proteins (CA) of the 3 viruses (York *et al.,* 1992). From the analysis of the genome sequence Pro, the protease is probably expressed as a fused Gag-Pro polypeptide as for MMTV (Jacks *et al.,* 1987). Pro (326 residues; calculated molecular weight 35kDa) consists of two parts: a pseudoprotease or protease-like (PrL) and an active protease (PR) which exhibits the core amino acid sequence of cellular aspartyl protease. Alignment of the JSRV PrL and PR domains against some lentiviruses shows that the PrL polypeptide is even more conserved than the protease. Pol is likely to be translated as a fused Gag-Pro-Pol polypeptide (York *et al.,* 1992).

Orf-x has a very unusual location and a codon usage; its predicted amino acid sequence is very hydrophobic and it doesn't show any similarities with other known proteins, with the exception of a low homology to a G-protein-coupled receptor (Bai *et al.*, 1999; Rosati *et al.*, 2000). The role of *orf-x* is unknown but it is conserved among various endogenous and exogenous JSRV isolates collected from different geographical locations (Rosati *et al.*, 2000).

Env ORF encodes the precursor of the viral envelope protein. Env is composed by two parts the outer membrane and surface protein (SU) and the cell membrane

spanning domain of the transmembrane protein (TM). The calculated molecular weight of the non glycosylated Env precursor is 69 kDa. The calculated molecular weight of mature SU protein is 47 kDa and that of TM is 33 kDa.

The complete genome sequence of JSRV enabled the expression of viral proteins in vitro and to overcome the inability of the virus to grow on cell culture.

Palmarini *et al.,* in 1999 established a convenient method to prepare infectious JSRV in vitro. This involved transfection of human 293T cells with a plasmid DNA containing the $JSRV_{21}$ provirus that had been modified by replacement of the U3 sequences in the upstream long terminal repeat (LTR) with the human cytomegalovirus immediate-early promoter ($pCMV2JS_{21}$). The in vitro-synthesised virus particles were called $JSRV_{21}$.

Proteins in $JSRV_{21}$ virions were labelled by incubation of transfected 293T cells for 24 h with [^{35}S]methionine. Labelled supernatant from transfected 293T cells was banded in an isopycnic sucrose gradient, and fractions corresponding to 1.14 to 1.16 g/ml were pooled and subjected to SDS-PAGE and autoradiography.

Several prominent labelled proteins were detected, while supernatant from mock-transfected and labelled 293T cells yielded no detectable radioactivity in material banding at 1.14 to 1.16 g/ml. By analogy to the sizes of other type D and type B retroviral proteins, it seemed possible that the 53- and 37-kDa proteins were envelope proteins and their diffuse migration in SDS-PAGE could be consistent with glycosylation (Dion *et al,* 1977; Li, 1987). Similarly, some of the lower-molecular-mass proteins (26, 23, 17, and 14 kDa) were detected and considered as mature Gag

proteins or proteins encoded by other viral genes (Bradac & Hunter, 1984; Hizi *et al.,* 1987; Hizi *et al.,* 1989). The 26-kDa protein had the same mobility as the 26-kDa CA protein present in lung fluid from SPA-affected sheep that is detectable by Western blots (Fig.1.16).

1.1.7.3 Homologies between JSRV and other retroviruses.

JSRV protein sequences were aligned to those of other beta retroviruses and scored for the percentage of amino acid identity.

There is more than 50% amino acid identity between JSRV, MPMV and SIV-1 Gag, Pro and Pol proteins but there is not reliable alignment of their envelope precursor proteins.

On the other hand , Env proteins of JSRV and MMTV are homologous, although there is less than 38% amino acid identity between their Gag proteins (Doolittle *et al.*, 1989; York, *et al.,* 1992).

The viral JSRV envelope is more similar to MMTV than MPMV exhibiting prominent surface spikes and an eccentric core. The MPMV-type morphology has less dense surface spikes and a cylindrical core. Phylogenetic analysis of the RT subdomain suggests that JSRV evolved from the type A (hamster intracisternal type A particles), B and retroviruses D and diverged between the B prototype MMTV and the D prototype MPMV (Chiu, *et al.*, 1985; McClure, *et al.*, 1988; York *et al.*, 1992).

JSRV is included in the genus betaretrovirus. Viruses assigned to this genus include

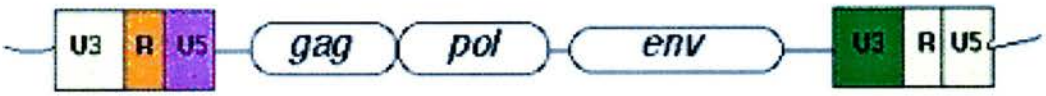


Fig.1.16. Major proteins encoded within the retroviral genome.

Gag: is processed to matrix and other core proteins that determine retroviral core.

Pol: is processed to reverse transcriptase, Rnase H and integrase functions.

Env: envelope protein, resides in the lipid layer.

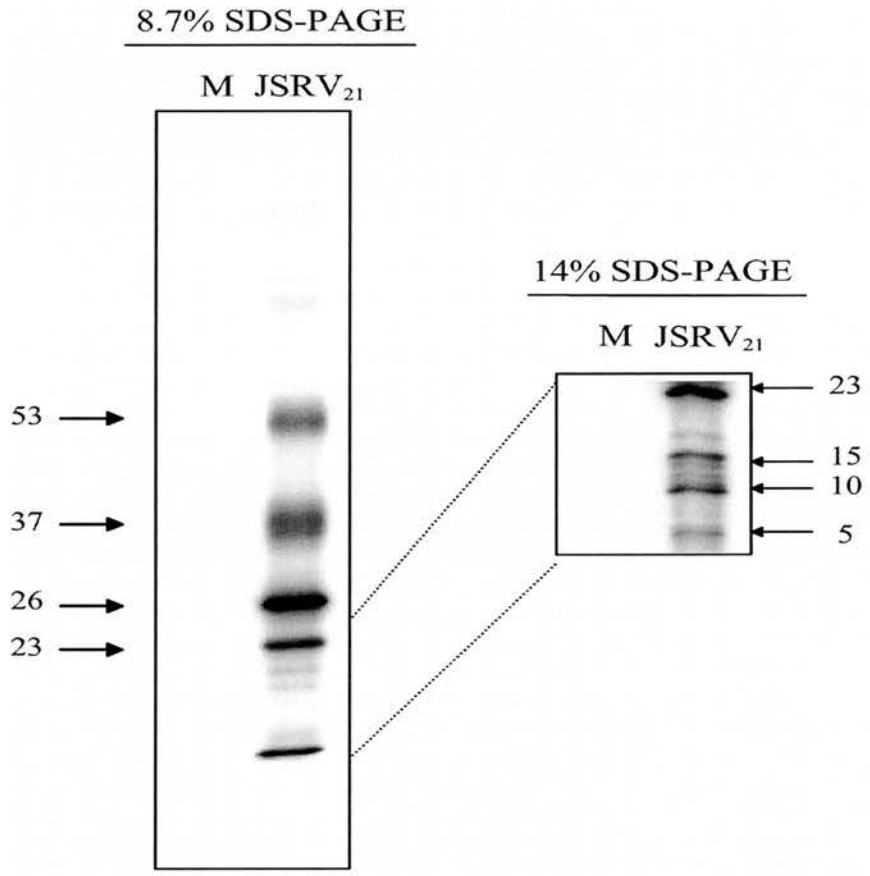


Fig1.17. Expression of proteins in $JSRV_{21}$ virions.

Palmarini *et al.,* in 1999 labeled $JSRV_{21}$ prepared from transfected 293T cells that had been treated with [^{35}S]methionine 24 h prior to supernatant harvest. Viral particles were purified by isopynic centrifugation, and the fractions corresponding to 1.14 to 1.16 g/ml were pooled and analyzed by SDS-PAGE on 8.7 and 15% polyacrylamide gels followed by autoradiography ($JSRV_{21}$). 293T cells that were mock-transfected were labeled and processed in parallel (M). No radioactivity banded at 1.14 to 1.16 g/ml from tissue culture supernatant from the mock-transfected culture. $JSRV_{21}$ virions contained major bands of 53, 37, 26, and 23 kDa visible in the 8.7% polyacrylamide gel (left). An additional band migrated at the bottom of the 8.7% polyacrylamide gel that resolved into three bands of 15, 10, and 5 kDa in the 14% polyacrylamide gel. The sizes of the radioactive proteins were calculated from the mobilities of protein size markers.

exogenous, vertically transmitted (milk), and endogenous viruses of mice, as well as exogenous, horizontally transmitted and endogenous viruses of New and Old World primates. Murine viruses are associated with mammary carcinoma and T-cell lymphomas, while the exogenous primate viruses are associated with immunodeficiency diseases (Table 1.3). No oncogene-containing member is known.

Table 1.3. Viruses included in the Genus Betaretrovirus.

Genus	Betaretrovirus
Type species	Mouse mammary tumor virus (MMTV)

Species in the Genus

Langur virus (LNGV)

Mason-Pizer monkey virus (MPMV)

Simian retrovirus 1 (SRV-1)

Simian retrovirus 2 (SRV-2)

Mouse mammary tumor virus (MMTV)

Ovine pulmonary adenocarcinoma virus

(Jaagsiekte sheep retrovirus) (JSRV)

Squirrel monkey retrovirus (SMRV)

1.1.7.4 Experimental transmission of OPA

The transmissibility of OPA to in-contact sheep was first demonstrated by de Kock in 1929; Dungal in 1946 proved that OPA could be transmitted by aereosol: affected

sheep were made to breath through a 20% solution of glycerine in normal saline for 30 minutes and this solution was used to inoculate intratracheally 3 lambs. Two of the lambs developed OPA four months after the experimental infection. The experiment was positively repeated after filtering the glycerol-saline solution through a gradacol membrane (pores 0.9μm in diameter). Several other authors reproduced the disease by aerosol spray or by intratracheal or intrapulmonary inoculation of lung tumour homogenates or lung fluid (Sigurdsson, 1958; Cuba-Caparo *et al.,* 1961; Wandera, 1968; Tustin, 1969).

When OPA lung fluid from field isolates concentrated by ultracentrifugation and injected intratracheally in new-born lambs, disease occurs extremely rapidly. In fact animals developed clinical signs of OPA between 3 and 6 weeks post-inoculation but some developed OPA within 4-10 days (Verwoerd *et al.*, 1980; Sharp *et al.,* 1983). Early experiment involving infection of mature sheep resulted in increased incubation time of OPA, more than 12 months, and in very small resulting lesions (Dungal, 1949; Wandera, 1968). Several authors demonstrated the experimental transmission of OPA to goats (Tustin *et al.,* 1977; Sharp *et al.,* 1986).

The virus cannot be cultivated on cell culture and transmission of OPA to laboratory animals failed with the exception of transplantation studies of OPA tumour cells into nude mice (Verwoerd *et al,* 1977; Zimber *et al.,* 1984; Jassim, 1987).

1.1.7.5 Involvement of JSRV with OPA

In the 1970s retroviral A-particles were observed in OPA tumour cells (Hod *et al.*, 1971, Perk *et al.,* 1971; Bucciarelli, 1973). The first report on the transmission of OPA with particles containing reverse transcriptase activity was by Martin *et al.,* 1976. Verwoerd *et al.,* (1980) succeeded in transmitting the disease with the cytoplasmic fraction of tumour cells that had a buoyant density in sucrose gradient of around 1.175 g/ml and which contained reverse transcriptase particles that resembled those of a retrovirus. The same group in 1981 showed that during experimental transmission there was an inverse dose relationship between the reverse transcriptase (RT) activity in the inoculum and the incubation time between inoculation and appearance of OPA symptoms (Verwoerd *et al.,* 1980). In 1983 Sharp *et al.,* confirmed that lung fluid from clinically OPA affected sheep contained both the virus and the viral particles carried RT activity. In the same year and from the Scottish group came the report of an immunological cross-reaction between the 27000 mol.wt. core protein of Mason-Pfizer monkey virus, mouse mammary tumour virus, and a 25000 mol wt. Protein in OPA tumours and lung fluid by Western blotting technique (Sharp & Herring, 1983).

In 1992 York *et al.,* released the complete genomic sequence of JSRV.

The JSRV genome was cloned from viral particles secreted in lung fluid of affected animals (York *et al.,* 1991 and 1992). The virus was produced in new born lambs by injecting Freon-extracted virus intratracheally. The inoculum was previously

obtained by lung lavages and then purified (Herring *et al.*, 1983; Verwoerd *et al.*, 1983). Polyadenylated RNA was isolated from isopynic-gradient-purified virus (York *et al.*, 1991); cDNA was synthesised with oligo (dT) as primer, and then cloned in appropriate vectors. Recombinant clones were selected by using either labelled random primed cDNA from JSRV polyadenylated RNA or JSRV specific clones as probes. Clones were ordered relative to each other by restriction mapping and by nucleotide sequencing of the extremities and alignment of the predicted translational products against MPMV and MMTV protein sequences.

The availability of a genomic sequence for JSRV facilitated the development of recombinant JSRV capsid antigens (CA). These were used to develop specific antisera and a blocking-ELISA to detect JSRV and to determine the anatomic distribution of JSRV in sheep. JSRV was detected only in lung fluid and tumours from affected animals and not in any other tissue from affected or unaffected sheep (Palmarini *et al.*,1995). Immunohistochemical examination of the same tissues, using the same antiserum to JSRV-CA, detected JSRV protein in the cytoplasm of the transformed epithelial cells in the pulmonary alveoli of both naturally and experimentally OPA-affected sheep. Although JSRV-CA was observed in all OPA tumours, not all tumour cells were positive, even in the same nodule. There was no staining of non-transformed epithelial cells nor of the stromal cells and specific staining was observed only rarely in the extracellular alveolar spaces. Specific staining was not demonstrated in any other tissue, with the exception of a few large lymphoblastoid cells in the paracortical zones and medullary sinuses of the tracheobronchial and mediastinal lymph nodes and some macrophages within the

pulmonary alveoli. These observations demonstrated that JSRV appears to replicate principally in the transformed epithelial cells in OPA tumours as well as a minor subset of lymphoid cells (Palmarini *et al.*,1995).

1.1.7.6 JSRV and its endogenous counterpart

However, confirmation of the pathogenic role of JSRV in OPA was complicated by the presence of 15-20 copies per sheep genome of JSRV-related endogenous sequences (York *et al.*, 1992).

To investigate this point York *et al.*, (1992) analysed genomic digests of an OPA derived tumour cell line, primary foetal fibroblastic type cells from sheep and goats and primary culture of choroid plexus. All the samples contained JSRV-like sequences as integrated proviral DNA without any detectable linear or circular unintegrated proviruses. Several sheep breeds in different places such as Kenya, Peru, Europe and north America were tested, indicating that all sheep populations contain these sheep endogenous retrovirus sequences (Hecht, *et al.*, 1994). Sheep harbour in their genome about 20 copies of endogenous betaretroviruses (York *et al.*, 1992; Hecht *et al.*, 1994 and 1996; DeMartini *et al.*, 2003).

These results did not rule out the possibility that JSRV might be acting as a helper virus for some other replication defective, acutely transforming retrovirus or represent an endogenous virus, reactivated as a consequence of neoplasia or, indeed, result from recombination with endogenous sequences as has been reported in other species. The identification of reagents and techniques to distinguish exogenous and

endogenous ovine retroviruses, therefore, was a critical development.

To investigate this relationship further, the distribution of JSRV transcripts by RT-PCR was examined in various tissues (lung, lymphoreticular tissues) from OPA-affected and unaffected animals (Palmarini *et al.,* 1996a). Clear evidence enJSRV transcripts in tissues of affected and unaffected animals was shown.

Differentiation between exogenous and endogenous RNA was evaluated by sequencing and/or restriction enzyme digestion of PCR products obtained from viral pellets and tumour of OPA sheep and tissues of unaffected sheep. DNA was collected from the same OPA animals and from control animals and tissues. The comparison of sequences derived from the RT-PCR products revealed a *Sca I* site in the *gag* region that was present only in lung fluid, tumours and viral pellets and mediastinal lymph nodes of affected sheep (Palmarini *et al.,* 1996a). The same result was obtained after analysis of DNA provirus in affected and not affected animals. So the *Sca I* site was correlated with the exogenous virus. The absence of the *Sca I* site was confirmed in a separate molecular characterisation of endogenous sheep retrovirus (Bai *et al.,* 1996).

The molecular characterisation of a number of endogenous sheep retrovirus loci (Bai *et al.,* 1996) and the identification of the major differences in the U3 region of the LTR of exogenous JSRV allowed the development of an hemi-nested PCR specific for the exogenous JSRV proviral DNA (Palmarini *et al.,* 1996b). Using this new assay the tissue distribution of JSRV in sheep with natural and experimentally induced SPA was analysed. Proviral DNA and JSRV transcripts were found in all

tumours and lung secretions of SPA-affected sheep (n = 22) and in several lymphoid tissues. The mediastinal lymph nodes draining the lungs were consistently demonstrated to be infected by JSRV (10/10). JSRV transcripts were also detected in spleen (7/9), thymus (2/4), bone marrow (4/8) and peripheral blood mononuclear cells (3/7). Proviral DNA was also detected in these tissues although in a much lower proportion of cases. JSRV was not detected in samples from unaffected control animals (Palmarini *et al.,* 1996b).

To rule out the possibility that the exogenous form of JSRV may have represented enJSRV that normally were transcriptionally silent but reactivated as a downstream event of neoplasia, it was necessary to demonstrate exogenous JSRV proviral DNA only in OPA-affected sheep and not unaffected animals.

1.1.7.7 Definitive proof that JSRV was the etiological agent of OPA

The identification of exogenous JSRV-specific restriction endonuclease polymorphisms (Bai *et al.,* 1996; Palmarini *et al,* 1996a) and the development of a diagnostic PCR specific for the exogenous JSRV DNA (Palmarini *et al.,* 1996b) opened the way for finally obtaining an infectious molecular clone of JSRV. A lambda phage library was constructed from a natural case of OPA (Palmarini *et al.,* 1999a). The tumour library was screened by sib-selection followed by plaque hybridization using JSRV DNA probe and specific PCR for the exogenous virus. A full length exogenous JSRV proviral clone was obtained (λJSRV21). The insert from this clone was subcloned in to a plasmid to give pJSRV21. Sequence analysis

confirmed that JSRV21 was indeed the integrated provirus, that it was full length and with expected open reading frames (ORFs). Recently another JSRV proviral clone has been isolated from an OPA lung tumour- derived cell line (JS7) that was originated from an OPA clinical case of UK origin (Jassim *et al.,* 1987; DeMartini *et al.,* 2001).

The U3 region of the upstream LTR in pJSRV21 was replaced with the human cytomegalovirus (CMV) immediate early enhancer/promoter. This plasmid, called pCMV2JS21, was successfully used to transfect 293T cells that released substantial amounts of JSRV21 virus (Palmarini *et al.,* 1999a). Four newborn lambs were inoculated with $JSRV_{21}$ particles produced in this manner, and two of them showed the classical signs of SPA 4 months postinfection. The resulting tumours were positive for JSRV DNA and protein. Thus, $JSRV_{21}$ was recognized as an infectious and pathogenic molecular clone, necessary and sufficient to induce sheep pulmonary adenomatosis (Palmarini *et al.,* 1999a).

1.1.8 Features of Endogenous JSRV (enJSRV)

Endogenous betaretroviruses of sheep are highly related to the exogenous and pathogenetic JSRV (York *et al.*, 1992; De las Heras *et al.,* 1993; DeMartini & York, 1997; Palmarini *et al.*, 1999b). Two approaches have been undertaken to identify and characterise the nucleotide sequence and genomic organization of enJSRV in the sheep genome: PCR amplification and molecular cloning of proviruses isolated from sheep lambda libraries (Bai *et al.,* 1996; Palmarini *et al.,* 1996b).

Recently three enJSRV proviruses have been molecularly cloned, sequenced and functionally characterized (Palmarini *et al.,* 2000a). The genome of the enJSRVs loci is highly related to exogenous JSRV with 90-98% identity at the amino acid level in most parts of the genome (Bai *et al.*, 1996, 1999; Palmarini *et al.*, 1996a, 2000b; Rosati *et al.*, 2000). One of the endogenous proviruses was full length and maintained open reading frames in all its structural genes but could not assemble viral particles when highly expressed in human 293T cells; by making chimeras between this provirus and JSRV 21 the defect for viral assembly was detected in the first two-thirds of the *gag* gene (Palmarini *et al.*, 1996a, 2000b).

Two short regions (VR1 and VR2) were identified to contain major differences between ovine endogenous and exogenous betaretroviruses. Besides VR1 and VR2, a third region (VR3) located in the carboxy-terminal portion of the transmembrane (TM) protein of the viral envelope is divergent between the exogenous JSRV and enJSRV sequences (Palmarini *et al.,* 2000a).

By PCR enJSRV RNA has been detected in many sheep tissues examined, including lungs, kidney, thymus, bone marrow, spleen, mediastinal lymph nodes, leukocytes and reproductive tract (Palmarini *et al.,* 1996a). However, in situ hybridisation indicates that enJSRV expression is mostly limited to the reproductive tract.

High levels of enJSRV expression are detectable in the endometrial luminal and glandular epithelium of the uterus and in the epithelia of the oviducts and in the cervix (Spencer *et al.*, 1999; Palmarini *et al.*, 2000b).

In situ hybridisation detected en JSRV RNAs in lymphoid cells associated with the

lamina propria of the small intestine and in the thymuses of sheep foetuses. Low level expression of enJSRV also have been found in the lung (Spencer *et al.,* 2002).

EnJSRV expression is not limited to RNA, in fact the epithelia of the lumen and glands of the endometrium of the uterus were positive by for enJSRV proteins by immunofluorescence when antiserum to the highly related exogenous JSRV CA and SU were used (Palmarini *et al.*, 2001b).

enJSRV RNAs are highly expressed in the epithelium of the uterus, while the exogenous JSRV appears to have a strict tropism for secretory cells of respiratory tract (De las Heras *et al.,* 1991; Palmarini *et al.,* 1995; Spencer *et al.,* 1999; Palmarini *et al.,* 2000a; Palmarini *et al.,* 2000b).

To further investigate the role of enJSRVs in sheep reproductive biology, studies were conducted to determine effects of neonatal age, estrus cycle, early pregnancy and progesterone on expression of enJSRVs in the ovine uterus.

By in situ hybridisation enJSRV expression has been studied in uteri of neonatal ewe lambs between postnatal (PND) day 1and 56 when the uterine gland morphogenesis occurs and no positive result was obtained at PND 1 while it was detected between day 7 and day 56 PND. In pregnant ewes expression of enJSRV RNAs was detected in endometrial lumenal epithelia and glandular epithelia of all regions of the uterus in the ampulla and isthmus regions of the oviduct, as well as in the cervix (Palmarini *et al.,* 2001b).

The oestrus cycle and pregnancy also influence the levels of enJSRV expression in the uterus. In cyclic ewes, endometrial enJSRV RNA is increased 12-fold between

days 1 and 13 and then decreased to day 15. The initial increase of enJSRV RNAs between days 1 and 13 of the oestrus cycle correlates with the formation of the corpus luteum after ovulation and consequent increase of progesterone in the blood (Palmarini *et al.,* 2001).

The high level and specificity of expression of enJSRVs in the endometrium of the ovine uterus might suggest physiological functions of this sequences in regulation of conceptus-endometrium interaction, as well as placental morphogenesis, during the peri-implantation period (Palmarini *et al.,* 2001b).

In OPA-infected sheep or OPA clinical cases there is not detectable antibody response toward JSRV (Sharp & Herring, 1983; Ortin *et al.,* 1998; Verwoerd *et al.,* 1980), consequently, it is conceivable that enJSRVs expression during foetal ontogeny leads to the tolerance of sheep towards the pathogenic JSRV.

EnJSRVs may interact and or interfere at different levels both with the host and with their exogenous and pathogenic counterparts (Spencer *et al.,* 2003; Palmarini *et al.,* 2004).

1.1.9 Molecular basis for the lung tropism of JSRV

JSRV appears to be unique among retroviruses in inducing transformation of the differentiated epithelial cells of the lungs (Rosenberg & Jolicoeur, 1997). The only sites where JSRV is highly expressed *in vivo* are the transformed epithelial cells of the lung (Palmarini *et al.,* 1995). Although it is also possible to detect JSRV DNA

and RNA by sensitive PCR assays in a variety of lymphoid tissues of SPA-affected sheep (Palmarini *et al.,* 1996b; Holland *et al.,* 1999). Proviral DNA has been found in adherent cells (macrophages), B lymphocytes, and $CD4^+$ and $CD8^+$ T lymphocytes of the mediastinal lymph nodes of SPA-affected animals (Holland *et al.*, 1999). Therefore, although JSRV is highly expressed only in the epithelial tumour cells of the lungs *in vitro* experiment JSRV infects several different sheep cell lines of various tissue origins (Palmarini *et al.,* 1999). *In vitro* observations suggested that the cellular receptor for JSRV is common to a variety of cell types; this data suggest that the restriction of viral expression to epithelial tumor cells in the lungs is likely not due to the presence of the JSRV receptor only on these cells and it could reside in the LTR region.

Palmarini *et al.,* (2000a) investigated whether JSRV-specific expression in the differentiated epithelial cells of the lung was due to lung epithelial cell-specific activation of the viral LTR. Reporter assays were performed with several cell lines originating from different cell types with a construct (pJS21-luc) in which the luciferase gene is under the transcriptional control of the JSRV LTR. JSRV LTR function was then studied by assaying the transcriptional activity of LTR deletion mutants and cotransfections with potentially activating transcription factors. By transient-transfection assays of 23 different cell lines with a reporter plasmid driven by the JSRV long terminal repeat (LTR), pJS21-luc, we found that the JSRV LTR is preferentially active in cell lines derived from type II pneumocytes and Clara cells (MLE-15 and mtCC1-2 mouse cell lines). Reporter assays using progressive 5' deletions of pJS21-luc allowed us to establish that the JSRV enhancers are able to

activate the JSRV proximal promoter in MLE-15 and mtCC1-2 cells, but they have very low activity in mouse cells of other lineages (e.g., NIH 3T3). The JSRV enhancers were able to activate heterologous promoters in both MLE-15 and 3T3 cells, although optimal activity was achieved in MLE-15 cells only with the homologous JSRV promoter. Thus, JSRV cell-specific LTR activity appeared to result from an interaction between the enhancer elements and the JSRV proximal promoter elements. After analysis of the U3 sequences in JSRV LTR for potential binding sites 2 putative enhancer binding motifs for transcription factors such as hepatocyte nuclear factor 3 (HNF-3) were found. HNF-3 is an important factor in lung-specific SP gene expression. Co-transfection experiments demonstrated that exogenous HNF-3 was able to enhance the expression of pJS21-luc in NIH 3T3 cells, which normally showed minimal enhancer activity for the JSRV LTR (Palmarini *et al.,* 2000a). Further studies, using a mouse cell line derived from typeII pneumocytes, indicated that JSRV LTR interacts with the hepatocyte nuclear transcription factor called HNF-3β (McGee-Estrada *et al.,* 2002). HNF-3β and its isoform, α, are highly expressed in hepatic and lung tissues (Costa *et al.,* 2001); HNF-3 isoforms are highly expressed in both type II pneumocytes and Clara cells and they regulate the transcription of several lung-specific genes, including surfactant protein B and CC10, a protein anchored on the surface of mature Clara cells (Clevidence *et al.,* 1994; Costa *et al.,* 2001).

Recently, the cellular receptor for JSRV has been identified as a hyaluronidase-2 (HYAL-2), a glycosylphosphatidylinositol-linked cell-surface protein (Rai *et al.,* 2001).

HYAL2 is a member of a protein family that includes the hyaluronidase present on the sperm surface (SPAM1, also called PH-20) that enables sperm to penetrate the hyaluronic acid-rich cumulus cells surrounding the oocyte, and a hyaluronidase found in lysosomes and serum, called HYAL1 (Lepperdinger, *et al.*, 1998); HYAL2 is widely expressed in most tissues, with the notable exception of brain (Csoka *et al.*, 1999). Therefore the distribution of HYAL2 does not drive the tissue tropism.

1.1.10 Mechanisms of oncogenesis in OPA

The mechanism by which JSRV transforms the target type II epithelial pneumocyte is not clear. In the following two paragraphs the hypothesis of JSRV as an acutely transforming retrovirus or as slow transforming retrovirus will be explored and supported by the data obtained by *in vitro* transformation of cell cultures and the features of the pathogenesis of the disease *in vivo*. So is JSRV:

a) an acute-transforming retrovirus

or

b) a slowly transforming retrovirus?

a) Is JSRV an acute transforming retrovirus?

JSRV can induce lung cancer in sheep quite rapidly, 4 months with the cloned virus and 3 to 4 weeks with the uncloned virus; moreover the pattern of tumour cells is close with multifocal disease (Verwoerd *et al.*, 1980; Sharp *et al.*, 1983).

This pattern of disease is almost always characteristic of retroviruses that carry oncogenes, viral versions of highly conserved cellular genes that play key roles in growth and development (Rosenberg & Jolicoeur, 1997), however, JSRV lacks an oncogene (Palmarini *et al.,* 1999).

One of the characteristics of acute transforming retroviruses is that they can morphologically transform cells in culture (Coffin *et al.,* 1997).

Maeda *et al.,* (2001) used the pCMVJS21 plasmid (in which the JSRV genome is driven by the human CMV immediate early promoter/enhancer (to assess the hypothesis that JSRV could transform NIH3T3 murine cell line). Many scientists have used these cells to test the presence of viral oncogenes or activated cellular proto-oncogenes (Shih & Weinberg, 1982). When pCMVJS21was transfected into NIH3T3 fibroblasts, foci of transformed cells were obtained; in contrast transfection with a control plasmid lacking JSRV sequences did not produced foci of transformation. Cells from the foci were analysed and JSRV DNA could be detected in all the transformants and also showed the capacity to grow in soft agar sospension (Maeda *et al.,* 2001). Similar results were obtained transfecting rat fibroblast cell line 208F (Rai *et al.,* 2001) and Rat6 (Maeda *et al.,* 2001).

At this point of the studies it was very interesting to understand which gene implicated in the transformation. The potential transformation power of *orf-x* region was checked using the plasmid pCMVJS21 in which to termination codons were inserted into the orf-x reading frame in positions where the amino acids of the overlapping integrase protein would not be affected; this plasmid was called

pCMVJS21ΔorfX and it was used to transfect NIH3T3 cells (Maeda *et al.,* 2001). pCMVJS21ΔorfX did not induce any decrease in the number of foci compared to the original plasmid pCMVJS21. Thus it could be deduced that *Orf-x* is not essential for transformation. Maeda *et al.,* (2001) then tested the possibility of that the *env* gene could be implicated in transformation. So it was generated a plasmid, pCMVJS21ΔGP, in which the coding sequences for *gag* and *pol* were eliminated, but the splice donor and acceptor sites for the envelope gene were maintained; it efficiently induced transformed foci. This result showed that JSRV envelope gene was responsible for the transformation of cell culture. Two additional plasmids for the expression of the truncated envelope protein were prepared for investigating which part of *env* was responsible for transformation. The results indicated that the truncated forms of *env* were not sufficient for transformation and that probably full-length envelope protein is required for transformation (Maeda *et al.,* 2001). The transformation of JSRV envelope protein has also been demonstrated using avian DF-1 cells (Allen *et al.,* 2002). This experiment used avian sarcoma virus (ASLV) derived vectors (pRCAS) and the avian fibroblast cell line, DF-1 (Federspiel & Hughes, 1997; Himly *et al.*, 1998; Schaefer-klein, *et al.,* 1998).

The pRCAS vectors were constructed by removing the *src* gene from the virus genome and replacing it with a cloning site (Federspiel & Hughes, 1997). JSRV *gag, pro, pro, pol, orf-x, env*, where cloned separately into the vector and their effects after transfection were monitored. Only cells transfected with the vector containing the JSRV *env* gene transformed. Cells transfected with PRCAS containing the JSRV *env* gene were inoculated into nude mice and they were tumorigenic (Allen *et al.*,

2002).

Palmarini *et al.,* (2001b) mapped the determinants of cell transformation of the JSRV envelope. A series of chimeric construct between the envelope of infectious molecular clone JSRV 21 and two JSRV-related endogenous retroviruses of sheep (enJS56A1 and en JS5F16) were constructed and used to transform NIH 3T3 cells. The VR3 region includes the putative membrane-spanning region and cytoplasmatic tail of JSRV TM glycoprotein; this suggested that the cytoplasmatic tail of JSRV ENV mediates transformation. Mutation of Y590 and M593 in the cytoplasmatic tail of the JSRV envelope were sufficient to inhibit the transforming abilities of these constructs. Y590 and M593 are part of a Y-X-X-M motif that is recognised by the phosphatidylinositol 3-kinase (PI-3K). PI-3K initiates a cell signalling pathway that inhibits apoptosis and is required for a number of mitogens during the G1-to-S-phase transition of the cell cycle (Palmarini *et al.,* 2001b). These data indicated that the cytoplasmatic tail of JSRV TM was necessary for cell transformation of rodent fibroblast. Further experiments (Maeda *et al.,* 2003; Liu *et al.,* 2003) showed that PI3K was not necessary for establishment of JSRV envelope-induced transformation, despite the requirements of the YXXM motif and that interestingly as already highlighted by Palmarini *et al.,* (2001b) the downstream AKT kinase is phoshorylated in JSRV transformed cells. The PI3K/Akt pathway has been implicated in several cancers and oncogenic systems. Avian and murine acute transforming retroviruses have transduced bot Akt (v-Akt in the AKT8 murine lymphoma virus) and the p110 of PI3K (v-P3k in avian S-7 virus) (Bellacosa *et al.,* 1991; Chang *et al.*, 1997). Amplification and/or overexpression of the Akt gene has

also been reported in a variety of human cancers (Vivanco & Sawyers, 2002).

The finding that JSRV envelope is able to transform cells opens further investigation for possible mechanisms for JSRV envelope induced oncogenesis.

JSRV envelope SU binds to its receptor Hyal-2 that could start a signal transduction cascade towards other regulatory mechanism. For example HIV-1 infects target cells that present CD4 surface molecules as receptor as well as another co-receptor. In the case of macrophage tropic HIVs, the coreceptor is the CCR5 chemokine molecule (Alkhatib *et al.,* 1996; Choe *et al.,* 1996; Deng *et al.,* 1996; Dragic *et al.,* 1996). The interaction of macrophages tropic HIV-1 with CCR5 leads to an intracellular signalling event involving calcium release (Weissman *et al.,* 1997). An argument against this theory is the fact that although rodent cells lack the JSRV receptor, they can be transformed by JSRV. Infact rodent cells show low levels of infectability by JSRV pseudotypes of retroviral vectors (Rai *et al.,* 2000).

JSRV envelope SU protein binds to a cell surface protein that is different from Hyal-2, and it stimulates cell growth through this other receptor. SU envelope proteins of MMTV and MuLV bind to the Toll-like receptor (TLR) 4 cell surface protein (Rassa *et al.,* 2002). TLR4 is present on the surface of B Lymphocytes, and its normal function is to mitotically activate them.

JSRV TM protein binds a cytoplasmic tail leading to a cascade signalling for cell growth. It is possible that a cellular protein could interact with the TM cytoplasmic tail. For example SIV and in particular the strain SIV macPBJ14 has a TM domain that can interact with cellular factors involved in the signalling for cell death (Fultz

et al., 1989).

b) Is JSRV a slowly transforming retrovirus?

However, JSRV lacks an oncogene, containing only *gag*, *pol*, and *env*, the three genes typically found in simple, replicating retroviruses. Non-acute transforming retroviruses are typically associated with tumours that arise after a long latent period. Invariably, such tumours contain an integrated provirus that alters the expression of cellular proto-oncogenes (Rosenberg *et al.,* 1997). The JS7 tumour cell line contains one integrated JSRV provirus (DeMartini *et al.,* 2001). The provirus from JS7 cells was molecularly cloned and was found in the surfactant protein A gene. The analysis of the JSRV insertion sites was greatly complicated by the presence in sheep genome of approximately 20 copies of enJSRV (DeMartini *et al.*, 2003; Palmarini *et al.,* 2004). Recently 70 JSRV integration sites from 23 sheep and also the integration site from the JS7 OPA tumour cell line were cloned (Cousens *et al.,* 2004). The chromosomal location of the integration sites was determined by PCR, using as a template DNA isolated from a panel of sheep-hamster somatic hybrid cells, each containing 1 or few of the 28 sheep chromosomes. After sequence aligning and Southern blotting techniques, the final data from this study suggested that there is a common integration site for JSRV on chromosome 16 in tumour DNA extracted from two sheep with OPA, neither of the insertion sites was found to be clonal (Cousens *et al.,* 2004). These data could support a role for insertional mutagenesis even if further evidence of integration sites in this region is required. The fact that none of the integration site was found to be clonal cannot exclude insertional mutagenesis infact multiclonality has been reported for tumours induced by MMTV

(Sarkar, 1995). The host background could play a strong effect on the frequency with which a particular gene is insertionally activated. In MMTV for instance, in CH3 mice wnt-1 is activated in 75% of tumours while in other mouse strains is activated only in 25-59% of the cases (Marchetti *et al.,* 1991). OPA affects an outbred sheep population, therefore analysis of a greater numbers of tumours is required to identify additional preferred sites (Cousens *et al.,* 2004).

How to link the insertional mutagenesis mechanism with the role of the recent identified JSRV receptor (HYAL-2) is still in the land of hypothesis (Rai *et al.,* 2000).

HYAL2 was previously described as a lysosomal hyaluronidase, but Rai *et al.*, (2000) showed that HYAL-2 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface protein. Furthermore, the hyaluronidase activity associated with or secreted by cells expressing HYAL-2 could not be detected, whereas such activity could be detected from cells expressing the related serum hyaluronidase HYAL-1 (Rai *et al.*, 2000). Although the function of HYAL-2 is currently unknown, other GPI-anchored proteins are involved in signal transduction, and some mediate mitogenic responses, suggesting a potential role of HYAL-2 in JSRV oncogenesis (Low, 1989; Lublin, 1992). Another interesting finding about this receptor is that the Hyal-2 gene resides in the 3p21.3 region (Rai *et al.,* 2000). The human chromosome 3p21.3 region has been studied in correlation with tumour suppressor gene loss in human tumours; infact one common method to scan tumour cells for tumour suppressor loss is to examine them cytologically and/or molecularly for chromosomal loss of heterozygosity (LOH), a prominent common site of LOH in lung cancers is on

human chromosome 3p21.3 (Sekido *et al.,* 1996). The HYAL-2 gene thus has been considered a candidate tumour suppressor gene for lung tumours (Rai *et al.,* 2000).

As has been shown for other retrovirus-induced tumours (Li *et al.,* 1990; Ruscetti *et al.,* 1990), OPA is likely to be a multistep process resulting from an accumulation of events. The recent data on the transforming activity of the transmembrane envelope protein in vitro suggest an important but not necessary exclusive role for the acute transformation by JSRV (Palmarini *et al.,* 2001a; Rai et al., 2001; Alberti *et al.,* 2002; Allen *et al.,* 2002; Chow *et al.,* 2003; Danilkovitch-Miagkova *et al.,* 2003; Liu *et al.,* 2003; Zavala *et al.,* 2003). In particular, an acute transformation mechanism does not fit with the long incubation periods between infection and OPA clinical signs seen in natural OPA.

At this stage insertional mutagenesis cannot be excluded. It has been shown that for certain acute transforming retroviruses, insertional activation of proto-oncogenes occurs as well. In particular Abelson murine leukemia virus, which carries the *v-abl* oncogene also requires insertional activation of proto-oncogenes for efficient tumorigenesis (Poirer & Jolicoeur, 1989).

1.1.11 OPA and bronchioloalveolar carcinoma in human

The histology and the ultrastructure of human bronchioloalveolar carcinoma (BAC) closely resembles OPA: similarities between OPA and BAC have been noticed for more than half a century (Bonne, 1939; Nobel & Perk, 1978; Perk & Hod, 1982; Gazdar & Linnoila, 1988). BAC is a subset of pulmonary adenocarcinoma arising from type II pneumocytes or Clara cells. BAC is now defined as a type of

adenocarcinoma with a pure bronchioloalveolar carcinoma growth pattern and no evidence of stromal, vascular, or pleural invasion; the neoplastic cells grow as a single layer along the walls of the terminal airways and alveoli (Liebow, 1960; Edwards, 1984; Travis *et al.,* 1999). Bronchioloalveolar carcinoma can be divided into non mucinous and mucinous adenocarcinomas.

In the non mucinous adenocarcinoma, the Clara cells and/or type II pneumocytes grow along the alveolar walls and there is no stromal invasion. The mucinous adenocarcinoma is composed of tall columnar cells with cytoplasmic mucin, which displace the nucleus to the base of the cell, growing along alveolar walls and without stromal invasion.

The existence of bronchioloalveolar adenocarcinoma as a distinct clinical and pathological type of adenocarcinoma has been somewhat controversial, mainly because of the difficulty in differentiating bronchioloalveolar carcinoma from other adenocarcinomas (Schraufnagel *et al.,* 1982; Sorensen *et al.,* 1993; Saleh *et al.*, 1998).

Lung cancer is the most common cause of cancer deaths: every year more than a million of people dye from lung cancer world-wide (Parkin, 1998; Greenlee *et al.,* 2000).

The majority of lung cancers are associated with cigarette smoking; however, about 10% of lung cancers in men and 20-25% in women are not associated with smoking (Shopland, 1995). Adenocarcinoma is increasing in frequency and accounts for almost half of lung cancers in some countries, and it is associated less strongly with

cigarette smoking (Greco *et al.,* 1986; Geddes, 1987; Morabia & Wynder, 1992).

Renewed speculation regarding the involvement of a retrovirus in BAC has been supported by the association of retroviruses with several diseases of humans, such as seminoma, undifferentiated germ cell tumours, multiple sclerosis, acute-onset type I diabetes and Sjögren's syndrome (Cremer & Gruber, 1992; Sauter *et al.,* 1995; Herbst *et al.,* 1996; Conrad *et al.,* 1997; Griffiths *et al.,*1997; Perron *et al.,* 1997).

Recently the evidence for a protein related immunologically to JSRV in some human lung tumour suggested a positive correlation between BAC and an endogenous retrovirus (De las Heras *et al.,* 2000). 274 samples from patients with BAC, other pulmonary malignancies, non malignant pulmonary lesions and normal lung were examined by immunohistochemistry using a rabbit antiserum toward the major capsid protein of JSRV The tumours included 129 bronchioloalveolar carcinomas, 65 adenocarcinomas, and 41 squamous cell carcinomas. Positive samples included 39 (30.2%) of 129 broncioloalveolar carcinoma and 17 (26.2%) of 65 adenocarcinomas.

No positive staining was observed in non tumour lesions or normal lung tissues.

The positive reaction was confined to the epithelial tumour cells and showed the same characteristics as that obtained with OPA tumours. These results support the view that the antigen in the human tumours may be a retrovirus protein although no other virologic or molecular data was obtained for supporting this observation (Yousem *et al.,* 2001).

The antigenic positivity observed might reflect cross reaction with the JSRV

antiserum of a protein whose expression is increased in the transformed cells or another hypothesis might be that a human endogenous retrovirus (HERV) is over expressed in the neoplastic cells. Recently JSRV endogenous related sequences have been obtained from human blood collected in Africa and Europe (Morozov *et al.*, 2004). The expression of some HERVs in human malignancies has been observed, but it is not clear if this expression is correlated with the etiology of the tumour (Löwer et al., 1996; Tonjes *et al.*, 1996).

1.1.12 Immuno and inflammatory responses to JSRV in OPA-affected and unaffected sheep

A peculiar feature of OPA is the absence of a specific antibody response to JSRV, despite the highly productive infection in the lungs and disseminated lymphoid infection. The reactivity to recombinant JSRV CA in sera from affected sheep, described in some works (Kwang *et al.*, 1995), was shown not to be specific and reflected the presence of antibodies to the GST fusion partner of the recombinant antigen used in the assays (Ortin *et al.*, 1998).

However, serum antibodies can be induced readily in sheep by immunisation with recombinant JSRV CA protein in adjuvant (Sharp *et al.*, unpublished data).

These results indicate that sheep are not inherently unresponsive to JSRV antigens. It is possible that the expression of endogenous JSRV during ontogeny or in neonatal life may interfere with the exogenous JSRV infection and disease outcome via the induction of immune tolerance. To date, specific cellular immune responses have not been demonstrated.

Another prominent feature of naturally OPA-affected sheep is the marked peripheral neutrophilia and lymphopaenia, particularly affecting CD4+ T lymphocytes as other subsets remained unaffected (Rosadio & Sharp, 1992; Holland *et al.,* 1999).

The persistent and disseminated infection of the lymphoreticular system by JSRV and dysregulation of the immune and inflammatory responses of infected sheep suggest that JSRV may interfere with the host immune responses (Palmarini *et al.,* 1996a). This notion is supported by studies demonstrating JSRV infection of a wide range of lymphoid cells. Holland *et al.,* (1999) showed that in naturally infected sheep, JSRV proviral burden was greatest in the macrophage/monocyte cell population [1/2500 cells], followed by B cells [1/3800 cells], CD4+ T lymphocytes [1/6800 cells] and CD8+ T lymphocytes [1/16700 cells]. Furthermore, dissemination of JSRV was an early event following experimental infection of young lambs. The virus was present in CD4+ and CD8+ T lymphocytes, B lymphocytes and adherent mononuclear cells in the pulmonary lymph nodes as early as 7 days post-inoculation, and was detected in peripheral blood leukocytes (PBLs) by 14 days post-inoculation. These observations are particularly significant as they indicate that dissemination of JSRV precedes transformation and, for the first time, that infected animals might be detected in OPA-affected flocks before the appearance of clinical signs.

In contrast to the changes in phenotypic frequencies observed in naturally-occurring OPA, no significant alterations were identified at any time during the first 20 weeks after inoculation in the blood of young lambs experimentally inoculated with JSRV, compared to age- and breed-matched controls. These observations indicate that the alterations in peripheral blood lymphoid cell subsets are not an early event and,

probably, do not occur in direct response to JSRV infection, but are a consequence of the superimposed bacterial infections that are common in natural cases of OPA.

Functional defects were also demonstrated in the immune responses of JSRV infected sheep. Lymphocytes from both natural cases of OPA and lambs inoculated experimentally with JSRV showed a significantly reduced proliferative response to concanavalina-A, a cell mitogen substance. The reduced response was detected in the lambs as early as eight weeks after inoculation and prior to clinical disease, when the response was only 33% that of the control lambs (Summers *et al.,* 2002). These observations have provided the first indications that JSRV may alter the functional activity of immune cells in infected animals, although the virus apparently does not alter the phenotypic profile of the peripheral blood.

1.1.13 Diagnosis and Control of OPA

OPA has been recognised by EU (Directive 91/68: Intra-community trade in ovine and caprine animals) as an important infectious disease of sheep because of its high prevalence in several member states. This disease results in large economic losses arising from its direct effects, as well as a loss of trade in live sheep.

Most information on the prevalence of OPA is based on clinical observations and histopathological diagnosis. Information of the true prevalence of JSRV infection in OPA-affected flocks has been a major gap in our knowledge. The detection of JSRV in the peripheral blood of experimentally infected lambs, before the development of tumour or onset of clinical disease, therefore, was an important finding that offered a

means to investigate the extent of JSRV infection in OPA-affected flocks (González *et al.,* 2001).

These findings have been extended in studies involving sheep from OPA-affected commercial flocks in Spain (García-Goti, 1999). Peripheral blood leukocytes (PBLs) and tissue samples from sheep were examined for JSRV by PCR. Overall, JSRV was detected in all OPA sheep, with either the classical or atypical form of OPA, as well as in 80% of OPA in-contact sheep. None of 71 samples from the control sheep was positive. Although an earlier report had indicated that as few as 1/250,000 PBLs might be positive for JSRV, in this study JSRV was detected in the PBLs of all classical OPA sheep, 83% of the sheep with atypical OPA, as well as 40% of lesion-free in-contact sheep. These results clearly demonstrated, for the first time, that JSRV can be detected in naturally infected live sheep before the onset of clinical OPA and even in the absence of discernible lung tumours. Although only a small number of in-contact sheep were examined, it seems clear that subclinical JSRV infection can reach a high prevalence in OPA-affected flocks.

To provide further information of the dynamics of JSRV infection in OPA-affected flocks, García-Goti *et al.*, in 2000 conducted a small prospective longitudinal survey in an OPA-affected flock. JSRV was detected by PCR in PBLs from 28% of the flock. Fifteen positive sheep and five negative were selected and PBLs examined by JSRV PCR at monthly intervals for the next four months. JSRV was detected in only nine of the original 15 positive sheep during this period and in four of those that were negative. These results demonstrated the fluctuation in detection of JSRV in blood and, more importantly, confirmed the high prevalence of JSRV infection in

OPA-affected flocks.

At present, the only means of control is regular inspection of adult sheep in affected flocks. Prompt culling of any suspicious animal is advisable, as well as the offspring of affected ewes, which frequently develop OPA. These methods have not been shown to eradicate OPA from a flock in which the disease is endemic, but a reduction in the prevalence of infection may be obtained. PCR techniques able to detect JSRV in the blood before the onset of clinical signs offer new opportunities that may lead to effective control. Currently, a large EU collaboration involving Scotland, Spain and Italy is undertaking research to improve diagnostics tests to detect JSRV during the preclinical stages of infection. New diagnostic procedures will change the overall approach to this disease. The epidemiological studies that are currently in progress in Scotland and Spain will report the rate of prevalence of JSRV infection in flocks with history of OPA.

Further aims of these studies are to understand what proportion of infected sheep will develop OPA, when the infection acquired and what are the routes of transmission (vertical transmission, *in utero*, milk). There is some preliminary evidence for the presence of JSRV in foetuses by PCR techniques (de las Heras *et al.,* 2000). This finding apparently conflicts with one study showing that embryo transfer can be used to obtain animals free of OPA. In this experiment 215 embryos recovered from 76 donor ewes from flocks with endemic OPA and mated with rams that did not have any sign of OPA were transferred to 131 recipients. 38 of 51 progeny from OPA positive donors and 55 of 74 progeny from donors in which no lesions of OPA were detected survived for at least five years. There was no evidence

of OPA in the recipients or their progeny on the basis of clinical and pathological criteria (Parker *et al.*,1998). Further investigations are necessary to clarify possible vertical transmission of the infection.

1.1.14 Aims of the project

OPA is an economically important infectious disease, endemic in several countries. OPA cannot be controlled because it is recognised only when the disease is obvious.

The present project has as a main goal the development of strategies for the control of the disease.

The base for OPA control programme will be epidemiology studies. The hypothesis is that improved understanding of routes of transmission will allow new approaches to management of OPA. Recent demonstration of JSRV in blood of sheep during the preclinical phase has created opportunities for prospective studies to describe the epidemiology of the infection (Gonzalez *et al.*, 2001).

The epidemiology project will consist of two steps:

first a high throughout robust and sensitive PCRs will be developed to detect JSRV in the blood of clinical and pre-clinical animals (Chapter 3); and then for the first time JSRV infection will be studied in longitudinal surveys in flocks with history of OPA (Chapter 4). Improved understanding of risk factors for transmission and maintenance of OPA will improve management control strategies to reduce the impact factor of the disease.

The other part of the project will create the basis for a vaccination strategy to control OPA. The first objectives for the long term JSRV vaccine development are:

to develop an experimental model for OPA in older lambs, so that the protective effect of vaccines can be evaluated (Chapter 3); this experiment has as a background the fact that OPA can be induced reliably in a high proportion of neonatal lambs by intratracheal inoculation of concentrated lung fluid (Sharp, 1983). Although this model has been critical to explain the role of JSRV and its pathogenesis, because of the age of the lambs, it clearly, is not suitable for assessment of potential vaccines. During this experiment the preclinical JSRV PCR will be used for monitoring the viraemia of experimentally infected lambs.

Part of the work has the aim to generate new forms of JSRV recombinant proteins to use in immunological techniques and immunisation trials (Chapter 2). Particular emphasis will be addressed to the production of the JSRV envelope protein.

The possibility to overcome the apparent tolerance to JSRV will be explored through the vaccination of JSRV viraemic and non viraemic animals with appropriate recombinant proteins (Chapter 2).

Chapter 2

Studying the pathogenesis of JSRV: synthesis of recombinant proteins and immunisation trials

2.1 Introduction

This chapter describes the development of new JSRV recombinant proteins to use for raising antibodies in rabbits and in sheep.

Research on the pathogenesis of OPA has been hampered for years by the lack of an *in vitro* system for the propagation of the virus and by the lack of appropriate immunological reagents for the detection of the virus.

The complete sequencing of JSRV (York *et al.*, 1992) offered new possibilities for the development of new JSRV antigens.

The first production of JSRV capsid recombinant proteins represented a crucial first step towards studying the distribution and site of replication of JSRV in OPA affected sheep (Palmarini *et al.,* 1995).

To date OPA tumours have been studied using polyclonal antibodies against JSRV capsid recombinant proteins; no study has been done using JSRV recombinant envelope proteins.

The aim of this part of the work was to produce new envelope and capsid recombinant proteins not only for immunological assays but also for preliminary immunisation trials in sheep. Essential features of the recombinant proteins for reaching these aims are solubility and high yield. According to these necessities, new protein production methods were attempted.

In OPA-affected animals there is no evidence of circulating JSRV-specific antibodies and the reduced response to ConA indicated an alteration in systemic immunity (Ortin *et al.,* 1998; Summers *et al.*, 2002). In this chapter it was investigated whether

sheep infected with JSRV can develop antibodies against JSRV capsid (CA) and envelope protein (SU) after immunisation with fusion proteins.

The lack of immune responses has been explained with two different hypotheses:

the virus could have a direct immunosuppressive activity on the immune system; previous work has shown that during JSRV infection the cellular immune-response can be compromised; in fact reduced responses to concanavalin A stimulation were demonstrated in the JSRV-inoculated lambs, prior to the onset of clinical disease, and also in the terminally ill adult sheep (Summers *et al.*, 2002);

Sheep may be tolerant to JSRV as a consequence of expression of related endogenous viruses in the foetus (Spencer *et al.*, 2003).

However, antibodies can be raised in sheep by immunisation with different forms of recombinant JSRV CA protein in Freund's complete adjuvant (Sharp & Dewar, unpublished results). This result was achieved in several sheep breeds.

Since it has been shown that uninfected sheep do respond to immunisation with CA in FCA, the ability of JSRV infected sheep to develop an immune response against JSRV CA was investigated. If antibodies to JSRV CA are produced in infected sheep this would indicate that JSRV infection does not directly render the immune system non-responsive to JSRV antigens.

If antibodies to JSRV-CA are not produced in infected sheep this would be consistent with the hypothesis that JSRV infection does have some immuno-inhibitory activity. Since an antibody response to JSRV proteins in sheep has only been successfully raised when FCA has been used as an adjuvant, FCA was used in this experiment (section 2.4.1). However, given the welfare concerns regarding the use of this

reagent, the effect of another adjuvant, Quil-A also was measured and the antibody response compared with that obtained with FCA (Kensil *et al.,* 2004; *Yu et al.,* 2004).

In the following experiment (section 2.5) sheep were immunised with JSRV SU recombinant proteins. No sheep immunisation trial with JSRV SU recombinant protein has been attempted before. It was possible only to immunise OPA negative sheep with JSRV SU recombinant proteins; budgetary and time constraints precluded studies with viraemic animals.

The results provided information about immune responses to JSRV. Such information is a necessary starting point for future rational design of vaccination protocols.

The aim of this chapter was to generate new JSRV capsid and envelope recombinant proteins to use in immunisation experiments with viraemic and non viraemic sheep for testing the hypothesis that JSRV viraemic sheep are immunocompromised.

In this chapter the following experiments were carried out:

-production of JSRV recombinant proteins;

-immunisation of rabbit with JSRV SU protein for obtaining an immune serum to use in immunohistochemistry studies;

-immunisation of OPA viraemic and non viraemic sheep with recombinant JSRV CA protein (paragraph 2.4);

-immunisation of non viraemic sheep with JSRV SU recombinant proteins (paragraph 2.5).

2.2 Production of JSRV recombinant proteins

2.2.1 Approach to the expression of JSRV recombinant proteins

Previous experiences of production of JSRV recombinant proteins had encountered solubility and concentration problems.

Recombinant JSRV antigens have been expressed previously in *E.coli* as β-galactosidase (βgal-gag) and glutathione-S-transferase (GST-gag) fusion proteins and used to generate specific antisera in rabbits and a new blocking-ELISA (Palmarini *et al.* 1995). Using these reagents, tissues from naturally and experimentally OPA-affected sheep, as well as age and breed-matched controls, were examined to determine the anatomic distribution of JSRV (Palmarini *et al.,* 1995). The specific antisera for recombinant JSRV CAs were used also in IHC studies: JSRV protein was detected only in the cytoplasm of neoplastic epithelial cells in the pulmonary alveoli of OPA affected sheep (Palmarini *et al.*, 1995, 1996a; Holland *et al.,* 1999; Platt *et al.,* 2002).

Currently, three strategies are used for protein production: chemical synthesis, *in vivo* expression, and cell-free protein synthesis. The first two methods have severe limitations. Chemical synthesis is not practical for the synthesis of long peptides (Blaschke *et al.,* 2000), and *in vivo* expression can produce only those proteins that do not affect the physiology of the host cell (Henrich *et al.,* 1982: Goff & Goldberg, 1987; Chrunyk *et al.,* 1993). These methods require transformation, cell maintenance, expression and optimisation procedures that can be both time-consuming and technically challenging. Due to fast growth, easy handling and low cost, *Escherichia coli* is the principal expression system of choice (Baneyx, 1999). However,

production of recombinant proteins with *E. coli* has many limitations: many of these proteins are insoluble, biologically inactive and in aggregated forms or inclusion bodies and do not undergo post-translational modifications, for example glycosylation. Other disadvantages are that the polypeptide product that may be unstable in a given cell, and in some cases the product is toxic to the cell. Traditional protein expression systems involve expressing recombinant proteins in intact eukaryotic or prokaryotic cells.

Wheat germ embryos store all the components of translation in a dried state, in high content, and are ready for protein synthesis after germination in response to transcription of mRNAs triggered by an environmental signal (Roberts & Paterson, 1973). The lack of a physical barrier, the cell wall, makes the reaction environment accessible to manipulation in the cell free system. The reaction conditions can also be varied over a wider range because living cells are not required (Alimov *et al.,* 2000). These cell-free translation systems can synthesize proteins with high speed and accuracy, approaching *in vivo* rates (Kurland, 1982; Pavlov & Ehrenberg, 1996), and they can express proteins that would interfere with cell physiology (Roberts & Paterson, 1973). Cell-free systems have great potential for large-scale protein synthesis and many efforts have been made to increase their efficiency. Spirin *et al.,* (1988) proposed a continuous flow cell-free (CFCF) system, in which a solution containing amino acids and energy sources is supplied to the reaction chamber through a filtration membrane. The CFCF system uses a continuous flow of the feeding buffer [including amino acids, adenosine triphosphate (ATP), and guanosine triphosphate (GTP)] through the reaction mixture and a continuous removal of polypetide product. The reaction works for about tens hours and can produce

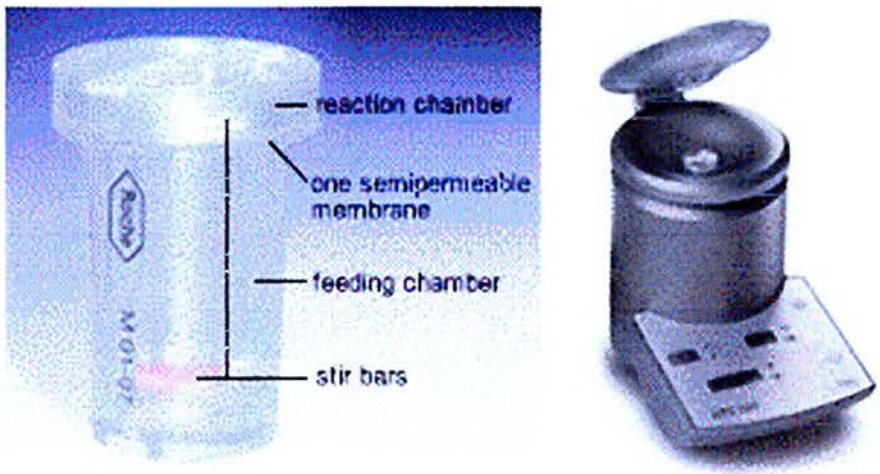


Fig. 2.1. Rapid translation system (RTS) technology (Roche).

On the left the RTS reaction device. Principle of Continous Exchange Cell-free protein synthesis (CECF) as invented by Spirin *et al.*, 1988. The RTS device is formed by a reaction compartment where transcription and translation occur and the protein product is retained and a feeding compartment into which inhibitory reaction by-products diffuse from which reaction substrates and energy components are continuously supplied for sustained synthesis. Once the RTS device has been filled with the appropriate solutions it can be inserted in the RTS 500 instrument (Roche).

hundreds of micrograms per milliliter reaction volume (Spirin *et al.,* 1988; Baranov *et al.,* 1989; Kigawa &Yokoyama, 1991; Endo et al., 1992). Recently, several modified versions of the Spirin system have been reported (Endo *et al.*, 1992; Kawarasaki *et al.,* 1995; Kim et al., 1996; Kigawa *et al.,* 1999). Cell-free translation systems can be generated from both prokaryotic and eukaryotic cells.

Although JSRV-CA has been expressed successfully in *E. coli* and it was used to raise antibodies, problems of solubility remained and no success had been obtained with the expression of JSRV-SU in *E. coli* (Dewar *et al.,* unpublished data) or in yeast systems (*Pichia pastoris*) (Philbey *et al.,* unpublished data). The availability of the new cell-free systems offered a different approach for the production of JSRV-CA and SU that may overcome the previous problems.

Therefore, this chapter reports results obtained with a commercial cell-free system, the Rapid translation System-500 (RTS-500) produced by Roche.

2.2.2 Materials and Methods.

2.2.2.1 Introduction to the RTS-500

The rapid translation system (RTS) *E. coli* circular template Kit (Roche Applied Science) was used to express recombinant CA and SU. It consists of the digitally controlled RTS 500 instrument, which ensures optimal expression conditions (Fig.2.1), and a reagent kit that utilizes coupled *in vitro* transcription/ translation for the expression of up to 5 mg of protein/ml reaction in 20-24 hours (Cho *et al.,* 2001; Murayama *et al.*, 2001; Fukushima & Kawaguchi, 2001; Betton, 2000).

The disposable RTS-500 reaction device has three components (Fig.2.1): the upper 1 ml reaction compartment, the semi-permeable membrane and the lower 10 ml feeding compartment. Transcription and translation take place simultaneously in the reaction compartment.

Substrates and energy needed for a sustained reaction are continuously supplied through the semi-permeable membrane. During coupled transcription and translation energy components, nucleotides and aminoacids are consumed, whereas waste products like inorganic pyrophosphate, phosphate, nucleotide mono- and diphosphates, and other degradation products are formed. The accumulation of inhibitory waste components and the depletion of substrates would quickly result in the stopping of the reaction. This is prevented by a continuous removal of waste and supply with substrate components by diffusion through a semipermeable dialysis membrane, which separates the upper reaction compartment from the lower feeding compartment (Spirin *et al.,* 1988).

2.2.2.2 Cloning of JSRV CA

The 660bp region of gag encoding the CA protein was amplified by PCR from pJSRV21 using primers p26CA-for (CGGAGCTCCCTGTTTTTGAAAATAA) and p26CA-rev (GCGAATTCAAGCAATACCTTGCATA). Primers lie between position 1029 and position 1689 of the JSRV genome. The forward primer contains a *SacI* site (GAGCT ^{v}C). This site was important to keep the frame in the correct position with

the upstream His-tag in the chosen final vector pIVEX 2.4a (Roche). The reverse primer contains a stoop codon (TCA).

PCR cycles employed were 94˚C for 1 min and 35 cycles of 94˚C for 45 s, 57˚C for 1 min, and 72˚C for 1 min, with a final extension of 72˚C for 2 min in a Perkin-Elmer GenAmp 2400 thermal cycler.

The PCR product was cloned into pT7blue-2 vector (Novagen) according to the manufacturer's instructions.

The CA gene was excised from pT7Blue-2 vector by restriction endonuclease digestion (*SmaI/Sac*I, Roche). The relevant 660 bp CA band was visualised after gel electrophoresis (1% agarose gel), by UV and then excised. The CA DNA was extracted from the gel using QIagen gel extraction kit (Roche)(Appendix II.3). Then CA DNA was inserted in pIVEX2.4 (Fig.2.2), previously cut with restriction endonucleases *Sac*I (Roche) and *Sma*I (Roche).

The insert (CA DNA) was added to the vector at approximate molar ratio of 3:1, 6:1, 9:1. One μl 2x ligase buffer and 1 μl of ligase were added, the final volume was made up to 20μl with distilled water (Rapid DNA Ligation Kit, Roche). The ligation reaction was incubated at + 4°C overnight before being used to transform competent *E.coli* JM109 (Promega). Transformants were selected by plating on to LB/Amp agar incubated overnight at 37°C. Single colonies were picked and grown overnight in 10 ml LB/Amp broth at 37°C with shaking. Plasmid DNA was extracted using QIaprep mini prep kit (QIagen), (Appendix II.4). The presence of the inserted CA DNA in pIVEX2.4a-CA was checked by restriction enzyme digestion and gel electrophoresis.

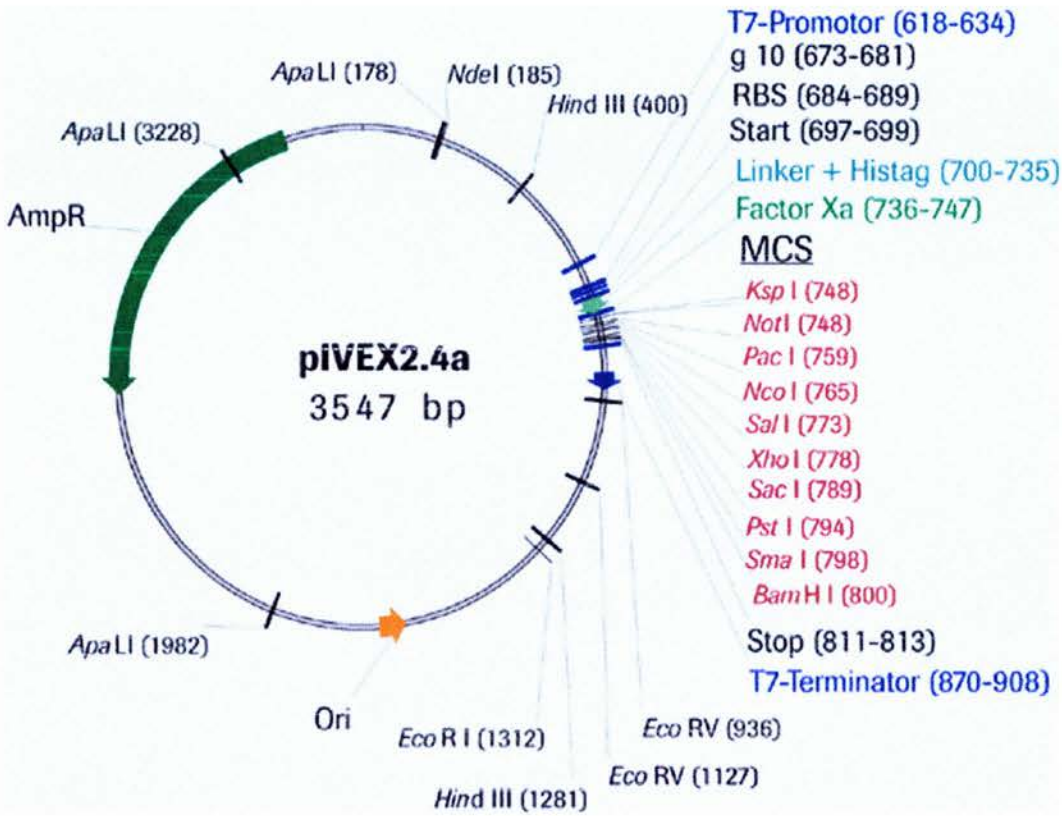


Fig 2.2. Map of pIVEX2.4a vector (Roche).

The pIVEX2.4a vector was used for subcloning and expression of JSRV CA and SU DNA. The coding sequence is cloned downstream from a hexa-His tag into a multiple cloning site. T7-P:phage T7 promoter; RBS: ribosomal binding site; Histag: tag sequence for purification; Xa: factor Xa restriction protease cleavage site; MCS: multiple cloning site; T7T: phage T7 polymerase terminator sequence.

The correct DNA sequence for CA was confirmed by automated sequencing using an ABI 700 (Applied Biosystem, MRI Functional Genomics Unit),(Sanger *et al.,* 1977). Glycerol stocks of pIVEX2.4a-CA were frozen at -70°C.

2.2.2.3 Cell free production of JSRV CA

The plasmid pIVEX2.4a-CA was expressed using the RTS 500 circular template kit (Roche) according to the product instructions (Appendix VIII).

In the first attempt with the RTS system, 15μg of pIVEX2.4a-CA was introduced in the reaction and the instrument was set at 30°C for 24 h at 120 rpm. These parameters had been commonly used for the expression of other proteins using the RTS system according to the manufacturer's protocol. The expression of JSRV capsid protein by the RTS system (JSRV RTS CA) was ascertained after purification (2.2.2.4) by SDS-PAGE stained with Coomassie and confirmed by Western-blot.

Thereafter JSRV RTS CA production was optimised. Different combinations of quantity of plasmid DNA (5-20μg) and reaction temperature (25°C and 30°C) were varied to determine the conditions that would result in the highest expression levels. The chosen time of protein expression was 24 h (Table 2.1) (Martin *et al.,* 2001).

Table 2.1. Optimisation of JSRV CA production with the RTS system.

DNA	temperature	time
5µg	25°C	24h
	30°C	24h
15µg	25°C	24h
	30°C	24h

The RTS instrument was set at a stirring speed of 120 rpm (Fig. 2.3).

The recombinant JSRV protein obtained with the RTS system was referred to as JSRV RTS CA.

2.2.2.4 JSRV RTS CA purification

The plasmid pIVEX2.4a-CA creates a his tagged protein, which allows easier detection and purification of the expressed protein because the his tag has a specific affinity for metal ions, such as nickel. A striking feature of the his tag system is that binding of the his-tagged protein to the solid phase metal is not affected by strong denaturants, allowing the protein to be soluble and purified in such i.e. agents as 8 M urea or 6 M guanidinium. Rapid and mild elution of the his-tagged protein is another key asset of the system because the bound protein can be displaced using basic or acid pH in imidazole or HCl. The ProBond™ Purification System protocol

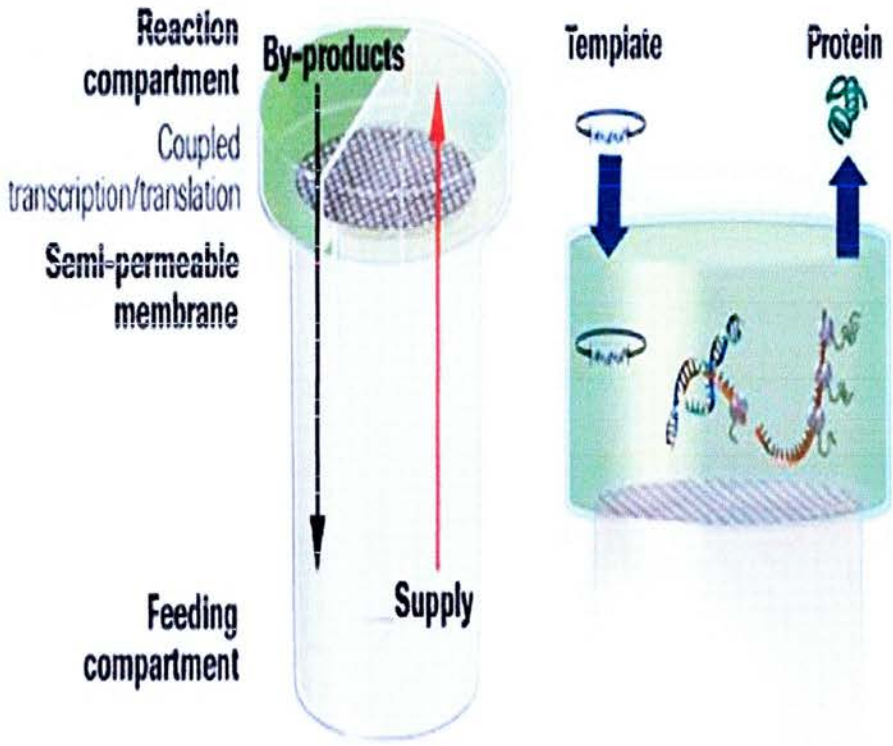


Fig. 2.3. Illustration of RTS protein expression system.

On the left a diagram of the 1 ml RTS vial; on the right a diagram of the reaction that happens in the upper compartment of the RTS device.Transcription and translation take place simultaneously in the 1 ml reaction compartment of the reaction device. Substrates and energy components essential for a sustained reaction are continously supplied via a semipermeable membrane.

First JSRV CA and SU were cloned in the template vector pIVEX2.4a and added to the reaction compartment. Then in a coupled in vitro reaction DNA is first transcribed from the template vector into mRNA by T7 polymerase, followed by translation by the ribosomal machinery present in the *E.coli* lysate. Expressed protein accumulates in the reaction compartment and is harvested after 24 hours run.

(Diagram adapted from Roche www.proteinexpression.com).

(Invitrogen) was used for the purification of recombinant RTS JSRV CA and SU. The kit utilizes nickel-chelating resin that is supplied with native and denaturing buffers for efficient purification of recombinant proteins under different conditions. The RTS system facilitates the purification because there is no bacterial cell lysis step and the 1 ml reaction fluid can be used straight away in the purification steps.

RTS- JSRV CA recombinant protein was purified following the "ProBond Purification System" (Invitrogen). JSRV RTS CA was purified using either

a) denaturing conditions or

b) native conditions.

The purification method under native conditions has the advantage to keep intact the functionality of the protein. When the protein has solubility problems, it is necessary to introduce denaturing agent (i.e. urea) in the purification steps (Basharov, 2003).

a) Denaturing method for the purification of RTS-JSRV CA

In general JSRV RTS CA was bound to nickel resin (Invitrogen) in 8 M Urea, 20 mM phosphate buffer, 500mM NaCl (pH 7.8). Other host cell proteins that have some affinity for the beads under these conditions can be removed by washing protein-bound beads with the same buffer but with a lower pH buffer (e.g. pH 6.0). The RTS-JSRV CA was eluted from the beads by washing with the buffer at pH 4.00 under denaturing conditions (see Appendix V.1).

The total 1ml content of the reaction compartment of the RTS device was collected and equal 500µl aliquots placed in two Eppendorf tubes and kept at 4°C until purification. Each aliquot was purified separately. One ml of nickel beads slurry (nickel beads in 50% ethanol, Invitrogen) was centrifuged at 800g for 2 minutes to remove the ethanol and then a further three times in 500µl of denaturing binding

buffer pH7.8. The supernatant was discarded after centrifugation to remove any possible trace of ethanol.

Then a 500µl aliquot of the RTS reaction was added to the Eppendorf tube containing the beads and 500µl of denaturing binding buffer pH 7.8.

The effect of incubation time was determined by incubation of the beads with the RTS reaction product in denaturing binding buffer pH 7.8 for 1 hour, 2 hours, 12 hours on a rocker at ambient temperature.

Then the beads were spun down as described previously and the supernatant was kept as "post-beads lysate". The beads were washed twice with denaturing binding buffer pH 7.8 by re-suspending the beads in 1ml of buffer, mixing well, spinning down the beads and discarding the supernatant. To wash, the beads were resuspended with 0.5 ml of denaturing binding buffer pH 7.8, centrifuged at 800g for 2 minutes and then the supernatant was discarded. The beads were washed twice with denaturing buffer pH 6.0 and twice with denaturing buffer pH5.3 and each time they were spun down by centrifugation to remove as much buffer as possible. The beads were re-suspended in 500µl of denaturing elution buffer pH 4.00 and mixed gently on a rocker at room temperature. (Ausubel *et al.*,1994*)*. The sample was spun down and the supernatant collected as "first eluate".

The effect of different incubation times was evaluated testing different incubation times (30 minutes, 1, 2, 12 hours) of buffer pH 4.00 with the beads.

The elution was repeated two more times with 500µl of denaturing elution buffer pH 4.00 to collect second and third eluates after mixing for 30 minutes on a rocker. A sample of the beads was kept to check how much of the protein remained bound to the beads. Elution samples were stored at -20°C.

b) Purification of RTS-JSRV CA under native conditions

Another approach for the purification of RTS-JSRV CA undertaken was the elution of RTS-JSRV CA under native conditions.

Nickel beads were used as previously described for the denaturing conditions protocol. The purification steps under native conditions were essentially the same described for the purification under denaturing conditions. The native purification buffers were:

- native binding buffer pH 7.2-7.6,
- native wash buffer pH 5.5,
- native elution buffer pH 4.0 (see Appendix V.2 for the preparations of the native purification buffers).

Only 2 washing steps were performed after the incubation of the RTS reaction with the nickel beads and before the elution step.

Imidazole was included in the native wash and elution buffers (final concentration 3M) to minimise the binding of untagged contaminating proteins and increase the purity of the target protein with fewer wash steps.

As already described the total 1ml content of the reaction compartment of the RTS device was collected and equal 500µl aliquots placed in two Eppendorf tubes and kept at 4°C until purification. One ml of nickel beads slurry (nickel beads in 50% ethanol, Invitrogen) were carefully washed with native binding buffer pH 7.2-7.6 as described during the denaturing method to remove any possible trace of ethanol.

Then a 500µl aliquot of the RTS reaction was added to the Eppendorf tube containing the beads and 500µl of denaturing binding buffer pH 7.8.

The effect of incubation time was determined by incubation of the beads with the RTS reaction product in native binding buffer pH 7.2-7.6 for 1 hour, 2 hours, 12 hours on a rocker at ambient temperature.

Then the beads were spun down as described previously and the supernatant was kept as "post-beads lysate". The beads were washed twice with native binding buffer pH 7.2-7.6 by re-suspending the beads in 1ml of buffer, mixing well, spinning down the beads and discarding the supernatant. To wash, the beads were suspended with 0.5 ml of native binding buffer pH 7.2-7.6, centrifuged at 800g for 2 minutes and then the supernatant was discarded. The beads were washed twice with native wash buffer pH 5.5. The beads were re-suspended in 500µl of native elution buffer pH 4.00 and mixed gently on a rocker at room temperature. (Ausubel *et al.,* 1994*)*. The sample was spun down and the supernatant collected as "first eluate".

The effect of different incubation times was evaluated testing different incubation times (30 minutes, 1, 2, 12 hours) of buffer pH 4.00 with the beads.

The elution was repeated two more times with 500µl of denaturing elution buffer pH 4.00 to collect second and third eluates after mixing for 30 minutes on a rocker. A sample of the beads was kept to check how much of the protein remained bound to the beads. Elution samples were stored at -20°C.

2.2.2.5 Analysis of JSRV RTS CA

The reaction product was analysed for the presence of recombinant CA by SDS-PAGE gel stained with Coomassie blue and by immunoblot (Appendix IV).

The protein yield was evaluated in the first eluate using Coomassie, SDS-PAGE gel and the BCA method.

An *E.coli* expressed his- tag CA was used as a positive control [gift of Van der Molen], (Summers *et al.*, 2002). The Western-Blot was performed using a previously produced anti-rabbit immune serum (Palmarini *et al.,* 1995).

2.2.2.6 Cloning of JSRV SU

DNA encoding the JSRV SU was amplified by PCR from an infectious molecular clone of JSRV ($pJSRV_{21}$), from nucleotide 5557 to nucleotide 6483 (York *et al.* 1992).

The primers sequences were as follows:

-forward primer: 5'- TACCATGGAAAATGGGGCAGCTG -3'.

-reverse primer: 5'- ATCTGCAGCTGAGCCGTATTAA. -3'. In the forward primer the CCATGG *NcoI* site was designed to keep JSRV SU coding sequence (which starts with ATG) in frame with the His-tag. In the reverse primer the CTGCAG *PstI* site was designed to keep JSRV SU in frame with the Stop codon encoded by the vector pIVEX2.4.

The PCR reaction was carried out in 2.5mM $MgCl_2$, 10mM Tris (pH 9.2)50mM KCl, 20mM $(NH_4)_2SO_4$, 10mM dNTPs (Roche), 10mM forward and reverse primers and 5 units of Taq polymerase (Promega) made up to 45µl with distilled water for each

tube. 5ng of the DNA template pJSRV$_{21}$ was used. A HyBaid gradient block was programmed to provide amplification cycles that consisted of 94°C for 1 min (1cycle), 94°C for 30 s, 67°C for 30s, 72°C for 45 s (30 cycles), and a 5 minute final extension at 72°C (1 cycle).

Ten µl of each PCR reaction was analysed by electrophoresis through a 2% agarose gel in 1X tris-borate-EDTA buffer in the presence of 0.5 µl g of ethidium bromide per ml. The length of the amplification product was of 826 bp. The remaining amplification product was loaded on a 2% agarose gel and electrophoresis was performed: the 826bp band was excised and DNA extracted with the QIAquick gel extraction kit (Roche). DNA concentrations were estimated by the use of a spectrophotometer. The SU PCR product was then inserted in pGEM-T Easy (Promega) vector to facilitate PCR cloning. Ligation reactions were set up at a molar ratios of approximately 3:1, 6:1, 9:1, of insert : vector. 50ng of pGEM-T Easy was used for each reaction. Ligation reactions were set up in a total reaction volume of 10µl. 5µl of 2X rapid ligation buffer (Promega) and 1µl of T4 DNA Ligase (3 Weiss units/ µl, Promega) were added for each reaction. Half of each ligation was used to transform competent *E.coli* JM109 (Promega). Transformants were selected by plating onto LB agar containing Ampicillin (50µg/ml), X-Gal, and IPTG. Colonies were picked and inoculated onto a fresh LB agar plate containing Ampicillin and into Universals containing 5ml LB/50µg/ml Amp and incubated overnight at 37°C. Plasmid mini-preps were prepared with QIAprep Miniprep kit (Qiagen). The presence of the insert of the right size was confirmed.

The insert SU fragment was excised from pGEM-T Easy using *Nco*I /*Pst* I (Promega) and it was subcloned into pIVEX2.4a cut with *Nco*I/*Pst*I (Promega) restriction enzymes (Roche). The ligation was performed with the rapid DNA ligation kit (Roche) at +4°C overnight. Half of each ligation was used to transform competent *E.coli* JM109 (Promega). Transformants were selected by plating onto LB agar containing Ampicillin (150μg/ml). Colonies were picked and inoculated onto a fresh Amp plate and into Universals containing 5ml LB/50μg/ml Amp and incubated overnight at 37°C. Plasmid mini-preps were prepared with QIAprep Miniprep kit and checked for the presence of the insert (Qiagen). The correct DNA sequence for SU was confirmed by sequencing the SU insert still present in the plasmid using an automated sequencer (MRI, Functional Genomics Unit).

2.2.2.7 Cell-free production of JSRV-SU.

The expression of JSRV SU in the RTS system followed the same production and optimisation scheme adopted for JSRV CA. During this work, the manufacturer of the RTS system produced a new system for higher yield of protein. This new system called RTS-500-HY (Roche) was also investigated.

2.2.2.8 JSRV RTS SU purification

RTS-JSRV SU was purified using Pro-bond nickel beads essentially as described for CA (section 2.2.2.4) using native and denaturing methods.

The RTS reaction optimisation for SU took advantage of the optimisation results already achieved for CA.

The samples kept from the different steps of the purification were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), stained with Coomassie blue and by Western-blotting.

The band of about 40 kDa that was assumed to be JSRV RTS SU and an upper band of around 50 kDa also present in the first eluate were excised from a SDS-PAGE and analysed by MALDI-TOF (Matrix assisted Laser Desorption Ionisation Time Of Flight) and submitted to database searches (MS-Fit Search). MALDI-TOF mass spectrometry is a highly sensitive technique (femtamole level) that can provide information on the molecular weight, structure, N and C terminal sequence and post-translational modifications of proteins.

2.2.2.9 Analysis of JSRV RTS SU

The 1ml reaction product was analysed for the presence of recombinant SU by SDS-PAGE gel stained with Coomassie blue and by immunoblot (Appendix IV). The concentrations of the proteins in the first eluate were measured by the BCA protein quantification method (Pierce method, see Appendix IX).

The Western-Blot was performed using a rabbit anti JSRV SU immune serum JS107 (Dewar *et al.,* unpublished data) and 3 different rabbit polyclonal anti JSRV SU immune sera obtained using synthetic peptides as immunogens (gift from Massimo Palmarini).

The JS107 serum was prepared in rabbits by inoculation of recombinant GST-SU and β-Gal-SU embedded in SDS-PAGE gel (Dewar & Sharp, unpublished data).

These proteins were totally insoluble and not quantified before producing the immune serum. Before use JS107 immune sera was absorbed over night with a lysate of *E.coli* JM109 cells that were previously transformed using the plasmid DNA of pIVEX2.4 without the SU DNA insert.

At the time this work was done no recombinant JSRV SU protein was available to use as a positive control in the Coomassie or in the Western blot.

2.2.3 Results

2.2.3.1 Production of JSRV RTS CA

PCR resulted in the production of a DNA fragment of the predictable size of 660 bp. The amplified JSRV CA DNA fragment was cloned into plasmid pT7blue. A clone was selected and the presence of the 660 bp insert was visualised after restriction endonuclease digestion on 1% agarose gel by electrophoresis.

The JSRV CA DNA fragment was then subcloned in pIVEX2.4a.

The presence of inserted CA-DNA in pIVEX2.4a after digestion with *Sac*I/*Sma*I was checked by gel electrophoresis. 24 colonies were picked and checked by gel electrophoresis, 8 colonies that were picked had an insert of the predicted size (Fig.2.4). One clone was selected for further studies. The nucleotide sequence for CA inserted in pIVEX2.4a confirmed that CA was in the right frame, orientation and corresponded to the expected sequence. Initially it was checked if it was possible to obtain RTS JSRV CA following the manufacturer's recommended protocol for the native method. The instrument was set at 30° for 24 h at 120 rpm and the 1ml reaction product was purified following the denaturing and native methods. A band of the expected size of 30 kDa was observed.

Very little JSRV RTS CA protein was observed in the first and second elutions following the native purification (Fig 2.5). The observation of the Coomassie stained SDS-PAGE gel(Fig. 2.5) revealed that JSRV RTS CA was not eluted from the beads. Infact lanes 5 and 6, first and second elution respectively, did not contain the CA 30 KDa band. Only a very weak line of 30 kDa in lane 7 could be observed. Analysis of the nickel beads at the end of native method purification, showed a thick 30 kDa band indicating that the protein was still bound to the beads. The presence of the 30kDa band in lane 2 showed that after one hour of incubation of the RTS reaction with the beads, protein is still free in the supernatant.

After purification of RTS JSRV CA with the denaturing method and staining of the SDS-PAGE gel with Coomassie, a clear band of 30 kDa was detected in lanes 5 and 6, related to the first and second elution (Fig. 2.6). A weak 30kDa line was observed in lane 7. In lane 8, the presence of the weaker 30 kDa band showed that still a small amount of JSRV RTS CA was bound to the beads even after the third elution. The presence of JSRV RTS CA in the different fractions was confirmed by Western blot using rabbit polyclonal anti CA antibodies (Fig 2.7).

Following these preliminary results it was decided to investigate whether the yield of JSRV RTS CA could be increased by altering the incubation times (30 minutes, 1, 2, 12 hours) of the beads with buffer pH 4.00 during the first elution step. The first eluate obtained from the 30 min , 1, 2 and 12 hours incubation time was analysed using SDS-PAGE gel (Fig 2.8). The bands related to JSRV RTS CA were evaluated after staining the gel with Coomassie Blue. One, 2 and 12 hours of incubation time (lanes 4,5,6, respectively) produced similar band densities. The band obtained after 30 minutes (lane 7) of incubation time was slightly less dense (Fig 2.8). The band that represented

the second elution after incubation for one hour (lane 3) was quite clean indicating that most of the JSRV RTS CA was collected in the first elution. These results were confirmed by Western blot, using rabbit polyclonal antibodies.

In all subsequent experiments the elution time was fixed at 1 hour. One hour incubation time allowed similar recovery of the protein to the longer incubation (2 and 12 hours) times and higher presence of the protein than lower incubation time (30 min.); in addition this incubation time allows to protein purification and quantification to be performed in one.

The optimisation of RTS JSRV CA yield proceeded according to the different parameters shown in Table 2.1. These results are summarised in Table 2.2 and in Figures 2.9a and 2.9b. The higher JSRV RTS CA yield was obtained using 15µg of DNA and setting the instrument at 30°C for 24 hours.

Table 2.2. JSRV RTS CA yield obtained using different RTS reaction conditions.

DNA	temperature	time	protein yield (mg/ml)
5µg	25°C	24h	0.2
	30°C	24h	0.4
15µg	25°C	24h	0.8
	30°C	24h	2.0

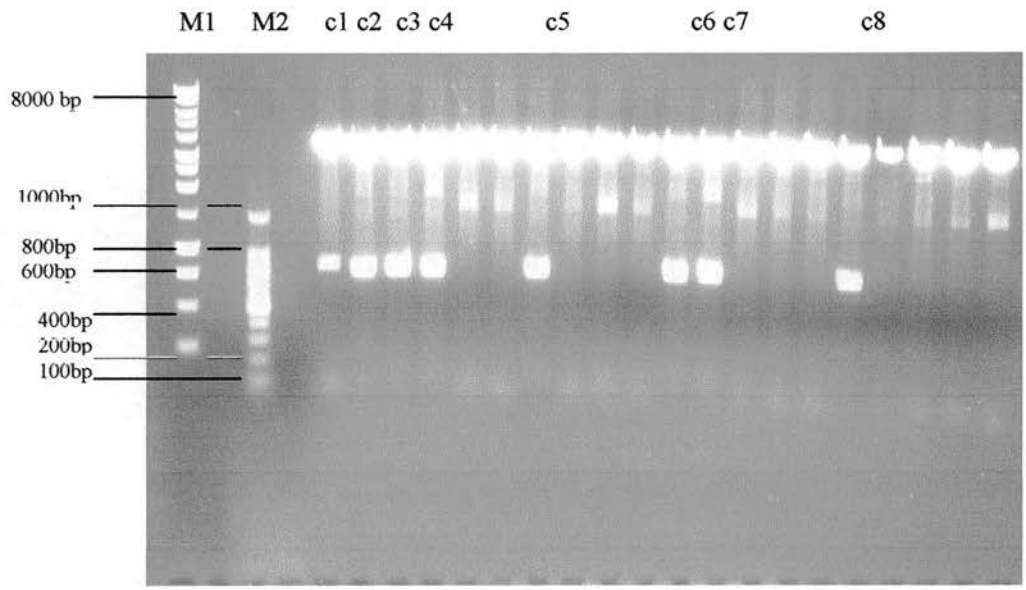


Fig. 2.4. Cloning JSRV CA.

Single colonies of E.*coli* JM109 transformed with pIVEX2.4a in which JSRV-CA was inserted.These were picked and grown in liquid broth overnight at 37°C; DNA was extracted and digested with *XhoI* and *SmaI* to check the presence of the insert by gel electrophoresis. The figure shows 8 colonies (c1 to c8) that contain an insert of the predicted size. M1= Hyperladder 1, range 200-10.000bp 1(Bioline); M2= Hyperladder IV, range 100-1000bp 1(Bioline).

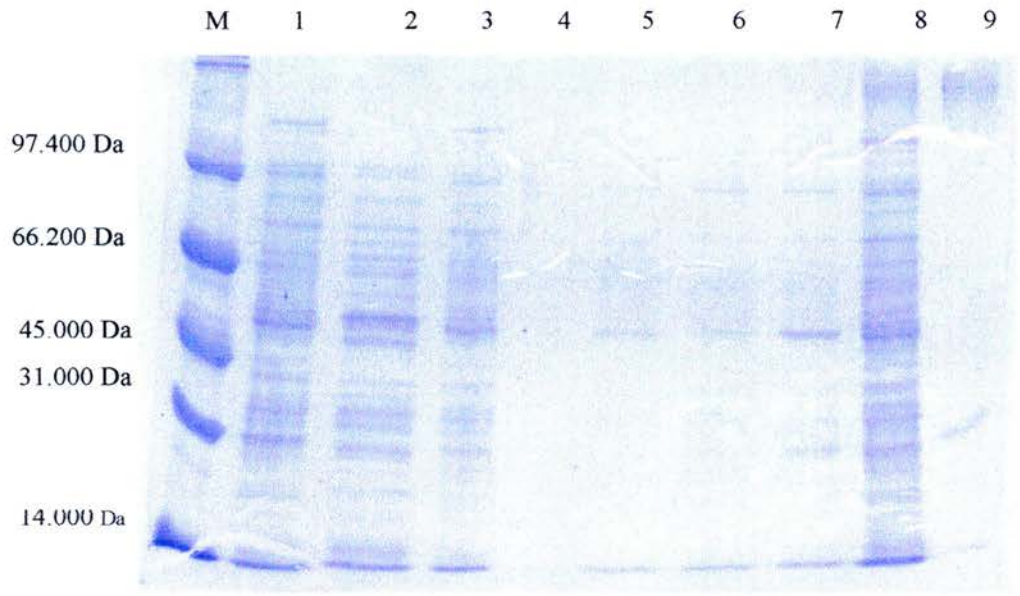


Fig 2.5. Purification of RTS-JSRV CA by native method.

Samples from different steps of the purification were analysed by SDS-PAGE gel stained with Coomassie blue.

M: molecular weight standard, low range (Biorad)

1: RTS reaction fluid before purification

2: analysis of supernatant after 1 hour incubation of the RTS reaction with the nickel resin (pH 7.6).

3: analysis of the nickel resin after 1 hour incubation of the RTS reaction with the nickel resin

4: analysis of the first wash collected after washing the nickel resin with native buffer pH 7.6

5: first eluate (elution buffer pH 4.00; elution time 1 hour)

6: second eluate (elution buffer pH 4.00; elution time 30 min.)

7: third eluate (elution buffer pH 4.00; elution time 30 min.)

8: analysis of nickel resin after the third elution

9: pBad-HisCA (+ve control)

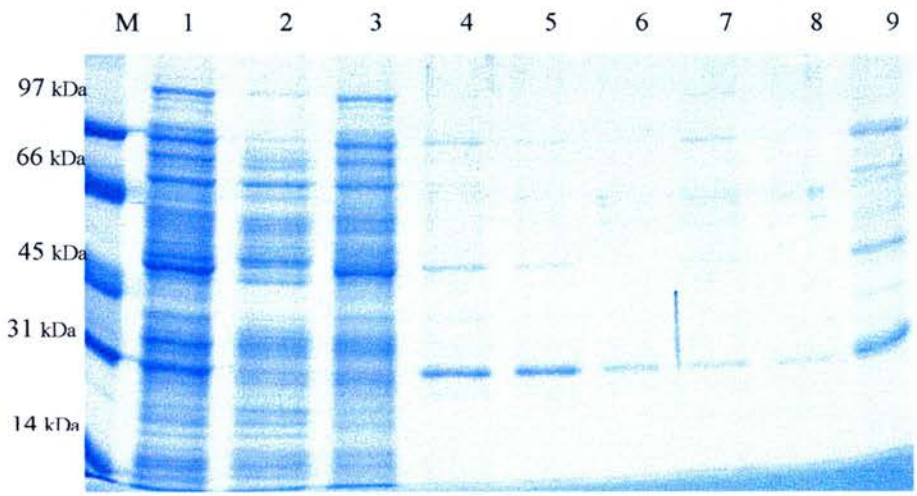


Fig 2.6. Purification of RTS-JSRV CA by denaturing method.

Samples from different steps of the purification were analysed by SDS-PAGE gel stained with Coomassie blue.

M: molecular weight standard, low range (Biorad)

1: RTS reaction fluid before purification

2: analysis of supernatant after 1 hour incubation of the RTS reaction with the nickel resin (pH 7.8).

3: analysis of the nickel resin after 1 hour incubation of the RTS reaction with the nickel resin

4: analysis of the first wash collected after washing the nickel resin with buffer pH 7.8.

5: first eluate (elution time, 1 hour)

6: second eluate (elution time, 30min.)

7: third eluate (elution time, 30min.)

8: analysis of nickel resin after the third elution

9: pBad-HisCA (+ve control)

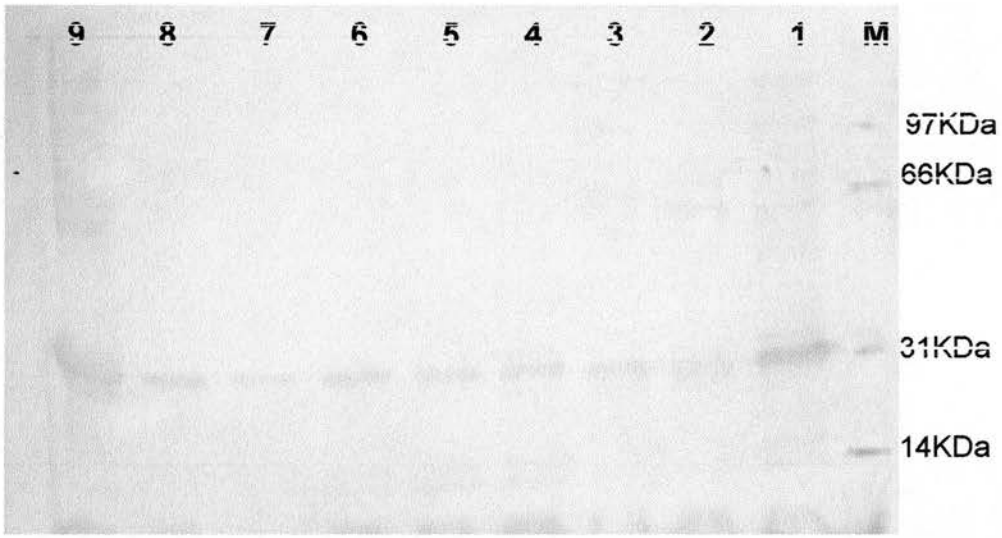


Fig. 2.7. Western blot of CA purified by " Pro-Bond denaturing method". The presence of JSRV RTS CA in the different phases of the purification was confirmed by Western-blot using rabbit polyclonal anti-CA antibodies

M: molecular weight standard, low range (Biorad)

1: RTS reaction fluid before purification

2: analysis of supernatant after 1hour incubation of the RTS reaction with the nickel resin (pH 7.8).

3: analysis of the nickel resin after 1hour incubation of the RTS reaction with the nickel resin

4: analysis of the first wash collected after washing the nickel resin with buffer pH 7.8.

5: first eluate (elution time: 1 hour)

6: second eluate (elution time: 30 min.)

7: third eluate (elution time: 30 min.)

8: analysis of nickel resin after the third elution

9: pBad-HisCA (+ve control)

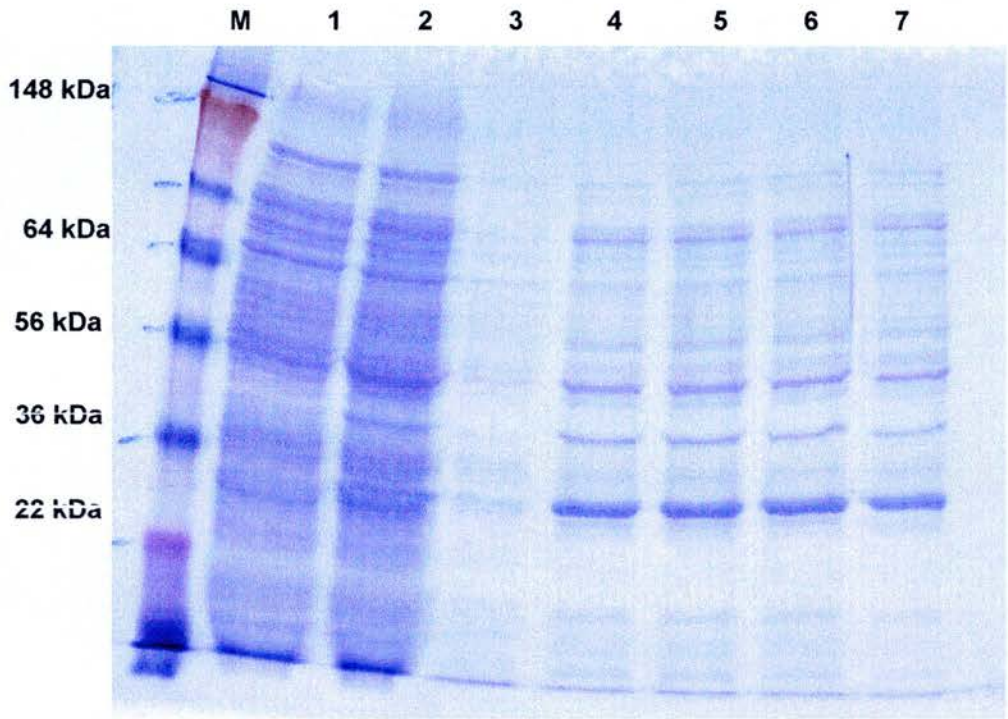


Fig 2.8. Optimisation of the denaturing method for the purification of RTS JSRV CA.

The first elution step was optimised using different incubation times of the elution buffer pH 4.00 with the nickel beads. The first elution from each incubation time was analysed by SDS-PAGE gel stained with Coomassie.

M: Pre-stained Protein standard (Invitrogen)

4,5,6,7: first eluate after incubation of the elution buffer ph 4.00 with the beads, for 12 h, 2 h, 1 h and 30 min respectively.

3: second elution from the sample that was incubated for one hour

2: nickel beads after 1 hour incubation of the RTS reaction with the nickel resin

1: supernatant after 1 hour incubation of the RTS reaction with the nickel resin (pH 7.8).

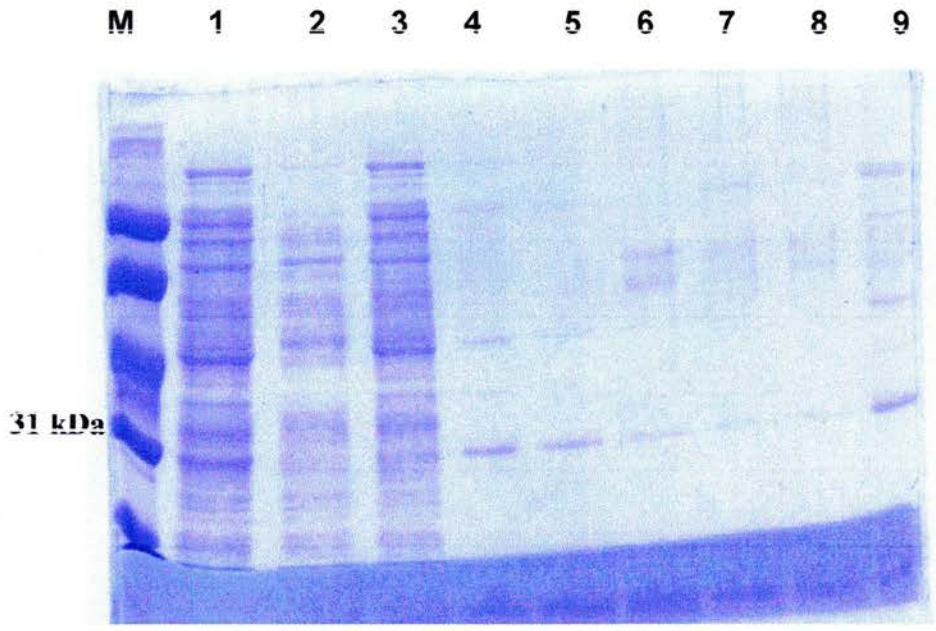


Fig. 2.9a. Optimisation of CA DNA amount and temperature in the RTS reaction.

5µl of DNA was included in the reaction and JSRV RTS CA protein was purified by denaturing method. This amount of DNA was used for two different RTS reactions: in the first one the temperature was 25°C and in the second one was 30°C.

The first eluate from each reaction was analysed by SDS-PAGE gel stained with Coomassie

M: molecular weight standard, low range (Biorad)

1: RTS reaction fluid before purification (5µl of DNA, 30°C)

2: supernatant after 1 hour incubation of the RTS reaction with the nickel resin (pH 7.8), (5µl of DNA, 30°C)

3: nickel resin after 1 hour incubation of the RTS reaction with the nickel resin (5µl of DNA, 30°C)

4,5,6: first, second and third eluate JSRV RTS CA (5µl of DNA, 30°C)

7,8: first and second eluate JSRV RTS CA (5µl of DNA, 25°C)

9: pBad-HisCA (+ve control)

The estimated total amount of CA protein in the first elution was about 2mg/ml (BCA, Pierce method).

2.2.3.2 Production of JSRV RTS SU

The JSRV SU DNA excised from pGEM-T Easy using *Nco*I /*Pst* I (Promega) was successfully subcloned into pIVEX2.4a cut with *NcoI/PstI* (Promega) restriction enzymes (Fig. 2.10 and 2.11).

The nucleotide sequence for SU inserted in pIVEX2.4a confirmed that SU was in the right frame, orientation and corresponded to the expected sequence.

pIVEX2.4a-SU DNA was then ready to be included in the RTS reaction.

According to the results from the optimisation of the production of CA, 15μg of pIVEX 2.4a-SU DNA was used, the reaction time was 24 hours and the temperature employed was 30°C. The 1 ml reaction collected after 24 hours was cloudy and flake shaped whitish formations were sticking to the semi-permeable membrane. It was not possible to recover any protein from this 1 ml reaction. It was attempted to open the RTS device and gently recover the flakes. This operation was hampered by the impossibility of opening the device without breaking the semi-permeable membrane.

Decreasing the reaction temperature to 28°C, as suggested in the manufacturer's protocol in case of insoluble proteins, avoided the formation of the flakey material; this temperature was employed for subsequent experiments to express JSRV-SU.

Native and denaturing methods were investigated for purifying the protein.

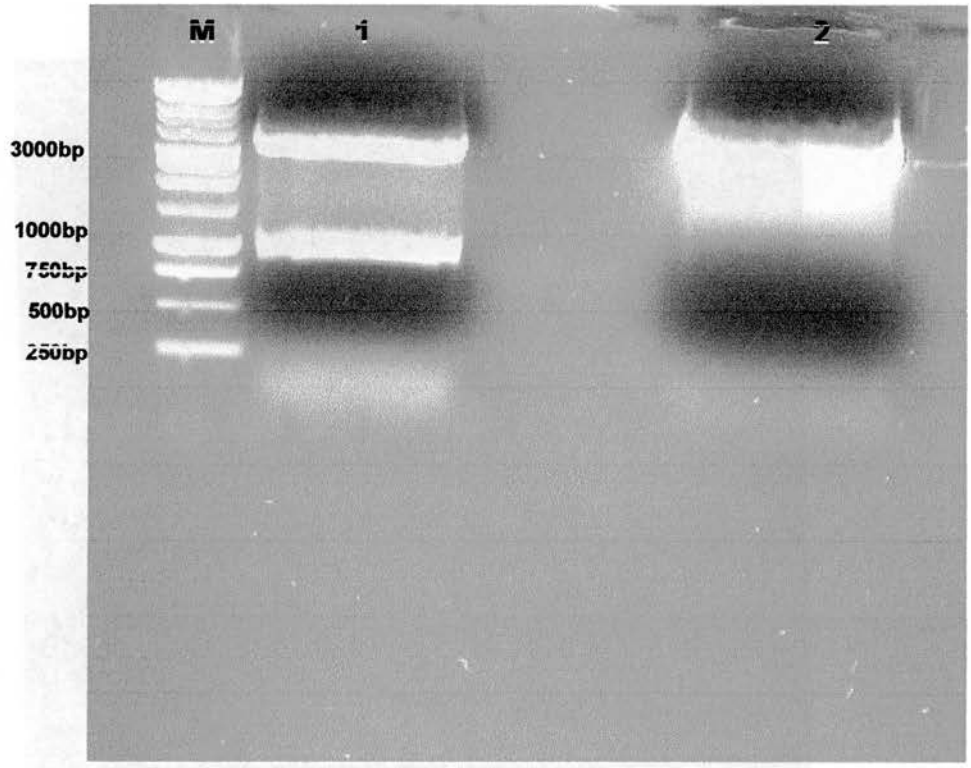


Fig 2.10. Cloning JSRV SU. Analysis by gel electrophoresis of SU DNA previously excised from pGEM-T Easy (*NcoI/PstI*) (lane 1, SU is 826 bp) and then inserted in the plasmid pIVEX2.4a(*NcoI/PstI*) .In lane 2 the DNA band is referred to pIVEX2.4a ready for the ligation reaction with SU DNA. M1=1Kb DNA ladder (bp range: 250-10.000)(Promega).

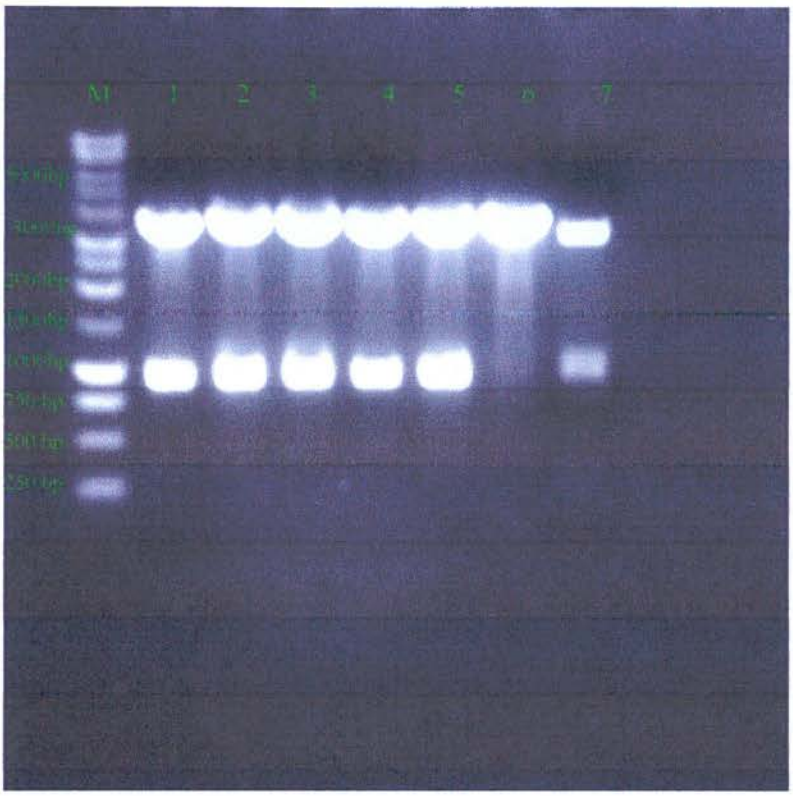


Fig. 2.11. Cloning JSRV SU. Gel electrophoresis of pIVEX2.4a (PstI/NcoI) DNA. JSRV DNA insert was excised from pGEM-T Easy and inserted in pIVEX2.4a

M1: 1Kb DNA ladder (bp range: 250-10.000)(Promega).

1-5 : pIVEX2.4a (PstI/NcoI) that contains SU insert DNA (826 bp band)

7: pGEM-T Easy (*NcoI/PstI*).

The different steps of the purification were analysed by SDS-PAGE stained with Coomassie and by Western Blot. The predicted size of JSRV RTS SU was 43.4 kDa but no bands of this size were obtained using the native method.

By the denaturing method of purification, a band of the expected size was observed (Fig. 2.12); and its identity was confirmed by Western Blot using JS107 serum (Fig. 2.13). The band containing the supposed JSRV RTS SU protein was excised from a SDS-PAGE gel, digested with proteolytic enzymes and then analysed by MALDI-TOF at the Moredun Functional Genomics Unit.

The MALDI-TOF application, followed by database search, confirmed that the 43 kDa band fitted the predicted sequence of JSRV SU protein (Appendix XII, Fig.XII.1 A & B).

The first eluate of RTS JSRV SU analysed by Coomassie (fig. 2.12, lane 5) contained not only the band related to JSRV RTS SU but another main band of about 56 kDa. By MALDI-TOF analysis the 56 kDa band was shown to be *E.coli* elongation factor TU (EF-TU).

The final SU concentration for 1ml RTS-500 reaction in the first eluate was about 0.1mg/ml. SU was produced with an advanced cell-free system called RTS-500-HY (Roche) and the final concentration measured with BCA method was 0.18mg/ml.

2.2.4 Discussion

CA was previously produced as GST-CA and β-gal-CA (Palmarini *et al.,* 1995) and His-CA (Summers *et al.*,2002). The final concentration of β-gal-CA was about 9mg/litre of transformed *E.coli* culture. The production and purification of GST-CA

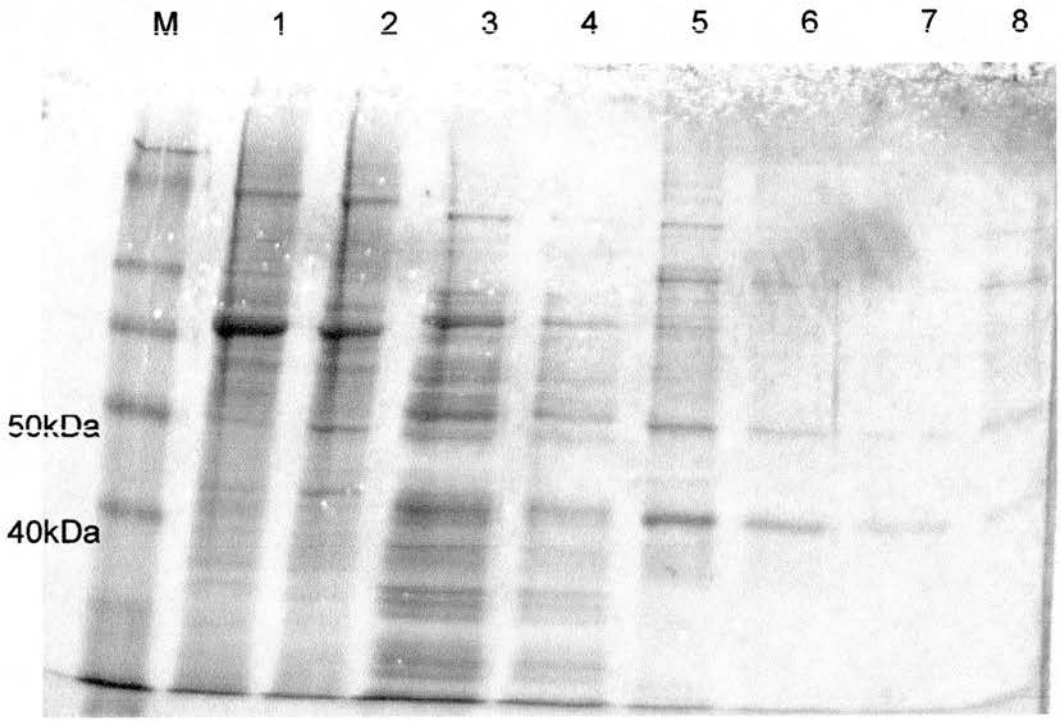


Fig 2.12. Purification of RTS-JSRV SU by denaturing method.

Samples from different steps of the purification were analysed by SDS-PAGE gel stained with Coomassie blue.

M: molecular weight standard (Invitrogen)

1: RTS reaction fluid before purification.

2: supernatant after 1 hour incubation of the RTS reaction with the nickel resin (pH 7.8).

3: nickel resin after 1 hour incubation of the RTS reaction with the nickel resin

4: first wash collected after washing the nickel resin with buffer pH 7.8.

5: first eluate. 6: second eluate .

7: third eluate.

8: analysis of nickel resin after the third elution.

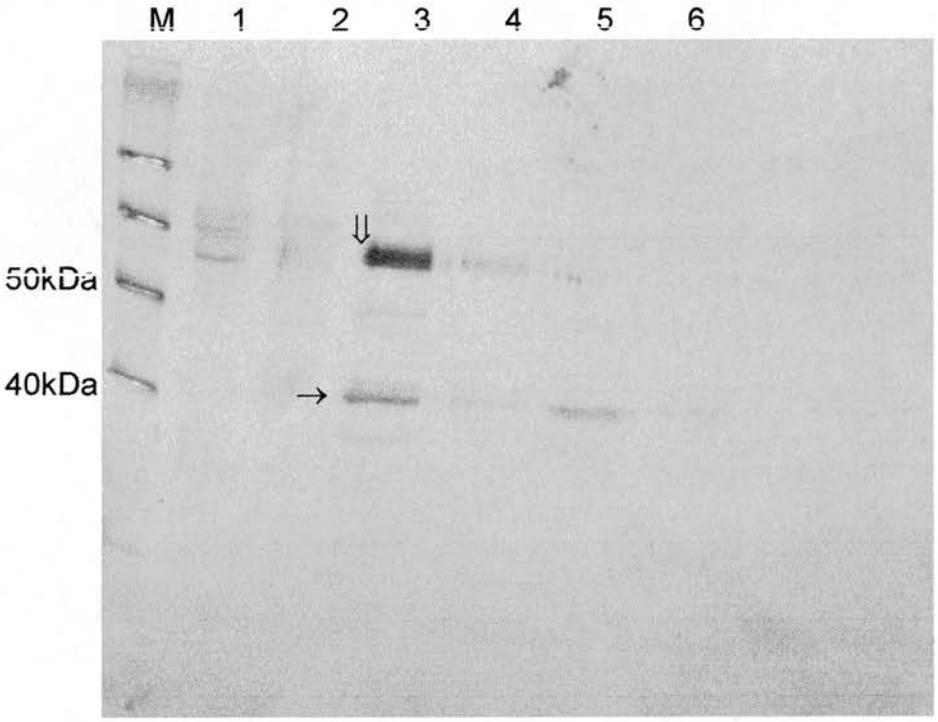


Fig. 2.13. Western blot of JSRV RTS SU purified by " Pro-Bond denaturing method".

After SDS-PAGE gel (Fig 2.12) and electronic transfer to a membrane. RTS JSRV SU was detected using polyclonal anti-SU antibodies (JS107 immune serum).

M: molecular weight standard (Invitrogen).

1: RTS reaction fluid before purification.

2: analysis of supernatant after 1hour incubation of the RTS reaction with the nickel resin (pH 7.8).

3: first eluate. JSRV RTS SU has an estimated molecular weight of 43kDa (→); the band of 56kDa belongs to E.coli elongation factor(⇓).

4: second eluate.

5: third eluate.

6: analysis of nickel resin after the third elution.

and β-gal-CA was prolonged and the final product was heavily contaminated by other *E. coli* proteins and the protein yield was variable. In contrast the JSRV RTS CA appeared much purer than these other recombinant proteins [Fig. 2.6, lane 6 (JSRV RTS CA) and lane 9 (pBad-HisCa)].

Considering that the RTS reaction takes 24 hours and that the denaturing method for protein purification takes no more than one working day, the RTS system represents a convenient method in terms of time and protein yield.

Previous attempts to express JSRV SU as β-gal or GST JSRV SU in *E. coli* resulted in production of insoluble protein which could not be purified (P.Dewar & J.M. Sharp, personal communications). Moreover the *E. coli* system did not give a constant protein yield.

Also attempts to express JSRV SU in the yeast *Pichia pastoris* (A. Philbey, unpublished data) were unsuccessful. The yeast *Pichia pastoris* was selected as a eukaryotic expression system to facilitate post-translational modifications including glycosylation and thus enhance antigenicity (Stubbs *et al.,* 2001).

The present experiments have been able to overcome the previously difficulties, although with low yields.

The RTS JSRV SU yield (0.18 mg/ml) was much lower than the yield obtained for RTS JSRV CA (2 mg/ml). This may be explained by the fact that SU has a hydrophobic nature (York *et al.,* 1992) or by the size of the protein which can influence the performance of the RTS system (Endo & Sawasaki, 2003).

JSRV SU RTS (43kDa) is bigger than JSRV CA (30kDa). In fact high productivity with the RTS system can be expected with fairly small proteins such as Ras protein (21 kDa) or chloramphenicol acetyltransferase (26 kDa). The problem with larger

proteins is that with the increasing molecular weight of the mRNAs, their degradation by endogenous *E. coli* ribonuclease(s) also increases (Endo & Sawasaki, 2003).

For future vaccination plans it would be worth investigating the possibility of expressing SU protein in vectors that can overcome some of these problems, e.g. poxvirus vectors.

A number of features make poxvirus recombinants suitable as vaccines: (i) the stability of freeze-dried vaccine, low cost, ease of manufacture and administration (Pastoret & Vanderplasschen, 2003). (ii) the vaccine can be administrated by several routes (Gherardi & Esteban, 1999). In the case of vaccinia virus it has even been shown that the virus can be administrated per os, this feature has been used for vaccination of wild fox and racoons (Pastoret & Brochier, 1996). (iii) The ability to induce both antibody and cytotoxic T cell responses against foreign antigen with long lasting immunity after a single inoculation (Smith *et al.,* 1983a; Smith *et al.,* 1983b). (iv) The packing flexibility of the genome, which allows large amounts of the genome to be lost or deleted and foreign DNA to be inserted (at least 25 kb), thus enabling multivalent vaccines to be created (Perkus *et al.,* 1985). Poxvirus vectors, such as avipoxviruses, have proved to be extremely safe and efficacious non replicating vectors when used in non avian species (Lee *et al.,* 2004; Wright *et al.,* 2004). Recombinant canarypox vaccines expressing the entire envelope proteins of FeLV and HIV have been successfully used for vaccination trials (Cox *et al.,* 1993; Tartaglia *et al.,* 1993).

More expensive, transgenic animal technology appears to be a good alternative for producing complex glycosylated proteins (Loftus & Rogers, 1997).

Rabbit can be used to produce proteins and vaccines. Rabbit mammary gland has the ability to make the post-translational modifications necessary for the functionality of human proteins. The rabbit is therefore suitable for the production of complex glycosylated proteins. Transgenic rabbits can be generated by micro-injection method, where the expression vector (gene constructs allowing the expression of the protein of interest under the control of a milk protein promoter) is directly injected into the pronucleus of a single-cell fertilized ovocyte. The transgenic embryos are then transferred to recipient female rabbits (Gibbs *et al.,* 1979). Several species of transgenic animals such as sheep, goat and cattle have been produced for production of physiologically active proteins in milk. This system is expensive but it could be approachable when high yield of protein is necessary and in the case of extensive vaccination plans.

At the moment cultivated mammalian cells are the dominant system for the production of recombinant proteins for clinical applications because of their capacity for proper protein folding, assembly and post-translational modification. Thus, the quality and efficacy of a protein can be superior when expressed in mammalian cells versus other hosts such as bacteria, plants and yeast. Recently, the productivity of mammalian cells cultivated in bioreactors has reached the gram per liter range in a number of cases, a more than 100-fold yield improvement over titers seen for similar processes in the mid-1980s (Wurm, 2004).

2.3 Production of SU immune serum in rabbit

2.3.1 Materials and methods

A specific JSRV-SU antiserum was prepared by immunising two rabbits with 500μg JSRV RTS SU combined with Freund's incomplete adjuvant. After 15 days, the rabbit was boosted with 500μg of GST-SU bound to the glutathione-sepharose beads (gift from P. Dewar, Moredun Research Institute).

A third injection of JSRV RTS SU was given after 4 weeks and the rabbit was bled 15 days after this last injection. The sera reactivity was tested by Western Blot against GST-SU blotted on a nitrocellulose membrane. Antiserum against GST-SU (JS107)(P. Dewar, unpublished data) was used as a positive control.

2.3.2 Results

The serum from one of the 2 immunised rabbits recognised the recombinant protein GST-SU (55 kDa) in Western blot analysis as shown in lane 2 (Fig. 2.14, Western Blot b). The immuneserum JS107 against JSRV SU used as a positive control produced a strong band (Fig. 2.14, Western Blot b, lane 1). The other rabbit serum was tested but no reactivity could be demonstrated.

The anti JSRV SU immune serum produced using JSRV RTS SU was used for the IHC studies described in chapter 3.

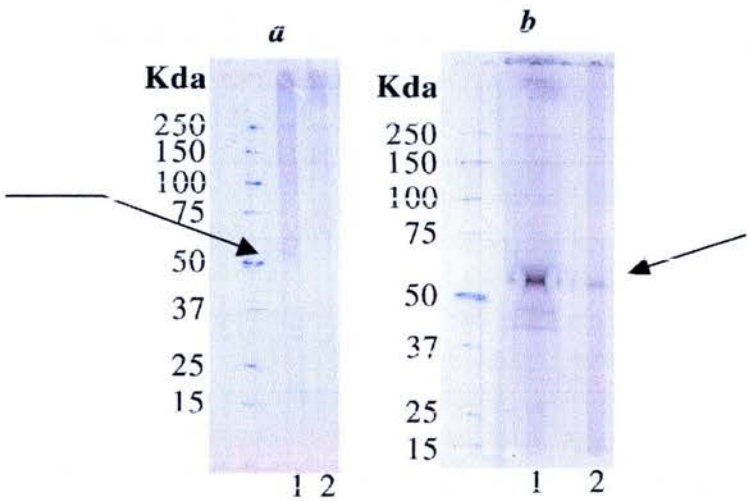


Fig. 2.14. Reactivity of antisera to RTS JSRV produced in sheep (a) and rabbit (b).

Western Blot a : Antibodies production in sheep was checked testing the sheep sera against GST-SU (55kDa) blotted on a nitrocellulose membrane. After blotting the nitrocellulose membrane was inserted in a multi-chamber apparatus in which it is possible to use different antibodies for different lanes of the same blot.

Lane 1(+ve control): GST-SU was revealed using JS107 serum as primary antibody and goat anti-rabbit IgG serum conjugated with horseradish peroxidase as secondary antibody (Sigma).

Lane 2: the serum from one of the two sheep was tested against GST-SU. Donkey anti-sheep IgG serum conjugated with horseradish peroxidase was used as secondary antibody (Sigma).

Western Blot b : Antibodies production in rabbit was checked testing the rabbit sera against GST-SU blotted on a nitrocellulose membrane.

Lane 1: GST-SU was revealed using JS107 serum (+ve control)

Lane 2: the serum from one of the two rabbit was tested against GST-SU.

2.4 Immunisation of viraemic and non viraemic animals with JSRV CA recombinant proteins

2.4.1 Rationale of the experiment

In OPA-affected animals there is no evidence of circulating JSRV specific antibodies and the reduced response to ConA stimulation indicates an alteration in systemic immunity. However, antibodies against JSRV can be raised by immunisation of sheep from flocks with no history of OPA with recombinant JSRV CA; indicating that sheep are not inherently incapable of recognising JSRV antigens and mounting an immune response. Therefore, the failure to respond to JSRV infection may be the result from infection by JSRV.

To test this hypothesis, JSRV infected sheep and JSRV non infected sheep were immunised with JSRV CA to determine if JSRV infected sheep were able to produce antibodies to recombinant JSRV proteins.

2.4.2 Materials and methods

2.4.2.1 JSRV recombinant proteins

The JSRV CA proteins used in this study were: β-Gal CA, GST-CA and RTS- JSRV CA. β-Gal CA and GST-CA were prepared and purified as described in Appendix VI. RTS JSRV CA was prepared and purified as previously described (section 2.2.2).

For each different recombinant protein a total of 15 mg of protein was prepared.

Each protein production batch was checked by Coomassie and Western blot.

β-Gal CA and GST-CA were inoculated into the sheep bound to the beads as described previously (Palmarini *et al.*, 1995).

2.4.2.2 Experimental plan

The JSRV status of the sheep in each group was confirmed by at least 3 blood tests at 3 month intervals. The test used was the JSRV U3-PCR test for detecting JSRV provirus in genomic DNA.

The JSRV positive animals were obtained from a flock with history of OPA in which the longitudinal epidemiological survey for JSRV infection (described in Chapter 4) was carried out. They were randomly selected from a group of approximately 60 sheep which were JSRV positive but clinically normal (i.e. had no signs of OPA and were not pregnant) (Table 2.3). The five sheep were brought to Moredun Research Institute animal facilities from the farm, at least one week before the start of the experiment to allow acclimatisation. In this JSRV viraemic group, three animals were 3 years old and British milksheep x Suffolk cross-breed; one animal was 4 years old and Milksheep x Texel cross-breed; the other was 3 years old and Suffolk x Texel cross-breed.

The JSRV negative sheep in groups 2 and 3 (group 2, n=5; group 3, n=5) were from the Moredun flock and were negative by JSRV U3 PCR test . The non viraemic animals were all one year old and Blackface breed.

The experimental design and immunisation regimes are summarised in Table 2.3.

Each group of sheep was subject to the same immunisation regime with recombinant CA (GST:CA fusion protein, Bgal:CA fusion protein or RTS JSRV CA) and ovalbumin (Sigma). Each sheep was immunised subcutaneously in areas of loose skin with 1 mg of each protein antigen and 100μg of ovalbumin.

For groups 1 and 2, the first inoculation was in 25% FCA formulated in a double emulsion with Tween 80. Subsequent inoculations used incomplete Freund's adjuvant. The immunisation regime took advantage of the different forms of CA so that responses were boosted to CA and not to the fusion partner of the recombinant proteins.

Sheep in group 3 were immunised using Quil-A adjuvant (5mg/ml) in order to compare the response with that of the animals in group 2.

The animals were inoculated once a month for 3 months with each different fusion protein in the following order: β-Gal CA, GST-CA, RTS JSRV CA.

The group of viraemic animals were kept separated from the two groups of non viraemic animals until the end of the experiment.

Blood was collected into EDTA vacutainers for JSRV U3 PCR tests from group 1, 2 and 3 sheep before starting the experiment as described and then once a month after each immunisation.

All of the animals were bled before and after each immunisation. The sera were examined by Western Blot for the presence of antibodies to JSRV CA and to ovalbumin. At the end of the experiment, each animal was bled.

Necropsy and histopathological investigation were carried out following standard techniques. Pieces of lung tissue were frozen in liquid nitrogen and then kept at –70°C for further PCR analysis.

Table 2.3. Immunisation of JSRV positive and negative sheep: experimental design.

GROUP	Treatment	Frequency of treatment
1 (JSRV+ve)	Inoculation of CA fusion protein and ovalbumin (in FCA)	Once a month (for 3 months)
2 (JSRV-ve)	Inoculation of CA fusion protein and ovalbumin (in FCA)	Once a month (for 3 months)
3 (JSRV-ve)	Inoculation of CA fusion protein and ovalbumin (in Quil-A)	Once a month (for 3 months)

2.4.2.3 Analysis of sheep serum

The Western blot method was used to analyse the humoral response to the fusion proteins. It was performed using the purified histidine tagged CA protein (Appendix IV). Briefly histidine tagged CA protein samples were transferred from SDS page gels on to nitrocellulose membranes (Bio-Rad) by electroblotting in Tris/glycine blot buffer, at a constant 100 V for 1 h. After staining with Ponceau red to confirm transfer of the separated proteins, the membrane was inserted in a multi-blotting apparatus (Bio-Rad). This apparatus creates compartments in the nitrocellulose membrane so that through different holes that are on the top lid, different sera can be tested. The sheep sera to test were diluted 1:50 in washing fluid B (WFB, 20 mM

NaCl, 20 mM Tris pH 7.5, 0.1 mM EDTA) and incubated for 1 hour at room temperature. After washing three times, rabbit anti-sheep horse radish peroxidase conjugate diluted 1: 1000 in WFB was added and incubated for a further hour at room temperature. After further washings, the reaction was visualised with diaminobenzidine (DAB, Sigma).

2.4.3 Results

One sheep in the viraemic group died after 2 weeks from the first protein inoculation due to causes unrelated to OPA.

In sera from all the groups analysed by Western Blot 30 days after the first inoculation, no antibodies to JSRV CA were detected. However, antibodies to JSRV CA were detected in sera analysed 30 days after the second and the third protein inoculation for all the animals included in the experiment (lanes 2 and 3, Fig 2.15). Antibodies to ovalbulmin were produced in all group of animals.

The JSRV negative animals remained PCR negative until the end of the experiment. The viraemic animals remained constantly JSRV U3 PCR positive during the whole experiment. At necropsy none of the viraemic animals nor the non viraemic animals had gross or microscopic evidence of OPA. Lung tissues from the viraemic animals were negative by PCR.

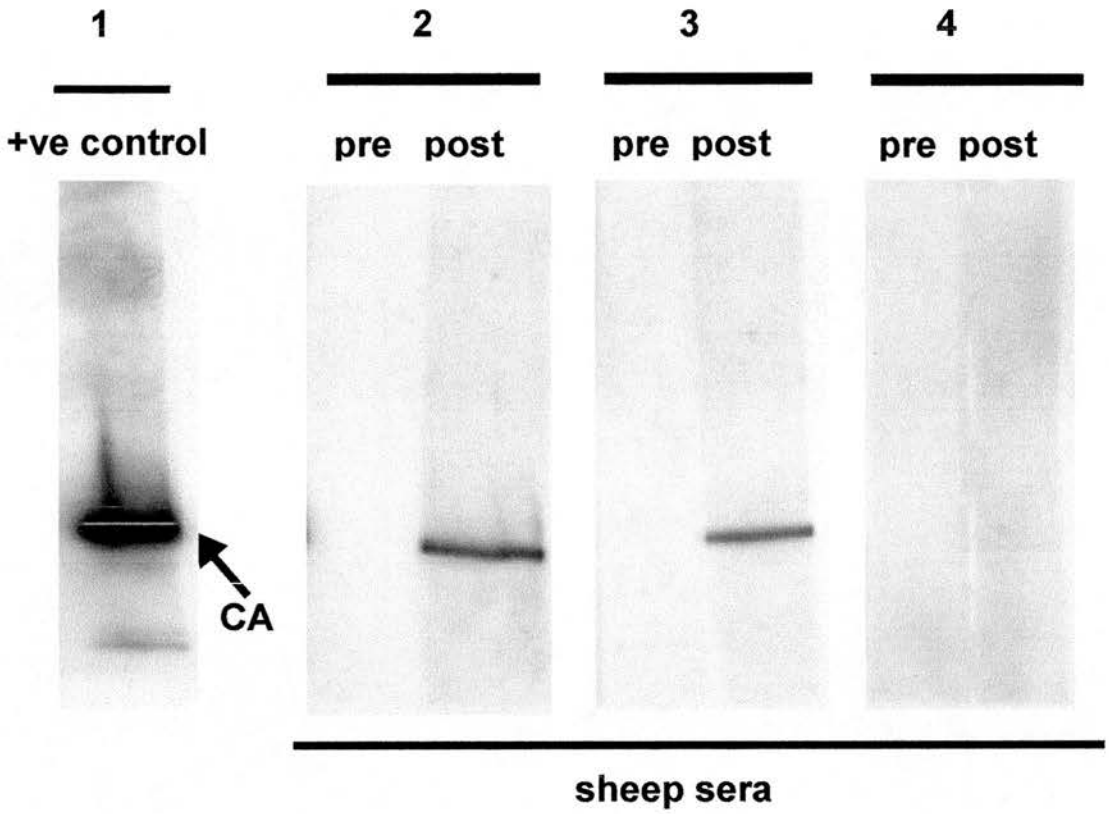


Fig 2.15. JSRV viraemic sheep can produce antibodies to JSRV capsid protein.

Antibody production in viraemic and non viraemic sheep was analysed by western blot, 30 days after the third immunisation with CA fusion proteins: Histidine tagged CA protein samples were transferred from SDS page gels on to nitrocellulose membranes.

In lane 1, the serum from a sheep previously immunised by Dewar *et al.*, (unpublished data) with GST-CA and β-gal-CA was tested (positive control); in lane 2 serum from a JSRV viraemic sheep was tested; in lane 3 and 4 sera were obtained from 2 different non JSRV viraemic animals. The non viraemic animal in lane 3 was immunised using as adjuvant CFA; the non viraemic animal in lane 4 was immunised using Quil-A. Rabbit anti-sheep horseradish peroxidase conjugate was used as secondary antibody.

2.5 Immunisation of OPA non viraemic sheep with recombinant JSRV SU protein

2.5.1 Rationale of the experiment

JSRV-SU interacts with the cellular receptor. JSRV envelope has been identified as a primary determinant of viral-induced transformation *in vitro* (Maeda *et al.,* 2001; Rai *et al.,* 2001, Hofacre & Fan, 2004). To further investigate the role of JSRV SU in OPA pathogenesis and the possibility of the tolerance, JSRV infected sheep were immunised with JSRV SU recombinant.

2.5.2 Materials and methods

2.5.2.1 JSRV SU recombinant proteins

The SU recombinant proteins used in this experiment were: the RTS JSRV SU produced as previously described and GST SU produced according to a previous protocol (Dewar & Sharp, unpublished data see Appendix VI.2).

GST-SU could not be eluted with free glutathione from the sepharose beads and therefore rabbits were immunised with the GST-SU coupled to the beads. GST-SU estimated concentration was 0.1mg/ml using the Pierce method.

2.5.2.2 Experimental plan

Before starting the immunisation with JSRV SU fusion protein, 2 JSRV negative sheep were selected according to the results obtained by JSRV U3 PCR test for the blood.

The JSRV status of the sheep in each group was confirmed in advance of this experiment by at least 3 blood tests at 3 month intervals. The two animals were from the Moredun flock, they were both Black-face breed and one year old. The sheep were immunised 3 times at one month intervals.

For the first inoculation 500μg of JSRV RTS SU per sheep was used; the second and third immunisation was with GST- SU and JSRV RTS SU respectively, following the same regime. Before inoculation the fusion proteins were combined with Freund's incomplete adjuvant. Blood was collected into EDTA tubes before starting the experiment as described and then once per month after each immunisation for JSRV U3 PCR tests. Clotted blood was taken from each sheep before and after each immunisation and examined for the presence of antibodies to JSRV SU by Western-blot. At the end of the fourth month all the animals were euthanased. Necropsy and histopathological investigation were carried out following standard techniques.

2.5.2.3 Analysis of sheep serum

Western blot was used to analyse the antibody response to the JSRV SU fusion proteins. It was performed on GST- SU protein (Appendix IV). Briefly GST- SU samples were transferred from SDS PAGE gels onto nitrocellulose membranes and Western Blot was performed following the same protocol described in section 2.3. The sheep sera to test were diluted 1:50 in WFB (WFB, 20 mM NaCl, 20 mM Tris pH 7.5, 0.1 mM EDTA).

2.5.3 Results

The 2 animals remained JSRV negative by PCR throughout the experiment.

No antibodies to JSRV SU production were detected in the sera collected at different times (Fig. 2.14). After necropsy the animal appeared perfectly normal.

2.6 Discussion

CA antibodies were produced in all of the JSRV viraemic and non-viraemic animals. The result in non viraemic sheep confirms the data obtained 10 years ago by Sharp & Dewar (unpublished data). In fact CA antibodies were obtained in non- viraemic and in different age- breed groups of sheep.

It is well established that sheep infected by JSRV do not develop detectable immune responses to this virus (Ortin *et al.,* 1998; .Summers *et al.,* 2002). This may be a consequence of central tolerance or may be due to peripheral tolerance.
Central tolerance concerns immature T or B cells as they differentiate in the primary lymphoid organs, the thymus or bone marrow (Ohashi, 2003; Venanzi *et al.,* 2004). Relevant antigens, then, would be those synthesized by nurturing stromal cells, circulating hematopoietic cells, or, ubiquitously, by all cells. The major mechanisms that come into play during central tolerance appear to be clonal deletion or inactivation of self-reactive lymphocytes, in particular the former. Peripheral tolerance (Walker & Abbas, 2002) on the other hand, relates to mature T or B cells after they have exited the primary lymphoid organs and are circulating through the blood, lymph, and secondary lymphoid organs or have accessed the parenchymal tissues in response to some stimulus. Antigens of concern would primarily be those

expressed in the tissues and not in the thymus or bone marrow. Clonal deletion and anergy are tolerance mechanisms also employed in the periphery, but a variety of other means are also exploited, including clonal ignorance, deviation, helplessness, and suppression (Mathis & Benoist, 2004).

It has been proposed that the failure to induce specific immunity during OPA is a consequence of the expression of endogenous retroviral sequences, closely related to JSRV, which can be expressed *in utero* (Palmarini & Fan, 2001b; Palmarini *et al.*, 2004), and may lead to central tolerance. Similarly, no JSRV-specific T cell responses in the blood have been found, although a reduced response to the mitogen Concanavalin A been observed in JSRV-inoculated lambs, prior to the onset of clinical disease, and in terminally ill sheep (Summers *et al.* 2002). Peripheral tolerance in OPA, perhaps could be induced by direct immunosuppressive effects of JSRV infection. Retroviruses may inhibit local immune responses through immunosuppressive domains contained within the Env proteins (Cianciolo *et al.*, 1980, 1985). Alternatively, the inability to detect systemic responses to JSRV may simply reflect the level of sensitivity of the JSRV-specific immunological assays that are currently available.

In this chapter anti JSRV CA antibodies could be induced in viraemic animals. This result would indicate that the ovine immune system is capable of recognising and responding to at least one of the JSRV proteins and specifically that the capacity of B cells to generate antibody responses to the virus is not impaired by infection with JSRV. On the other hand the findings do not permit a conclusion to be made about the responsiveness of virus-specific T cells, since help for antibody production could have been provided by T cells responding to the non-viral part of the fusion protein

Further studies using viral polypeptides on their own as immunogens are necessary to investigate the possible role of T cell tolerance.

The immune system in previous studies was approached by studying naturally infected adult sheep in the terminal stages of OPA and lambs infected with concentrated lung fluid so that the changes noted have been due to secondary infections, known to be common in OPA cases (Cutlip & Young , 1982; Garcia-Goti *et al.,* 2000) or to the high dose of the *inoculum*. In order to minimise any immunological effects caused by concurrent infections a recent study (Summers *et al.,* 2002 and 2004 submitted) used only lambs born and reared under SPF conditions, experimentally infected with JSRV. Summers *et al.,* (2004 unpublished results) found strong evidence for a local immune response during OPA mediated predominantly by infiltrating macrophages that produced high levels of IFN-γ. No significant lymphocyte influx was detected in the lungs of the experimentally infected lambs, as might be expected during viral infection or oncogenesis. The most striking observation was the presence of strongly positive MHC Class II cells, which were found within the tumours as well as in the alveolar spaces located within close proximity to the tumour nodules. On morphology alone, the MHC Class II-positive cells at these two locations appeared to be different and it was suggested that they were primarily fibroblasts, with a minority being macrophages. Based on these findings the authors suggest that a peripheral tolerance rather than a central tolerance should be considered as a mechanism contributing to the apparent lack of an immune response during JSRV infection and in animals with OPA.

It is not known what is the consequence of JSRV provirus in the lymphoid cells and mononuclear phagocytes. In previous studies it has been shown that in experimentally

infected lambs, JSRV proviral DNA can be detected in lymphoid cells as early as 7 days post infection when no histological signs of OPA are present (Holland *et al.*, 1999). This would indicate that infection of lymphoid cells precedes the neoplastic transformation. This conclusion has been supported by findings in naturally infected flocks that indicate that proviral DNA can be detected in the blood before the onset of the clinical signs (see chapter 4). The lack of antibody production after immunisation with recombinant JSRV SU protein could be due to the amount of protein that was injected (500μg each *inoculum*) or to tolerance.

The lack of antibody production after immunisation of JSRV non-infected sheep with recombinant JSRV SU proteins requires further studies. The lack of immune response could be due to the amount of protein that was injected (500μg each *inoculum*). Moreover, for supporting the tolerance theory it would be useful to repeat the immunisation in viraemic animals.

Chapter 3

Induction of ovine pulmonary adenocarcinoma (OPA) in lambs of different ages and detection of viraemia during the pre-clinical period

3.1 Introduction

Natural OPA has a long incubation period with most clinical cases occurring in 2-4 year old animals. However, in experimentally inoculated new born lambs, JSRV induces multifocal tumours in a few weeks (De las Heras *et al.*, 2003). Early transmission studies using tumour extracts were performed in sheep older than one year by several routes of parenteral inoculation or aerosol exposure, (Dungal, 1946; Wandera, 1968). In these experiments, clinical disease was seldom achieved, with incubation periods usually longer than one year and pathological changes consisting of confined small tumour nodules. In contrast, later experiments demonstrated that OPA could be induced much more rapidly and efficiently by intra-tracheal inoculation of newborn lambs with concentrated lung fluid or less efficiently with tumour extracts from OPA affected sheep (Verwoerd *et al.*, 1980; Sharp *et al.*, 1983; DeMartini *et al.*, 1987). Newborn lambs inoculated with lung fluid developed clinical signs characteristic of OPA in 3-6 weeks post infection (p.i.) or even earlier and histopathological confirmation was achieved in 70-100% of inoculated lambs. However, when a similar intra-tracheal challenge was conducted in 10 week-old lambs with tumour extract, only 25% of them developed scattered neoplastic nodules and none showed clinical disease after 8 months, suggesting a possible age effect. (Rosadio *et al.*, 1988). The neonatal lamb model has been very useful in determining the aetiological role of JSRV in OPA and some aspects of its pathogenesis. However, due to the very young age of the lambs, it is unsuitable to evaluate the efficacy of potential vaccine preparations and to study pathogenic mechanisms of JSRV infections occurring later in life. For example the long incubation period in natural OPA points to insertional activation of cellular proto-oncogenes as a mechanism of

oncogenesis (Rosenberg & Jolicoeur, 1997); on the other hand the rapid induction of the disease in experimentally inoculated neonatal lambs suggests that JSRV is an acutely transforming retrovirus (Sharp & DeMartini, 2003). The study of the pathogenic mechanisms of JSRV also has been hampered by the absence of detectable antibodies to JSRV in affected animals (Palmarini *et al.*, 1996b; Ortín *et al.*, 1998), a feature of OPA that also has compromised the pre-clinical diagnosis of the infection and the undertaking of epidemiological studies. More recently, JSRV-specific polymerase chain reaction (PCR) techniques have been used to identify infected sheep during clinical and subclinical stages of both natural and experimental OPA (García-Goti *et al.*, 2000; González *et al.*, 2001).

The main aims of this work therefore were:

1) to investigate whether JSRV infection, OPA lesions and clinical disease could be induced in older lambs (Age experiment, paragraph 3.3);

2) to determine the dynamics of the infection by PCR testing of peripheral blood samples. During this experiment DNA extraction from blood and high throughput PCRs assays were optimised. The techniques developed in this chapter (blood collection, DNA extraction, PCR tests, paragraph 3.2) will be used to study JSRV infection in natural OPA affected flocks. For this reason high throughput assays of clinical samples were needed to support the extensive molecular epidemiological survey that will be described in chapter 4.

3) a secondary aim was to determine the expression of JSRV SU in the lungs of inoculated lambs to better understand the interaction of JSRV with pulmonary epithelial cells in the neoplastic process (Age experiment, paragraph 3.3). *In vitro* studies recently demonstrated that envelope (Env) proteins of JSRV can transform

rodent and chicken fibroblasts in culture, suggesting a role for Env proteins in oncogenesis (Maeda *et al.*, 2001, Rai *et al.*, 2001; Allen *et al.,* 2002). From these studies a new mechanism of transformation for JSRV has been postulated, so that functional Env proteins can serve as oncogenes in addition to their primary function of mediating viral entry into cells (Alian *et al.,* 2000; Alberti *et al.,* 2002; Dirks *et al.,* 2002). Examination of JSRV genome revealed no apparent oncogene (York *et al.,* 1992), although *orf-x* had been suspected but later excluded (Palmarini *et al.,* 2001a).

The prelude to the experiments using animals was the development of methods to obtain DNA from ovine blood and to optimise the detection of JSRV in these samples by PCR (Section 3.2: description of Blood collection, DNA extraction and PCRs test).

The optimised procedures where then used as part of the subsequent experiment to investigate the the susceptibility to JSRV in animals infected at different ages (Section 3.3: induction of OPA in older lambs).

3.2 Blood collection, DNA extraction, PCR tests

3.2.1 Material and methods

3.2.1.1 Blood collection and DNA extraction

Blood collection and DNA extraction were first optimised on blood samples from the Moredun flock (Blackface sheep) and then applied to the samples from the experimental animals.

Two different approaches were considered for extracting DNA from the blood:

3.2.1.1a) <u>extraction of DNA from the whole blood;</u>

3.2.1.1b) extraction of DNA from PBLs (peripheral blood leukocytes).

3.2.1.1a) Extraction of DNA from the whole blood:

Ten ml blood samples were taken from 15 different animals in EDTA vacutainers. From each different animal blood sample, DNA was extracted by six different commercial kits following the manufacturer's protocols; 1)Flexigene (Qiagen), 2)Puregene (Gentra system,MN), 3)Genisol (ABgene), 4)DNAzol (BD), 5)Dneasy tissue kit for whole blood samples (QIAgen) and 6)Ultraclean (Flowgen) as shown in Table 3.1. Aliquots of fresh blood were taken from the 10 ml tubes and DNA was extracted on the same day. The rest of the blood was stored at -20°C for one week and then DNA was extracted (Steinberg *et al.*, 1997). Each DNA extraction kit was run sequentially.

Table 3.1. Different methods for extracting DNA were applied to individual blood samples following manufacturers' instructions.

Kit	Starting volume of whole blood	Number of samples tested
Flexigene	1ml	15 (fresh blood) 15 (frozen blood)
Puregene	600 μl	15 (fresh blood) 15 (frozen blood)
Genisol	1ml	15 (fresh blood) 15 (frozen blood)
Dnazol	0.5 ml	15 (fresh blood) 15 (frozen blood)
DNeasy	100 μl	15 (fresh blood) 15 (frozen blood)
Ultraclean	200 μl	15 (fresh blood) 15 (frozen blood)

The different protocols for DNA extraction are briefly reported in this chapter; more details are described in Appendix XI.

1) FlexiGene DNA Kits provide a method for purification of DNA from human whole blood (1 ml starting material, 30-35ng/μl expected DNA concentration, processing time 30 minutes). The procedure can be scaled up or down, allowing

purification from variable amounts of starting material. Purification is performed in a single tube, to minimise the risk of sample mix-up and to reduce waste. Lysis buffer is added to the sample. Cell nuclei and mitochondria are pelleted by centrifugation. The pellet is resuspended and incubated in denaturation buffer, which contains a chaotropic salt, and protease. This step should efficiently remove contaminants such as proteins. DNA is precipitated by addition of isopropanol, recovered by centrifugation, washed in 70% ethanol, dried, and resuspended in hydration buffer.

2) Using the Puregene kit the average DNA yield expected is 200 ng/µl and the processing time is 25 minutes. First, 600 μl whole blood are aliquoted into 2.0 ml microfuge tubes containing 900 μl Red blood cell lysis solution. The next steps are summarised in the diagram below:

RBC Lysis: lyse red blood cells

⇩

Cell Lysis: lyse nucleated cells

⇩

Protein precipitation:

Pellet proteins

⇩

DNA recovery:

Precipitate DNA

⇩

DNA hydration:

Rehydrate DNA

3) The Genisol kit is designed for the rapid isolation of high molecular weight DNA from a variety of mammalian sample types without the use of phenol or chloroform (typical expected yield up to 40ng per µl of blood, execution time ~20min).

No specialised plastic consumables or columns are required and all centrifugation steps can be performed in a low-speed centrifuge. The protocol is based on salting-out of protein contaminants using the Miller technique (1998). This method does not require chaotropic reagents (guanidine, urea, etc.)

The components of the kit are: Lysis Buffer, Digestion Buffer, RnaseA, Precipitation Buffer, DNA Buffer. All reagents were stable at room temperature and the quantity of the different solutions can be scaled up or down according to the amount of starting material. It was decided to start with 1 ml of whole blood. The method could not be carried out in microcentrifuge tubes.

4) DNAzol is a reagent specifically formulated for the isolation of genomic DNA from whole blood. The DNAzol procedure is based on the use of a guanidine-detergent lysing solution (DNAzol) which hydrolyzes RNA and allows the selective precipitation of DNA from the lysate. Blood samples are mixed with DNAzol and DNA is precipitated from the resulting lysate with isopropanol. The DNA pellet is washed successively with DNAzol BD and ethanol, and solubilized. The entire procedure can be completed in 30 minutes and the estimated DNA yield was 10 - 20 ng/µl. The protocol includes the following steps:

1. LYSIS: 1 ml DNAzol BD + 0.5 ml of whole blood.

2. DNA PRECIPITATION: lysate + 0.4 ml isopropanol at 6,000 g x 6 min.

3. DNA WASH: 0.5 ml DNAzol BD at 6,000 g x 5 min.; 1 ml 75% ethanol at 6,000 g x 5 min.

4. DNA SOLUBILISATION: 8 mM NaOH or water.

5) Dneasy tissue kit method for the whole blood This method can be used for whole blood, PBLs, fresh or frozen tissues.

DNeasy Tissue Kits are based on a silica-gel–membrane technology for purification of total cellular DNA without organic extraction or ethanol precipitation. The buffer system is optimised to allow direct cell lysis followed by selective binding of DNA to the DNeasy membrane. After lysis, the DNeasy procedure can be completed in about 20 minutes. By centrifugation, contaminants and enzyme inhibitors such as proteins and divalent cations are removed. Samples are first lysed using proteinase K. Buffering conditions are adjusted to provide optimal DNA-binding conditions and the lysate is loaded onto the DNeasy Mini spin column. During a brief centrifugation, DNA is selectively bound to the DNeasy membrane and contaminants pass through. Remaining contaminants and enzyme inhibitors are removed in two efficient wash steps and DNA is then eluted in water or buffer, ready for use.

This protocol is essentially the same for the different sources of DNA (cultured cells, tissues, whole blood), only the first steps differ according to the material from which DNA needs to be extracted.

6) Ultraclean: This kit has been conceived for isolating DNA from 200µl of whole blood using a spin filter method, with similar steps to the Dneasy Tissue kit (see appendix for details).

In general DNA was eluted or resuspended in 150µl of the appropriate solution.

3.2.1.1b) Extraction of DNA from PBLs.

In previous work, lymphocytes were obtained using Lymphoprep TM (Axis-Shield,PoC.As, Oslo) following the manufacturer's instructions (Palmarini *et al.,* 1996b). This method is labour intensive and requires multiple tube transfers that increase the risk of loss of DNA and unwanted contaminants of DNA material. It therefore is not practical for use in large scale experiments and/or epidemiological studies (Miller *et al.,* 1973; Gross-Bellard *et al.,* 1994; Hawkins *et al.,* 1994). In this study a method based on PBLs lysis was performed. The interval between blood collection and PBLs separation was 24 hours maximum during which the tubes were stored at ambient temperature. No reduction in DNA yields, as a result of keeping samples at ambient temperature for up to 3 days have been reported (Madisen *et al.,* 1987; Polakova *et al.,* 1989; Schunemann *et al.,* 2000).

Blood samples were obtained in EDTA vacutainers from the same 15 animals used for the previous study; from each animal 2 EDTA tubes were taken. Each vacutainer tube contained 10 ml of blood.

Blood tubes were centrifuged at 800g for 15 minutes. The buffy coat layer was removed with a 1ml Gilson pipette and transferred into a 1.7 ml Eppendorf tube containing 700µl of NH_4Cl solution (RBC Lysis Solution, Puregene, Gentra Systems, MN, USA), at room temperature for at least 5 minutes. The tubes were centrifuged for 1 minute and the pellet suspended in 1 ml PBS. The wash with PBS was repeated twice. The pellet obtained from each blood tube was divided in two: one sample was used fresh for extracting DNA and the other one was stored at -20°C for one week before DNA extraction. This buffy coat extraction method was

followed by DNA extraction using QIAgen Dneasy tissue kit (method for cultured animal cells). DNA was eluted in 150µl of the appropriate solution.

3.2.1.2 DNA concentration and DNA quality

DNA concentration was measured for all the samples obtained from the whole blood and from the PBLs extraction (details of methods in appendix III). All the DNA samples were stored at -20°C until further use. The quality of the DNA was tested with GAP-DH PCR (for method see appendix X). Each sample was tested in triplicate and was considered positive when at least one of the replicates was positive.

3.2.1.3 JSRV U3 PCR

A single-round PCR for JSRV detection was developed based on the primers PI and PIII used for the hemi-nested protocol reported previously (Palmarini *et al.,* 1996a, b). The new PCR was developed using as positive control the OPA cell line JS7 (Jassim *et al.,* 1987), which contains a single copy of JSRV provirus per cell (Bai *et al.*, 1999; Holland *et al.,* 1999; DeMartini *et al.,* 2001); DNA from Icelandic sheep PBLs was used as negative control.

Assuming that each JS7 cell contains one copy of JSRV provirus and that 100 ng of DNA correspond to about 14,000 cells, the sensitivity of JSRV U3 PCR was estimated using serial dilutions (10,000- 0.1 copies) of JS7 DNA in a background of 800 ng of DNA from Icelandic sheep.The optimised PCR conditions were as follows: 5µl of 10X PCR Buffer (Qiagen), 200µM dNTPs (Roche Diagnostics Ltd, East Sussex, UK), 200 nM of each primer (MWG, Milton Keynes, UK) and 1.25 U of Hot

Start Taq polymerase (Qiagen) were added to ultra-pure water (Sigma, Surrey,UK) to a final 50µl volume. To this pre-mix, 800 ng of test DNA were added and incubated at 94°C for 16 minutes, followed by 35 cycles at 94°C/30 seconds, 59°C/30 seconds and 72°C/30 seconds, with a final extension step of 5 minutes at 72°C. PCR products were visualized following electrophoresis in a 2% agarose gel containing 100µg-ml ethidium bromide. The test samples were analysed in triplicate and considered positive if one or more of the replicates gave a band of the correct molecular weight. To confirm the integrity of the DNA, samples that were negative in the JSRV-U3-PCR were tested for the house-keeping gene GAPDH transcripts (Palmarini *et al.*, 1996b).

The efficiency of JSRV-U3-PCR was first tested on blood from sheep with clinical evidence OPA, submitted to Moredun from Scottish farms (n=18), on blood samples from New Zealand (n=8) and from Iceland (n=50). DNA from Icelandic sheep was used as negative control in each batch of PCR reactions. The New Zealand sheep were recently imported to Great Britain. The Icelandic samples were shipped at different times by express courier. The minimum amount of DNA, from OPA clinical cases, to give a visible amplification product on 2% agarose gel was determined by testing 3 different concentration of DNA, 1000ng, 500ng and 300ng from five different OPA clinical cases confirmed positive after necropsy.

3.2.2 Results

3.2.2.1 Blood collection and DNA extraction

The six different commercial kits for extracting DNA from the whole blood were in general very easy to use. Microcentrifuge tubes could be used for all the kits with the exception of the Genisol kit.

When DNA extraction was performed using whole blood as starting material the best DNA yield was obtained using DNeasy and Ultraclean kits (Table 3.2).

The GAP-DH results from the same samples are summarised in Table 3.2. The higher number of positive samples were obtained with Puregene and DNeasy kits (7/15 and 8/15 respectively). Genisol method gave only one positive result out of 15, Dnazol gave 2 positive out of 15, Flexigen and Ultraclean 4 out of 15.

The GAP-DH results from frozen samples are summarised in Table 3.2. All the DNA samples obtained by Genisol gave negative results. 3 positive samples out of 15 were obtained with Puregene and Dnazol, respectively. Ultraclean gave 4 positive out of 15, Flexigene and Dneasy 5 out of 15.

DNA extraction from fresh and frozen PBLs with the Dneasy tissue kit gave a mean DNA yield of 133 and 155 ng/μl respectively. GAP-DH was positive in all the 15 fresh and frozen tested samples. The DNA samples extracted from PBMC with Dneasy tissue kit gave the highest number of positive replicates by GAP-DH PCR without any difference between fresh and frozen samples (15/15) (Table 3.2). Due to the consistency of results this extraction method from WBC was used for the remainder of the experiments.

Table 3.2 (a & b): Evalution of different methods for extracting DNA from fresh and frozen blood. DNA yield (ng/μl) in the final DNA solution, per ml of initial whole blood, was expressed as mean of 15 samples and the standard deviation (SD) and the standard error (SE) were calculated. DNeasy method was used to extract DNA from PBMC derived from 1 ml of blood, which contains approximately $7.8x10^5$ of PBMC. Data from frozen and fresh blood samples are compared using an unpaired t-test, assuming unequal variances. The results are summarised in the following tables:

a)

	Fresh blood				**Frozen blood**		
Kit	Mean DNA yield (ng/μl)	SD	GAPDH n. of +ve		Mean DNA yield (ng/μl)	SD	GAPDH n. of +ve
Flexigene	12.2	2	4/15		16	4.7	5/15
Puregene	37.1	3	7/15		38.6	3.3	3/15
Genisol	11.7	1.9	1/15		14.9	3.9	0/15
Dnazol	44.6	3.6	2/15		44.8	3.6	3/15
DNeasy	171	19	8/15		223	29	5/15
Ultraclean	100	15.5	4/15		136	10	4/15
DNeasy(*)	133	33.1	15/15		155	35.5	15/15

b)

Test	**Mean Difference (Frozen-Fresh)**	**SE(Difference)**	**t_{14}-statistic**	**p-value**
Flexigene	3.8	1.3	2.88	0.01
Puregene	1.5	1.2	1.30	0.21
Genisol	3.2	1.1	2.86	0.01
Dnazol	0.2	1.3	0.15	0.88
DNeasy	52	9.0	5.81	<0.001
Ultraclean	36	4.8	7.56	<0.001
DNeasy	22	12.5	1.76	0.10

3.2.2.2 JSRV U3 PCR

From the JSRV U3 PCR a product of 176 bp was expected. This JSRV U3 PCR successfully amplified between 5-10 copies of JSRV template integrated in genomic DNA from JS7 cells (Fig. 3.1). All 18 OPA clinical cases gave positive results when their blood was examined with this test. Negative results were obtained with the blood from the 8 New Zealand and 50 Icelandic sheep (Fig. 3.2). Three different amounts of DNA, 800, 500 and 300ng from five different OPA animals were tested in triplicate by the JSRV U3. The 300ng DNA concentration gave negative results in all the samples tested. The minimum amount of DNA necessary for giving the expected amplified product was 500 ng; 800ng of total DNA did not inhibit the PCR reaction on the contrary the amplified 176bp band appeared stronger in 2% agarose gel (Fig 3.3).

3.2.3 Conclusion

The kits for DNA extraction from the whole blood offered a number of advantages. The kits for extracting DNA from the whole blood required at least 30 minutes less than the method in which isolation of PBMC was performed. The use of microcentrifuge tubes in the whole blood kits (with the exception of the Genisol Kit) allows decreasing time of centrifugation and easier storage of the obtained DNA. However, each kit had a major disadvantage that made them unsuitable for the proposed application: the poor quality of the DNA. The low number of positive samples by GAP-DH highlights the presence of inhibitors in the DNA. The quality of DNA was a crucial factor for choosing the DNA extraction method that had to be applied in a large epidemiological survey in which PCR test for each animal could

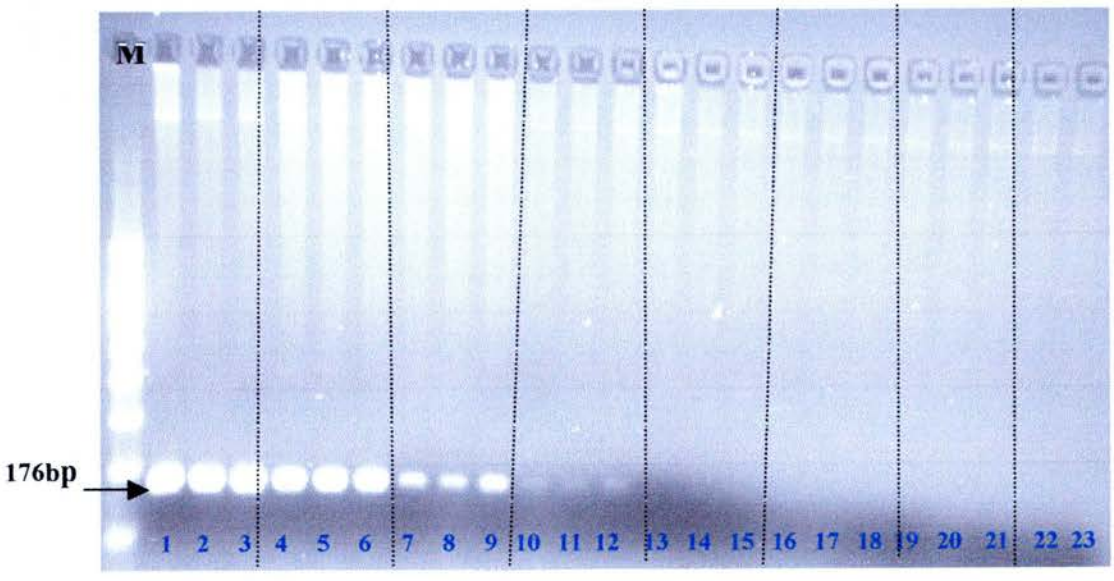


Fig 3.1. Sensitivity of the JSRV U3-PCR.

The sensitivity of JSRV U3 PCR was performed using serial dilutions (10,000- <5 copies) of JS7 DNA in a background of 800 ng of DNA from Icelandic sheep.

1-3: 10,000 copies of JSRV template; 4-6: 1,000 copies of JSRV template; 7-9: 100 copies of JSRV template; 10-12: 10 copies of JSRV template; 13-15: 5 copies of JSRV template; 16-18: less than 5 copies of JSRV template; 19-21: icelandic blood sample; 22-23: water. Lane M: contains molecular mass DNA marker IV(Bioline).

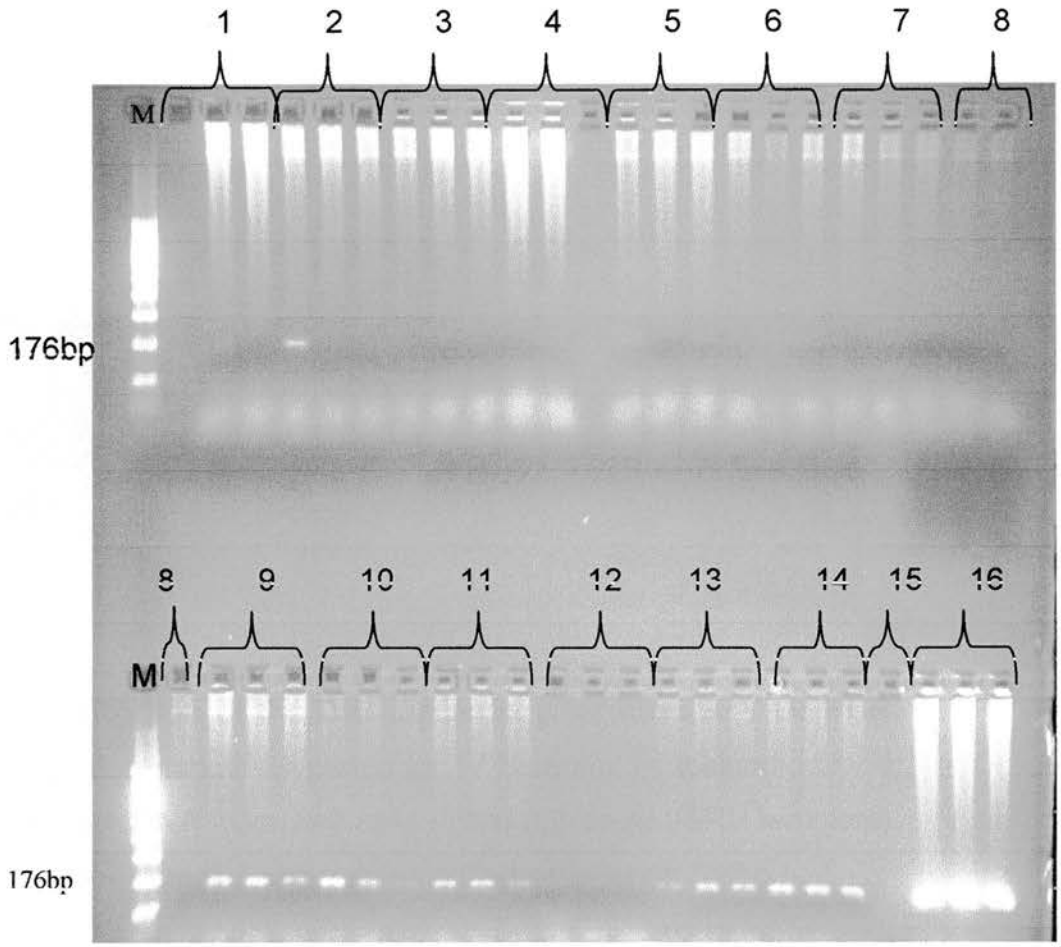


Fig 3.2. Detection of JSRV provirus in sheep blood by JSRV U3-PCR.

M: molecular marker (Hyperladder IV Bioline). Each blood sample was tested in triplicate. 3,4: samples sheep imported from New Zealand; 1, 2,5,9,10,11,12,13, 14: samples from OPA clinical animals; 6,7,8: samples from Icelandic sheep; 15: water; 16: DNA from JS7 cells (1000 copies of JSRV template). The positive samples show an amplified product of 176bp.

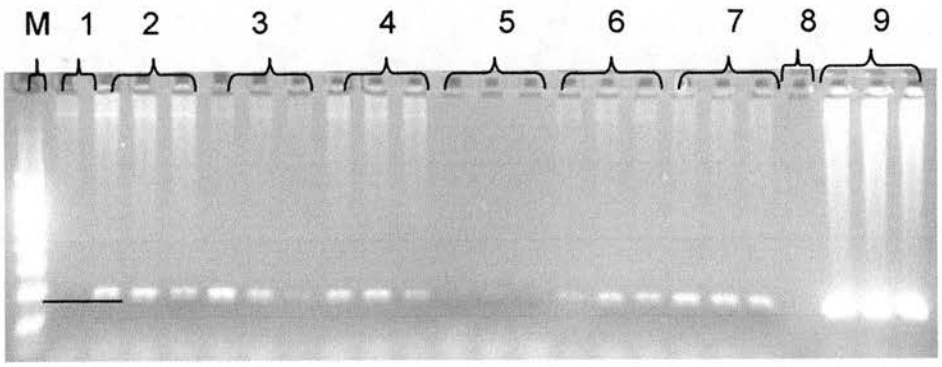


Fig 3.3. Titration of the DNA necessary for the JSRV U3 PCR. M=molecular marker (Hyperladder IV Bioline). 1= Icelandic blood; 8=water. 9= DNA from JS7 cells (1000 copies of JSRV template). The positive samples show an amplified product of 176bp.

5,6,7: blood DNA from one OPA clinical case; in the triplicates panel 5 the amount of DNA for each replicate was 300 ng, in panel 6 was 500ng and in 7 was 800ng.

Each of the replicates 2, 3 and 4 contains DNA from three OPA clinical cases animals; for each of the replicates 2, 3 and 4 a total DNA amount of 800ng was used.

include only three replicates. These limitations were due to concerns regarding the available budget and the management of a consistent number of samples to test.

After DNA extraction from the PBMC with Dneasy tissue kit a good DNA yield and a significant improvement in terms of DNA quality were achieved. DNA extraction from PBMC gave 100% percentage of positive samples by GAP-DH PCR.

The storage of PBMC at –20°C did not influence the quality of the DNA and the DNA yield (Table 3.2).

The previous heminested PCR protocol for detecting the provirus from the blood used a minimum amount 500 ng of DNA per PCR reaction (Palmarini *et al.,* 1996b; Gonzalez *et al.,* 2001). Each sample has to be repeated in triplicate in the GAP-DH and U3 JSRV PCRs. After these considerations the highest DNA yield and the best DNA quality were obtained with DNA extraction of the PBMCs with Dneasy tissue kit. The amount of time necessary for PBMC isolation from the whole blood was kept to a minimum by devising a method to perform the red cell lysis in a microcentrifuge tube. PBMC DNA extraction using the Dneasy tissue kit allowed a high degree of confidence, based on the DNA yield and DNA quality, for applying this method to the experimental samples and samples from the epidemiological survey.

3.3 Induction of OPA in older lambs

3.3.1 Materials and methods

3.3.1.1 Experimental infection

Four groups of Scottish Blackface lambs of different ages were inoculated with JSRV-containing lung fluid, as confirmed by western-blot and reverse transcriptase PCR (Palmarini *et al.*, 1995). The ages at inoculation were: one week (n=5), one month (n=10), 3 months (n=10) and 6 months (n=10). All the animals were from an OPA-free flock at Moredun Research Institute (Edinburgh, UK). Six lambs from the same source were used as unchallenged controls.

Lung fluid (LF) collected from sheep naturally affected with OPA was first filtered through a double layer of sterile gauze and clarified by centrifugation at 11,000 X *g* for 60 minutes at 4°C and stored at -70°C. In order to remove variation in JSRV titres between batches of LF and ensure that each lamb received the same inoculum, several batches of LF from different sheep were thawed, pooled together, aliquoted and stored at -70°C. Immediately before inoculation of the lambs, the appropriate number of aliquots were thawed and concentrated by centrifugation at 100,000 X *g* on a glycerol cushion, and the resulting pellet suspended in TNE (0.01M Tris pH7.5, 0.1M NaCl, 1mM EDTA) buffer to give a 12.5 fold concentrate of the original volume (Sharp *et al.* 1983). Each lamb in each age group received 5 ml of this concentrated lung fluid pool by the intra-tracheal route, as previously described (Sharp *et al.*, 1983).

3.3.1.2 Blood collection and DNA extraction in the experimental animals

Peripheral blood samples were collected immediately prior to experimental JSRV challenge and at intervals until necropsy. From each animal, a final blood sample was taken immediately before necropsy. Ten ml of blood was collected by venipuncture into individual EDTA vacutainers (BD) (Wang *et al.*, 1992; Holodniy *et al.,* 1991; Lam *et al.,* 2004).

3.3.1.3 Clinical examination, culling and post mortem examinations

After experimental infection, animals were monitored regularly for clinical signs of respiratory disease suggestive of OPA including tachypnoea, dsypnoea, noticeable movement of the abdominal wall (abdominal lift), moist rales and nasal discharge of pulmonary fluid. According to their nature and severity, the clinical signs were described as highly suggestive or compatible with OPA. As soon as these signs were identified, lambs were euthanased by an intravenous overdose of pentobarbitone. Lambs not showing any clinical signs were kept for a maximum of six or seven months p.i. and then culled. The unchallenged control animals were retained until the end of the experiment and culled at the same time as the last inoculated lambs.
At necropsy, visual examination of the lungs was performed to identify any gross lesions suggestive of OPA. Neoplastic-like areas or, in their absence, a range of sites from all pulmonary lobes were taken for microscopic examination. Samples were fixed in 10% neutral buffered formalin, routinely processed, sectioned at 5μm, and stained with hematoxylin and eosin (HE). Sections from selected lung samples were

subjected to immunohistochemical examination for JSRV capsid protein (JSRV-CA) as described by Palmarini *et al.,* 1995, and for JSRV surface glycoprotein (JSRV-SU) using a newly developed antiserum (Chapter 2).

Incubation period (days from experimental infection to post-mortem) of OPA in four groups of sheep challenged at different ages, all of which showed clinical signs of the disease and histopathological confirmation of pulmonary neoplasia was further anlysed using a statistical method (Fig.3.4). The comparison of incubations periods between groups was done using the "unpaired test". The following comparison were carried out:

1.- 1 week versus 1 month-old lambs: the non-parametric, unpaired t test (Mann-Whitney test) was used; this is because there were only four sheep in the 1 week-old group and this figure was too low to tell if the incubation periods were arranged in a normal (Gaussian) distribution.

2.- 1 month versus 3 month-old lambs: the unpaired t test with Welch correction was used; this was because the two groups had significantly different standard deviations.

3.- 3 month versus 6 month-old lams: the unpaired t test was used; this time the normal t test was included because the data followed a Gaussian distribution and the two groups had similar standard deviations.

3.3.1.4 Immunohistochemistry (IHC)

Tissue samples were fixed in 10% neutral buffered formalin, processed routinely in an automatic tissue processor, embedded in paraffin wax and sectioned at 4-6μm. Lung tissue sections were microwaved at 800 w for 10 min. in citrate buffer pH 6.0 and endogenous peroxidase was inhibited by immersion in 1% vol (v/v) hydrogen

peroxide in methanol for 25 min. The sections were then incubated overnight at 4°C with either anti-JSRV-SU or anti-JSRV-CA polyclonal antibodies, both at 1/1000 optimised dilution. As a additional control, slide sections were incubated with the relevant rabbit pre-immunisation serum. Section were then washed 3X10 minutes with TBS/Tween (TBS, 0,5% [v/v]Tween) and incubated with a biotinylated goat anti rabbit immunoglobulin (1: 100; Vector Laboratories). After 3X10 min washings with TBS/Tween, the ABC Vectastatin complexTM (Vector-elite ABC kit; Vector Laboratories, Peterborough) containing avidin and biotinylated horse radish peroxidase was added to the sections and incubated for one hour at room temperature. Washing were then performed as above and the reaction was developed after incubation with DAB substrate solution (0.05 DAB[w/v] in TBS, activated with 0.15, v/v H_2O_2). After one minute, the reaction was stopped with tap water
At the end, sections were counterstained with Carazzi's haematoxylin. Sections from natural OPA cases were used as positive controls.

3.3.2 Results

3.3.2.1 Situation in the different age groups

Unchallenged controls

None of the uninoculated control lambs developed clinical signs of respiratory disease and their PBMCs were negative by JSRV-U3-PCR throughout the experiment. These lambs were kept until 14 months old and neither histopathological lesions of typical OPA nor proviral DNA were detected in lung samples or mediastinal lymph nodes taken at necropsy.

One week old lambs

Severe clinical signs highly suggestive of OPA were observed in four lambs of this group, and two of them showed nasal discharge of lung fluid. They were killed between 70 and 74 days p.i. (Fig.3.4). One lamb (1W1, Fig. 3.5) died 28 days after experimental challenge of an unrelated illness without obvious OPA clinical signs. All five lambs showed gross lesions highly suggestive of OPA, which were confirmed by microscopic examination (Fig. 3.5). All lambs were PCR negative at the time of infection but their PBMC were consistently positive from there onwards (Fig. 3.2), including the lamb that died from an unrelated cause.

One month old lambs

Nine out of ten lambs in this group showed clinical signs highly suggestive or compatible with OPA, with incubation periods ranging from 92 to 209 days (average 152, Fig. 3.4). The remaining lamb (1M10, Fig. 3.5) was asymptomatic when culled at almost seven months p.i. All ten lambs had macroscopic pulmonary lesions histologically confirmed as OPA (Fig. 3.5). The PBMC of all lambs also were JSRV-U3-PCR positive throughout the experiment with the exception of one (1M7, Fig. 3.5). This lamb was positive only in the sample taken immediately before necropsy, even though it had developed clinical signs of OPA with lung fluid production.

Three month old lambs

Nine of 10 lambs challenged at 3 months of age developed clinical signs highly suggestive or compatible with OPA, with incubation periods ranging from 159 to 192 days (average 185, Fig. 3.4). The other animal (3M10, Fig. 3.5) remained asymptomatic until it was culled at 192 days p.i. Macroscopic lung lesions were confirmed histologically as OPA in all 10 lambs. PBMC from all 10 also were JSRV-

U3-PCR positive at some point during the experiment. While JSRV viraemia was consistently detected in six of them, including the one that remained asymptomatic, the other four (3M2, 3M3, 3M5 and 3M8 in Fig. 3.4) gave some PCR negative results.

Six month old lambs

Five of the 10 animals in this group developed clinical signs of respiratory disease mainly manifested as tachypnoea and dyspnoea after exercise and never with production of lung fluid. The incubation period ranged from 164 to 169 days (average 182, Fig. 3.4). Amongst the five other sheep, two (6M1 and 6M2 in Fig.3.5) were culled and died respectively from OPA-unrelated conditions at 104 and 145 days p.i. The other three (6M8, 6M9 and 6M10) were asymptomatic when culled at 190-192 days p.i. All five sheep with clinical signs of OPA showed characteristic gross lesions confirmed by histopathological examination. Neither macroscopic nor microscopic lesions of OPA were found in the two sheep that died from unrelated conditions and in one of the three asymptomatic sheep (3M10, Fig. 3.5). The other two asymptomatic sheep showed pathological evidence of neoplasia although in one of them (6M9, Fig.3.5) only microscopic lesions were detected. Within this 6 month-old group, PBMC from all animals were JSRV-U3-PCR negative at 3 weeks p.i., but, with the exception of a single sheep (6M9 in Fig. 3.5) they were consistently positive thereafter.

Figure 3.9 presents an overview of the results obtained in the different age groups of animals.

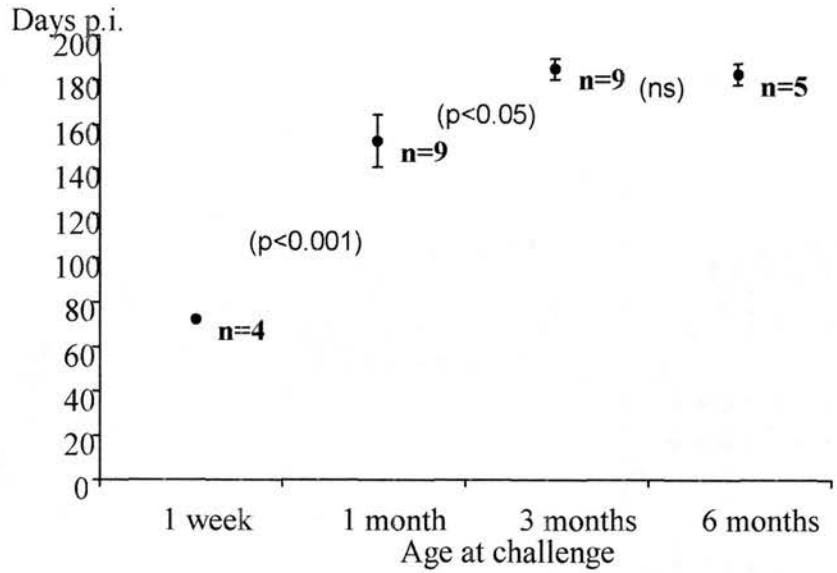


Fig. 3.4. Incubation period of experimental OPA depending on the age at challenge. test". Incubation period (days from experimental infection to post-mortem) of OPA in four groups of sheep challenged at different ages, all of which showed clinical signs of the disease and histopathological confirmation of pulmonary neoplasia. Results expressed as average values, bars indicating standard errors; statistical differences between groups appear in brackets.The comparison of incubations periods between groups was done using the "unpaired t test".

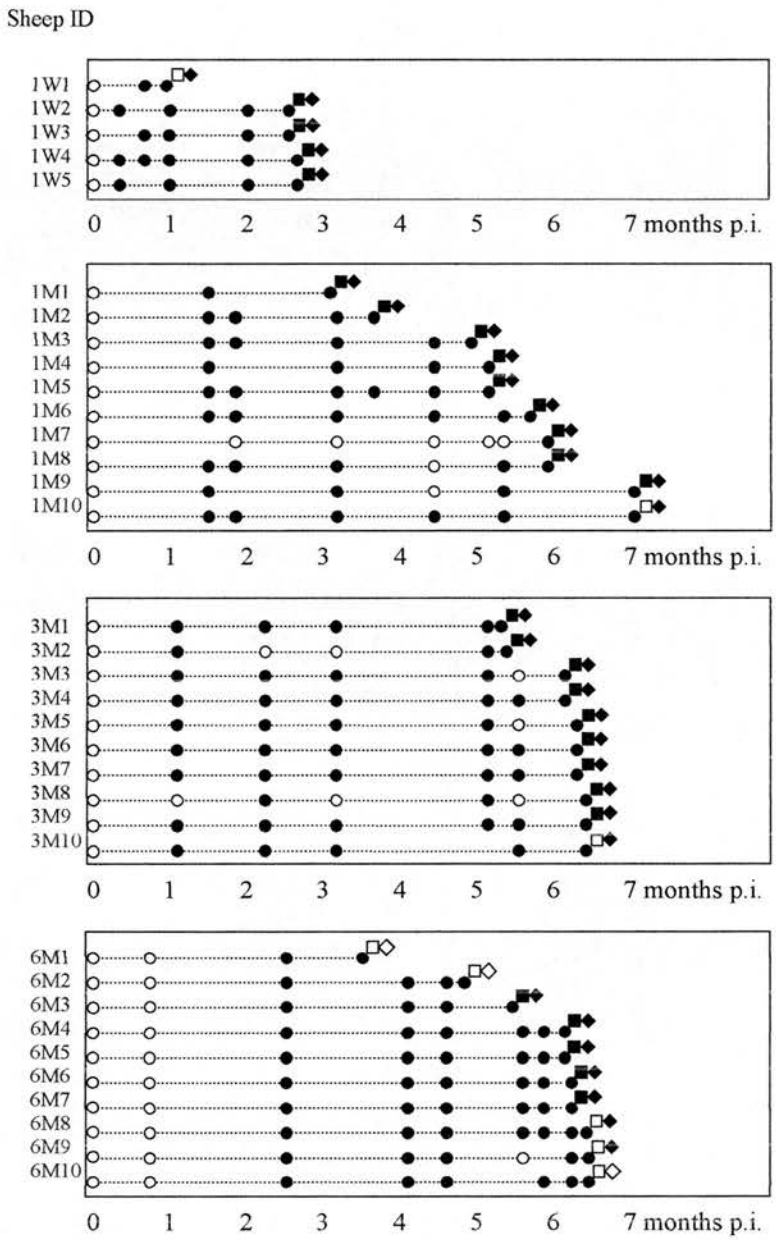
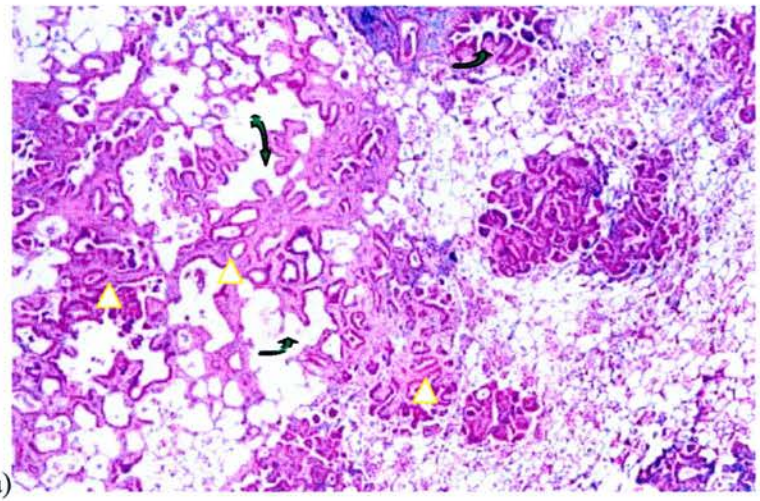


Fig.3.5. Evolution of JSRV viraemia as detected by U3 PCR (○ , negative; ● , positive) during the course of the experimental infection and at culling of sheep showing clinical signs of OPA (■) or not showing such signs (□), and in which gross and/or histopathological lesions of OPA were confirmed (◆) or absent (◇).

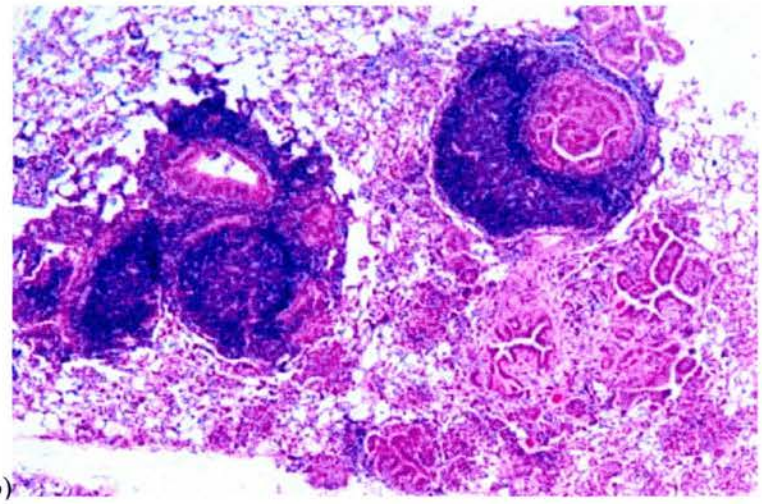
3.3.2.2 Histopathological and IHC observations and PCR examination of tissue samples

In all sheep with histologically confirmed OPA, regardless of their age at challenge, the tumour was predominantly a papillary adenocarcinoma of the alveoli even if in some cases an acinary pattern was also present (Fig.3.6a). Papillary neoplastic growths into the lumena of bronchioles and alveolar nodules of acinary and bronchioloalveolar appearance were found inconsistently and were unrelated to the age at infection. Fibrosis of the stroma of the neoplastic nodules and presence of spiral shaped myxoid tissue were rare. Infiltration of the tumour stroma by mononuclear cells was relatively frequent, though mild, in the lambs challenged at one and six months of age, but inconspicuous in the other two groups, particularly in the one week old lambs. Also in the animals inoculated at one month, hyperplasia of the bronchiole associated lymphoid tissue was common and prominent in half of them (Fig. 3.6b). Desquamation of enlarged alveolar macrophages in para-neoplastic alveoli was a common finding in all sheep groups, while suppurative exudates in tumour and non-tumour areas were very variable between individuals within the same age group.

IHC examinations showed JSRV-CA protein in the cytoplasm of a proportion of ATII neoplastic cells and nodules as already described in previous studies (Palmarini *et al.,* 1995; Platt *et al.,* 2002). As expected JSRV CA was identified mainly in tumour cells (Fig 3.7a and Fig 3.8a). In some tumour nodules individual cells or group of cells stained intensely yet were adjacent to cells that did not stain at all. There was no staining of non transformed epithelial cells nor of the stromal cells.



a)

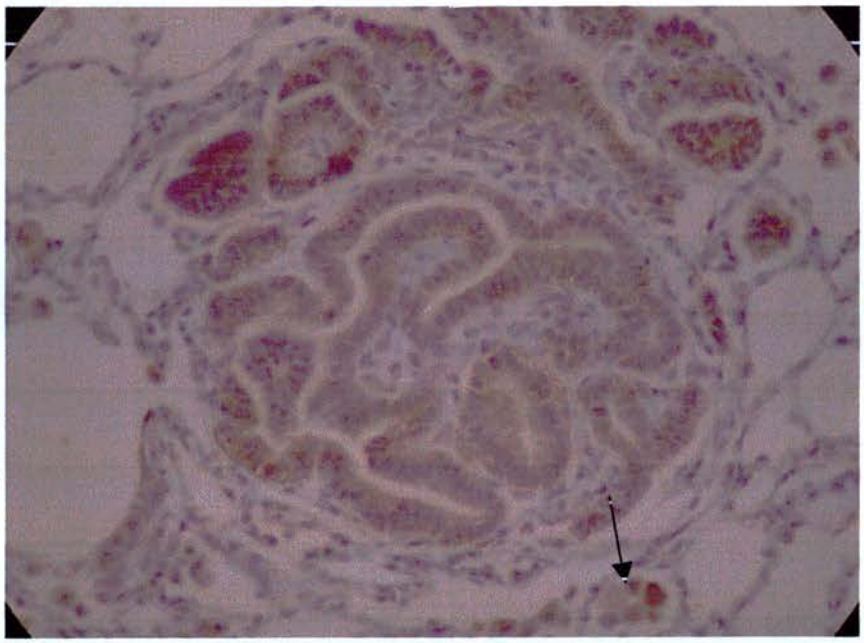


b)

Fig.3.6. Histopathological lesions in JSRV experimentally inoculated lambs.

a) Co-existence of papillary (arrow) and acinary (triangle) forms of the tumour in the lung of a lamb inoculated at three months of age. H-E x4. b) Papillary neoplastic proliferations involve alveoli and bronchioles, which also show hyperplasia of associated lymphoid tissue. Para-adenomatous change is also evident. Lamb challenged at 3 months of age. H-E x8.

a)



b)

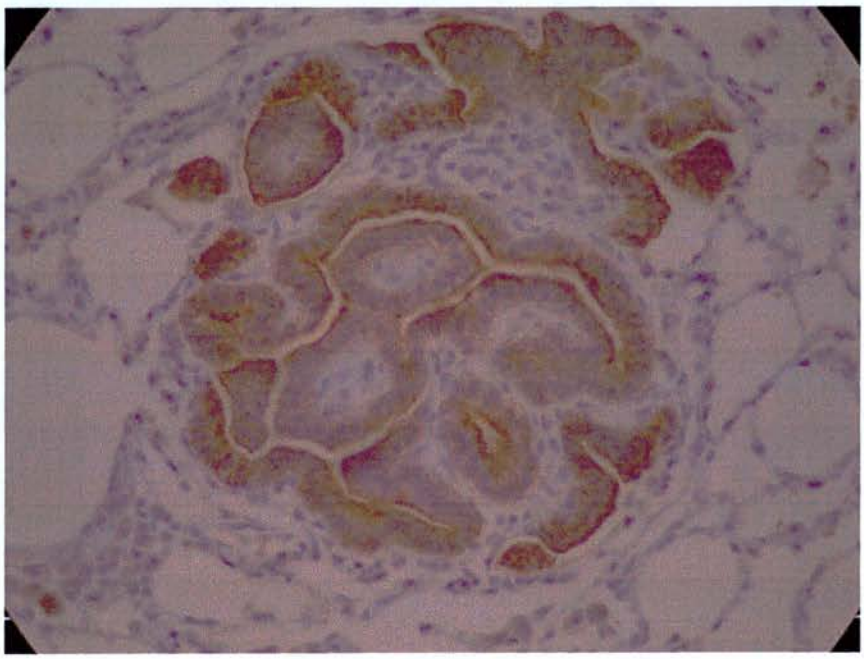


Fig 3.7. IHC of experimental OPA.

Papillary alveolar tumour showing:

a)strong immunoreactivity for viral capsid protein in the cytoplasm of the transformed cells. The arrow in fig 3.9a indicates the presence of desquamated alveolar macrophages in the alveoli

b) strong immunoreactivity for viral surface protein mainly in the apical border of transformed ATII cells (magnification 400x).

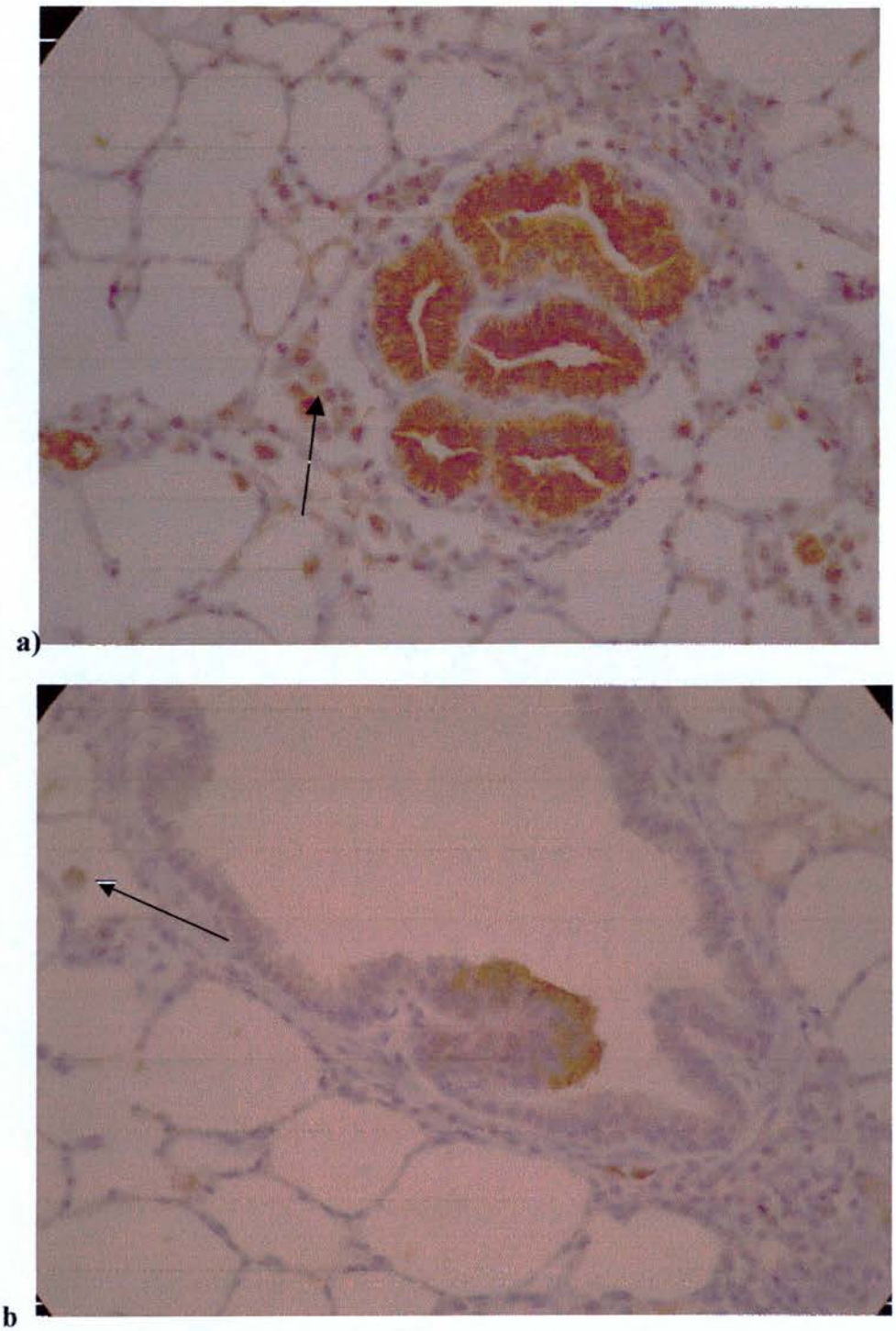


Fig 3.8. IHC of experimental OPA.

Proliferating cells in a bronchiole showing a) staining confined to the cytoplasm using anti JSRV CA immune serum; b) immunoreactivity for JSRV-SU protein, which is most evident in the apical cytoplasm, while non-transformed cells are negative (a,b). The arrows in fig 3.8a&b indicate the presence of desquamated alveolar macrophages in the alveoli. Magnification 400x.

Detection of IHC reactivity to JSRV-SU was consistent in epithelial cells lining neoplastic alveoli (Fig. 3.7b) and in cells lining neoplastic polypoid structures in the bronchioles (Fig. 3.8b). In both cases, immunolabelling was localised in the cytoplasm but was most intense at the apical surface of the tumoral cells. On some occasions, isolated cells with morphology of ATII cells also showed cytoplasmic immunolabelling. While normal epithelial cells of the alveoli and bronchioles were not labelled, a few interstitial cells (macrophages-like) within the bronchiole-associated lymphoid tissue or in the stroma of the alveolar tumours were JSRV-SU positive.

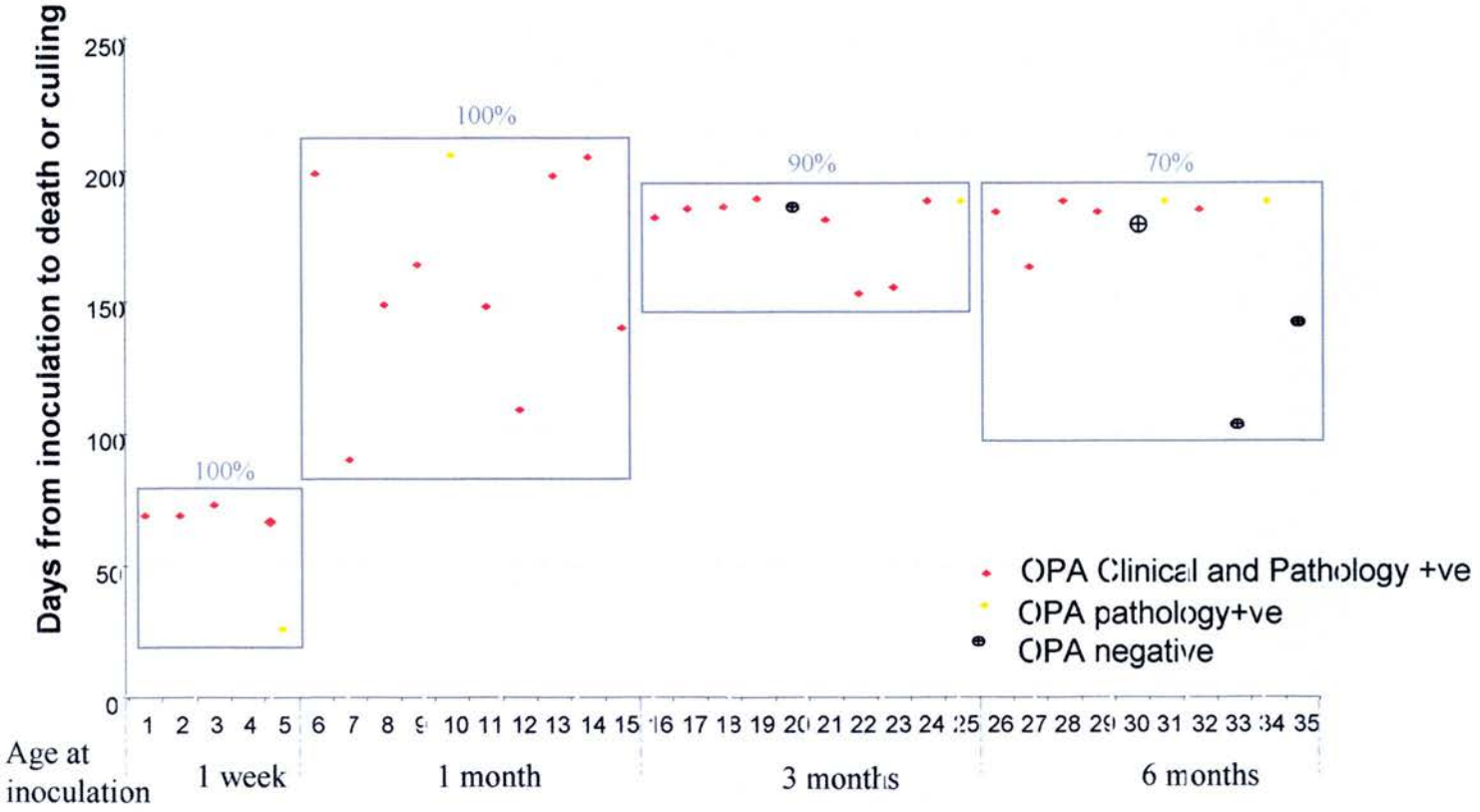
The same pattern of JSRV SU and JSRV CA staining was observed in tumours with papilliform and/or acinary formations. IHC results did not show any difference between natural and experimentally infected animals.

Proviral JSRV DNA was detected by U3-PCR in all mediastinal lymph node and lung tissue samples collected from the 35 challenged sheep.

3.3.3 Discussion

The present study has demonstrated clearly that JSRV infection can be induced in 100% of lambs aged one to six months at the time of inoculation and that a high proportion of these animals develop clinical signs (62-90%) and lesions (87-100%) of OPA (Fig.3.9). These findings are in contrast with a much smaller-scale previous study that employed tumour extract as inoculum, in which only 25% of older lambs developed neoplastic lesions (Rosadio *et al.,* 1988). One explanation for the success of the current experiment would be a large amount of JSRV in the inoculum, as it has been demonstrated that LF contains more JSRV than tumour extracts (Herring *et al.,*

Fig 3.9. Experimental infection of different age group of lambs with LF.
Summary of clinical and pathological results in the different age groups of animals.



1983) and that there is an inverse relationship between incubation period and amount of JSRV in the inoculum (Verwoerd *et al.*, 1980). Nevertheless some features of the present study support the notion of an age-related resistance to OPA.

First, within those animals that developed clinical signs of OPA, the incubation period was significantly shorter ($p<0.001$) in the lambs inoculated at one week of age than in the older lambs (Fig.3.4). It also was significantly shorter ($p=0.03$) in the animals challenged at one month than in those inoculated at 3 months.

However it is possible to argue that the age effect was so remarkable because the dose was not adjusted to weight size of the animals. It would have been interesting to have had time to investigate a possible dose effect. An important prelude to such studies would be the development of a quantification system for JSRV in the LF and the dose-effect experiment could be carried out challenging lambs that are 3 months old with 3 or 4 different concentrations of JSRV virions.

In the family Retroviridae, other examples of age-related susceptibility can be found. The two factors that determine whether a cat exposed to FeLV will become viraemic or recover are the age at which the cat becomes infected (Hoover *et al.*,1976; Flynn *et al.*, 2000) and the dose of virus to which it is exposed. Kittens that lack protective maternal antibodies and that are infected within the first 4-5 months of life have a high risk of becoming permanently infected. After this period, resistance to infection develops rapidly so that by the time cats are 6 months of age only about 15% develop persistent viraemia following exposure to high doses of virus. The effect of dose is seen dramatically in the contrast between the prevalence of infection in multicat households in which the virus is enzootic and in free ranging cats, generally from single-cat households. In multicat households, the dose of virus to which the cats are

exposed is high due to continuous contact with viraemic cats, so in this population of cats there is a high prevalence of viraemic cats (30-40%) which have a high risk of developing FeLV-associated disease. By contrast, animals from single-cat households have a prevalence of only around 1% and the incidence of FeLV-related disease is very low (Jarrett, 2001).

Certain avian leukosis viruses are able to induce rapid lymphomas after inoculation of 9 to 13 day-old chickens, while neoplasia in mature chickens takes longer to develop (Pizer *et al.,* 1992; Stedman *et al.,* 2001). Another example is provided by Maedi-visna virus (MVV) infection, which usually leads to long incubation periods and low rate of lesions and disease in adult animals. Experimental infection of young lambs, however, can result in acute disease with very high mortality (Lairmore *et al.*, 1986; Andresson *et al.,* 1993). These observations could be explained by differences in the ability of neonates and older animals to mount an immune response against MVV, as some authors found that the appearance of circulating anti-MVV antibodies was correlated with a decrease in viraemia titres (Haase *et al.,* 1977). This explanation, however, would not seem likely for JSRV infection, in view of the lack of a specific humoral immune response (Ortín *et al.*, 1998; Sharp & Herring, 1983), perhaps due to the early expression of closely related JSRV-endogenous retroviruses (Palmarini *et al.,* 2004).

A more plausible explanation for the age effect in OPA would be that the target cells for primary JSRV infection and oncogenesis are present in higher numbers in newborn lambs than in older animals. The major target cells for JSRV replication in the lung are the ATII and Clara cells of the bronchioles (Palmarini *et al.,* 1995) in which the active LTR causes the enhanced JSRV gene expression (Palmarini *et al.,*

2000b). In rodents and ruminants the proportion of ATII cells changes with age. At birth, the ratio of type I pneumocytes to ATII cells is around 1:2, and ATII pneumocytes account for approximately 60% of alveolar cells (Kahwa *et al.*, 1997; Otto, 1997; Weibel & Taylor, 1996). During the first month of life, the number of ATII cells declines progressively and dramatically, so that they represent only 6% of alveolar cells at 7 days, 1% at 3 weeks, and 0.2% at one month, with no further significant changes occurring later (Wright & Alison, 1984). Similarly, Clara cells retain foetal characteristics in calves and goat kids that are up to 30 days old, but lose them and become fully differentiated after this time (Castleman & Lay, 1990; Kahwa *et al.*, 2000).

The hypothesis proposed is that following intra-tracheal inoculation of JSRV, primary viral replication would occur in the ATII pneumocytes and Clara cells and that the magnitude of such replication would depend on the availability of those target cells. Thus, a higher viral production could be expected in newborn lambs, leading to earlier detection of viraemia and development of neoplastic lesions (and hence shorter incubation period). As the number of available target cells decreases, initial viral replication would occur at a lower rate resulting in delayed viraemia and development of lesions (and hence longer incubation period). This age effect would be particularly noticeable during the first month of life and, as far as viraemia is concerned, needs to be confirmed by new experiments involving more exhaustive PCR testing of blood samples.

The persistent viraemia and the delayed development of OPA in older lambs parallel epidemiological observations from naturally affected flocks where JSRV could be detected in the blood before the onset of clinical OPA and even in absence of

discernible lung tumours (García-Goti, *et al.,* 2000; González *et al.,* 2001). It is also noticeable that in flocks with endemic OPA only a proportion of sheep (up to 30%, but usually less) develop neoplastic lesions (Sharp & DeMartini, 2003), which may or may not lead to overt clinical disease, while JSRV infection appears much more widely disseminated (around 75%, as judged by PCR; García-Goti, 1999). These observations on the natural disease would suggest that JSRV is a slow-transforming retrovirus, raising questions about the *in vivo* role of JSRV *env* gene in transformation (Fan *et al.,* 2003), even though recent *in vitro* studies have indicated that JSRV Env protein is capable of transforming rodent and avian cells (Maeda, *et al.*, 2001; Zavala, *et al.*, 2003). However, our finding of consistent expression of SU protein in tumour cells is consistent with a role in proliferation of these cell. This hypothesis is reinforced by the fact that positive IHC results have been obtained using the anti JSRV-SU polyclonal rabbit serum on nasal gland tissues obtained from animal with natural ENA (de las Heras *et al.,* 2003). The mechanisms of *in vivo* oncogenesis in OPA appear therefore to be more complex than those of cell transformation *in vitro*, with several steps being probably necessary to achieve viral-induced cell transformation.

In conclusion a new experimental model to induce JSRV infection and OPA in aged lambs has been developed. Infection also can be monitored by an efficient PCR technique using blood samples. Apart from providing new insights into the pathogenesis of the disease, confirming an effect of the age in the time scale of OPA-related events, the model will be useful to test the efficacy of potential vaccines. Finally, our PCR results reinforce previous studies on detection of JSRV infection in

subclinically infected sheep, providing a further opportunity for epidemiological research.

Chapter 4

Epidemiological and transmission studies in sheep naturally infected by JSRV

4.1 Introduction

Currently there is no treatment or pre-clinical diagnosis for OPA and it is invariably fatal. OPA has a widespread prevalence throughout the world (Sharp & DeMartini, 2003). Economic losses due to OPA can vary. For example in Scotland some farms report losing 5 – 10% of sheep per year to this disease (Sharp & De Las Heras, 2000). However all available data on the prevalence and distribution of OPA are based on retrospective histopathological reports. Information of the true prevalence of JSRV infection is a major gap in the knowledge of the disease.

In UK most of the data are limited to reports on cases submitted to Veterinary Investigation centres. For example, of 653 lung samples submitted to the MAFF veterinary laboratory in Lasswade, Midlothian during 1975-1979, 191 presented lesions of OPA (Hunter & Monroe, 1983), (Table 4.1). In this study and that of Harbour & Jamieson, (1946), the OPA cases came particularly from the East of Scotland and the Borders. Recent reports of OPA in the Borders, Dumfries, Caithness and Orkney suggest a wider distribution across Scotland (SAC veterinary services monthly reports Jan02-July03). Farming groups, veterinarians and regulatory authorities all recognise OPA as an important target for control or eradication. Currently farmers attempt to control OPA by managerial measures such as reductions in animal density or culling of animals showing signs consistent with the disease. However, much of the information required to develop and implement a rational control or eradication strategy is lacking.

The detection of JSRV in the peripheral blood of experimentally infected lambs, before the onset of clinical disease (Holland *et al.,* 1999) was an important finding

Table 4.1. Occurrence and Distribution of OPA in Scotland based on histopathological diagnosis (Hunter & Munro, 1983).

year	Lung samples examined	No. of OPA positive	No. of affected farms
1975	100	24	unknown
1976	95	24	unknown
1977	128	46	34
1978	178	45	33
1979	152	52	41
1980/81	526	99	63

that offered a means to investigate the extent of JSRV infection in OPA affected flocks. These findings were extended in studies involving sheep from OPA-affected commercial flocks (Gonzalez *et al.*, 2001). Peripheral blood leukocytes (PBLs) and tissue samples from 36 sheep were examined for JSRV by hemi-nested PCR. Animals were classified according to the status of OPA which was confirmed by pathological examination, as follows: (i) sheep with classical OPA (cOPA, n=10), (ii) sheep with atypical SPA (aOPA, n=6), (iii) non-affected sheep from OPA-affected flocks (in-contact, n=10) and (iv) non-affected sheep from OPA-free flocks (control, n=10). JSRV proviral DNA was detected in the PBLs of 10/10 cOPA, 5/6 aOPA, 4/10 in-contact and 0/10 control sheep. The number of positive PCR results was greater for sheep in the cOPA group than for those in the aOPA and in-contact groups. These results showed that JSRV can be detected in naturally infected sheep before the onset of clinical disease and even before the development of discernible tumours.

Further information of the dynamics of JSRV infection in OPA-affected flocks were provided by a small prospective survey in an OPA-affected flock (Garcia Goti., 1999). JSRV was detected by hemi-nested PCR in PBLs from 28% of the flock. Fifteen positive sheep and five negative sheep were selected and PBLs were examined by JSRV PCR at monthly intervals for the next 4 months. JSRV was detected in only 9 of the original 15 positive sheep during this period and in 4 of those that were negative (Table 4.2).

The development during this study of a one step PCR test (JSRV U3 PCR) for JSRV infection (Chapter 3) gave the possibility to start the first prospective and

Table 4.2. Longitudinal survey of JSRV infection in a small OPA affected commercial flock. (Garcia-Goti, 1999).

Fifteen positive sheep and five negative sheep were selected and PBLs were examined by hemi-nested PCR at monthly interval for 4 months.

JSRV status	No. of sheep	JSRV positivity during time (months)					Necropsy	Total
		0	1	2	3	4		
PCR(+ve)	15	15	9	5	1	4	6	11
PCR(-ve)	5	5	2	3	1	1	2	4

comprehensive study of JSRV infection following JSRV incidence within a single flock with previous history of OPA cases.

The longitudinal survey of JSRV infection that is presented in this study lasted two and half years. Ewes of different ages were bled at 3-4 months intervals. Lambs from selected JSRV positive and negative ewes also were tested at same time.

The overall aim of the work was to characterise the epidemiological features of JSRV infection, particularly its routes of transmission in OPA affected flocks, which can be used to identify the risk factors for transmission and maintenance of OPA in flocks. In particular the aims of the study were i) to determine the ranges of prevalence and preliminary indications of the influence of the breed and the management system, ii) determine the times and ages at which infection first occurs, iii) to evaluate JSRV infection in sheep of different ages, iv) determine the proportion of JSRV viraemic sheep that eventually develop OPA i.e. the prognostic significance of viraemia, v) determine the presence of a familial trend that could predispose descendants of infected sheep. The analyses presented in this chapter will form the basis for a larger epidemiology survey aimed at control and eradication of OPA.

The longitudinal study of the offspring of JSRV viraemic and non-viraemic ewes was planned to provide preliminary indications of the occurrence or absence of JSRV transmission from dam to lamb. If maternal transmission is epidemiologically relevant, the control strategies will be designed taking this into consideration.

Maternal transmission was further investigated studying the presence of JSRV in foetuses from OPA clinically ill sheep that did not belong to the flock under epidemiological survey.

All the samples from the epidemiological studies were tested using the JSRV U3 PCR developed in Chapter 3, although a real-time PCR approach was evaluated and developed during the survey. Real-time PCR will represent the next step forward for testing large numbers of blood samples and possibly milk samples for a future OPA control and eradication programme.

4.2 Materials and Methods

4.2.1 Experimental plan

A farm with a history of OPA over many years was recruited. The commercial flock was located in England, and the productive attitude was for meat. A schematic of the experimental design is presented in Fig. 4.1.

Samples were taken from an initial number of 194 ewes; the following test was repeated in the same group of animals 3 months after, in February 2003. An initial selection of the dams was made on the basis of PCR results obtained in the pre-lambing period (December 2002 and February 2003) in May 2003.

Each single ewe was considered negative when the PCR result was negative in both pre-lambing bleed and was considered positive when it was JSRV positive in one of the two bleeds. The positive and negative group of dams included different age groups of 2, 3 and 4 or more years old; each age group was composed of animals selected randomly.

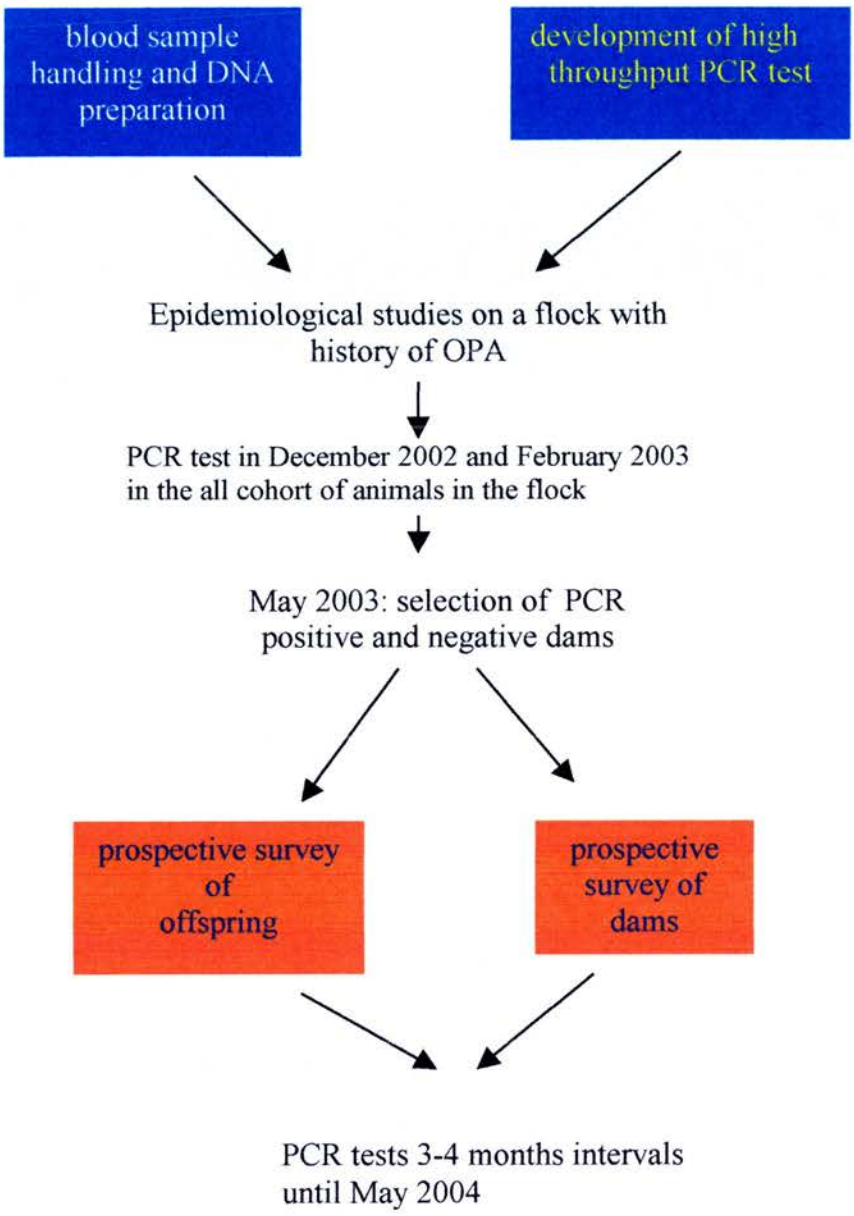


Fig. 4.1. Design of the JSRV longitudinal survey.

The selection of the study groups was made after lambing, to account for the sex and viability of the offspring, as only those dams whose progeny were kept for replacement were eligible.

The initial group of positive animals included 50 JSRV positive ewes and the negative group included 49 JSRV negative ewes. In total 117 lambs were selected for the prospective survey. It was not possible to include equal number of dams from the different age groups.

All ewes and lambs, both the negative and positive animals, were part of the same flock and they were managed together. There was no introduction of new animals within the flock during the experiment. After May 2003 it was necessary to reintroduce in the study ewes that were negative or positive in December 2002 and February 2003 to balance the age and breed composition of the ewes.

The dam:offspring ratio was set in most of the cases at 1, only in few cases more than one lambs from multiple births were included.

In the lambing period of May 2003, blood samples were tested from 24 rams that generated the offspring obtained in May 2003. At that time the animals were two years old and they were Suffolk or Texel pure breed.

The tissues from those animals that died during the survey were collected and subjected to histopathological studies. The survey lasted until May 2004, the ewes and the lambs were bled every 3-4 months until May 2004.

4.2.2 Sample handling and JSRV U3 PCR test

Blood was collected, DNA was extracted from fresh PBLs with the Dneasy tissue kit (Qiagen)) and PCR tests were performed as described in Chapter 3. The performance of the PCR test was first evaluated in a group of 18 OPA clinical diseased animals, a group of 50 animals from Iceland and 8 animals from New Zealand (section 3.2.2.2). The provirus could be detected in all diseased animals. The result obtained was always negative for the 58 animals from Countries without history of OPA.

The JSRV U3 PCR test was evaluated under experimental conditions in a total number of 35 lambs of different ages that had been inoculated i.t. with infectious lung fluid (Fig.3.7). During the course of the experiment, viraemia was detected consistently by the PCR test. Viraemia could already be detected at one month after infection, with the exception of the 6 months old group, in which a delay was registered for all the lambs in the group. The prognostic value of the test in the experiment was considerable; in fact viraemia could be detected in all the infected animals before the onset of the clinical signs.

The aim of the JSRV U3 PCR test in the epidemiological survey was to detect the viraemia in a flock that had a previous history of OPA, but with no clinical cases at the time at which the survey started.

The number of blood samples collected for each bleed was considerable and had to be done in such a way that created minimal disruption to the management of the flock. For this reason the adopted strategy was to bleed all the animals in one day and to dedicate the day after the bleed to extract the white cells from all the blood samples, storing them at -20°C and then during the following days proceed with DNA extraction. The samples therefore were processed in random order regarding

breed, age or previous OPA test result. A positive result was assigned where one or more of the triplicate PCR reactions produced a visible product upon agarose gel electrophoresis.

4.2.3 Data exploration

4.2.3.1 Collection of the PCR test results

The results for each single test of the ewes and the lambs are summarised in tables (from Table 4.3 to 4.15) in which the number of positive animals related to age and breed are highlighted.

4.2.3.2 Statistical analysis of the data

A further statistical analysis was necessary to understand which factors could have influenced the PCR test results. This statistical analysis took into consideration the data collected for each single bleed and the whole data set. For this purpose the results from each single test were summarised in Excel format. The data were analysed with the statistical support from BioSS, UK. The final Excel format required some manipulation prior to analysis. For example lambs that did not have any ewe associated with them were removed from the analysis. In addition the data were checked for missing values, and the column names were standardised. An example of the working data is shown in Appendix XIII, Table XIII.1.

First it was calculated for each single bleed of ewes and lambs the number of new JSRV positive animals (JSRV incidence).

The second approach undertaken for the evaluation of the PCR test result aimed to connect the PCR results over time. In other words it was evaluated which factors

would influence the fact that an animal in a given time could become positive when in previous tests it was negative or alternatively in which an animal can become JSRV negative although in previous tests it was JSRV positive. This kind of evaluation is called JSRV conditional incidence. This approach took into account the fact that animals could give fluctuant results during the survey. In statistical terms JSRV conditional incidence uses the concept of "conditional probability". Conditional probability calculates the probability of an event occurring given that another has already occurred (Papoulis, 1984).

The statistical calculation of JSRV incidence included all the sheep being present at all sampling times; it was relevant that a number of animals were removed from the sampling group during the study when JSRV incidence was calculated. Summaries for the calculation of JSRV incidence therefore were restricted to animals which had a complete set of observations over the study. This was done for removing some sources of errors in the elaboration of the data.

In contrast a higher number of sheep could be used in the conditional incidence approach. In fact animals with no missing values plus animals with missing values that were subsequent to a positive observation where included. Hence when a missing observation was followed by a subsequent negative it was not assumed that the missing value was negative; however, where the missing values were after a positive detection of the virus, the missing value was filled in as positive.

The data were analysed using Generalised Linear Mixed Models (GLMMs) (Brown & Prescott, 1999). Subsets of data also were analysed using Fisher Exact Test. These statistical calculations were carried out by Dr I. McKendrick (Bioss) using Genstat for Windows Version 7.1 (Payne, 2000). Confidence intervals for binomial

proportions were calculated using the exact procedure defined by Armitage & Berry (1987).

4.2.4 Collection of fetuses and DNA extraction from foetal tissues

The tissues of 22 fetuses from OPA clinical ewes, that were pregnant at the time of necropsy, were collected and stored at -70°C. The foetuses were first carefully removed from the uterus to avoid contamination by maternal blood. Foetal lungs, thymus and kidney were collected. The ewes were not part of the flock used in the epidemiological survey, but they were kindly donated by the farmers to the Moredun Research Institute. DNA was prepared from the collected tissues using Dneasy tissue kit (Qiagen) and analysed by JSRV U3-PCR as described in Chapter 3.

4.3 Results

4.3.1 JSRV infection in the ewe and lamb groups for each single bleed

The following data consider the population of animals analysed for each single test. At the beginning of the survey (December 2002) 194 animals were included. The flock was composed as follows: 96 animals were Milksheep x Suffolk cross breed, 50 animals were Milksheep x Texel cross breed, 48 were Suffolk x Texel cross breed. 96 animals were 2 years old, 67 were 3 years old, 23 were 4 years old and 8 were 5-7 years old. Sixty of the 194 animals (31%) were positive in the first sampling (Table 4.3). In February 2003, only 187 ewes could be bled; 80 of the187 (43%) ewes were JSRV positive (Table 4.3). The increase in positivity occurred in 2

Table 4.3. Detection of JSRV positive and negative animals in December 2002 and February 2003.

Bleeds	Tot. no. of animals	JSRV+ animals	JSRV- animals
Dec 2002	194	60 (31%)	134 (69%)
Feb 2003	187	80 (43%)	107 (57%)

Table 4.4. Age distribution of JSRV infection at the two sampling times December 2002 and February 2003.

	2 years	3 years	4 years	5-7 years
Dec 02	23/96 (24%)	14/67 (21%)	18/23 (78%)	5/8 (63%)
Feb 03	31/92 (34%)	27/65 (42%)	17/22 (77%)	5/8 (63%)

and 3 years old sheep (Table 4.4). In fact increases of positivity of 10% and 21% were registered in the 2 years and 3 years old group of animals respectively.

In December 2002, 51 out of 98 (52%) animals with Texel genes were JSRV positive, whereas only 9 of 96 (9%) ewes that did not have Texel genes were positive.

In February 2003, 48 animals were JSRV positive on a total of 95 (51%) ewes with Texel genes; 32 of 92 (35%) ewes without Texel genes were JSRV positive (Table 4.5).

Of the 185 sheep that were tested twice, in December 2002 and February 2003, 100 were negative and 50 were positive at both samplings and 35 were positive at only one of the two (Table 4.6). Seven animals that were positive at the first test were negative at the second test (Table 4.6).

After the first two bleeds (December 2002 and February 2003) a positive and a negative group of ewes were selected to proceed with the epidemiological survey over the following year of the ewes and their female lambs. Female lambs were selected to enable a long-term study, whereas the male lambs were not retained.

The positive ewes were selected from the animals that were JSRV-positive at least once in December 2002 and February 2003 tests. The negative ewes were animals constantly negative in the December 2002 and February 2003 tests. Following this criterion, in May 2003, 50 positive ewes and their 65 offspring, were bled together with 49 negatives ewes and their 52 offspring.

When blood was collected in May 2003 the lambs were between 20 –30 days old. At the May 2003 test the 50 positive ewes remained positive; 14 of their 65 lambs

Table 4.5. Cross breed distribution of JSRV positive ewes in December 2002 and February 2003.

	Dec 2002	Feb 2003
Cross breed	JSRV+/ JSRV- sheep(%)	JSRV+/ JSRV- sheep(%)
Milksheep x Suffolk	9/96 (9%)	32/92 (35%)
Milksheep x Texel	32/50 (64%)	28/45 (62%)
Suffolk x Texel	19/48 (40%)	20/50 (40%)

Table 4.6. Comparison of results for individual animals over the December 2002 and February 2003 bleeds.

Dec 2002	Feb 2003	No. animals	%
-	-	100	54%
+	+	50	27%
+	-	7	4%
-	+	28	15%

(22%) were positive; 4 ewes in the negative groups converted to positive (Table 4.7c); 9 of the 52 lambs (17%), were positive (Table 4.7a & b).

Tables 4.7. Summary of the results obtained in the selected positive and negative cohorts of ewes and their respective offspring in the May 2003 JSRV U3 PCR test.

Table 4.7.a. In May 2003, 50 positive ewes and 65 lambs were bled. Positive results were confirmed in all the ewes; the presence of positive lambs related to the age and cross breed status of the mother it is shown between brackets.

	AGE			
Cross breed	2 years	3 years	4 years	6 years
Milksheep x Suffolk	11 ewes (2+ve lambs)	8 ewes (1+ve lambs)		
Milksheep x Texel	2 ewes (3+ve lambs)	5 ewes (2+ve lambs)	14 ewes (4+ve lambs)	2 ewes
Suffolk x Texel	8 ewes (2+ve lambs)			

Table 4.7.b. In May 2003, 49 negative ewes and 52 lambs were bled. Between brackets it is shown the presence of positive lambs related to the age and cross breed status of the mother. Four ewes that were negative in the December 2002 and February 2003 test resulted positive the May 2003 sampling (Table 4.7.c).

Cross breed	AGE 2 years	3 years	5 years
Milksheep x Suffolk	12 ewes (3 +ve lambs)	8 ewes (3 +ve lambs)	
Milksheep x Texel	7 ewes	11 ewes	1 ewe (1 +ve lambs)
Suffolk x Texel	8 ewes (1 +ve lambs)	2 ewes (1 +ve lambs)	

Table 4.7.c. Four ewes that were included in the negative cohorts (Table 4.7.b) of the dams became positive after the May 2003 test. Two lambs, offspring of these ewes were JSRV U3 PCR positive.

Ewe No.	Cross breed	Age	+ve lambs
12	Milksheep x Suffolk	2	1+ve lambs
24	Milksheep x Suffolk	2	
26	Milksheep x Suffolk	3	1+ve lambs
50	Milksheep x Suffolk	2	

A total number of 24, 2 years old rams which were the fathers of the May 2003 offspring were tested in May 2003. Eight out of the 24 rams were JSRV positive. Five of the 8 positive were pure Suffolk breed and other 3 were pure Texel Breed. After May 2003, the positive and negative cohorts of dams and their offspring were tested in September 2003, January 2004 and May 2004.

In September 2003 it was necessary to add other 39 ewes to the survey. The 39 ewes were already tested in December 2002 and February 2003. This was necessary to keep the different age and cross-breed groups of ewes balanced enough to carry out statistical calculation.

In September 2003, 78 of 138 (57%) ewes were JSRV positive. 32 of the 71 (45%) ewes with non Texel genes were JSRV positive; 46 animals of the 67 (68%) ewes with Texel genes were JSRV positive (Table 4.8). 33 of the 67 (49%) 2 years old animals were JSRV positive, 26 of the 50 (52%) 3 years old animals were positive; 19 of the 21 (90%) ≥4 years old animal were JSRV positive (Table 4.9).

Table 4.8. JSRV positive ewes in the September 2003 test.

The number of positive ewes is given for each different cross-breed group.

	September 2003
Cross breed	JSRV+ve / Total No. of ewes
Milksheep x Suffolk	32/71
Milksheep x Texel	30/37
Suffolk x Texel	16/30

Table 4.9. JSRV positive ewes arranged by age and cross-breed according to the September 2003 test results.
The number of JSRVpositive animals is indicated over the total number of ewes for each age and breed class of ewes.

	AGE			
	2 years	3 years	4 years	≥ 5 years
Cross-breed				
Milksheep x Suffolk	17/37	15/33	0/1	
Milksheep x Texel	5/6	7/12	13/14	5/5
Suffolks x Texel	11/24	4/5	1/1	

In January 2004, 69 of 124 (56%) ewes were JSRV positive. 27 of the 69 (39%) Milksheep/Suffolk cross-breed ewes were JSRV positive; 42 of the 55 (76%) ewes with Texel gene were JSRV positive (Table 4.10). 22 of the 58 (38%) 2 years old animals were JSRV positive; 30 of the 48 (63%) 3 years old ewes were JSRV positive and 17 of the 18 (94%) ≥4 years old animals were JSRV positive (Table 4.11).

Table 4.10. JSRV positive ewes in the January 2004 test.
The number of positive ewes is given for each different cross-breed group.

	January 04 test
Cross-breed	JSRV+/ total No. of ewes
Milksheep x Suffolk	27/69
Milksheep x Texel	33/33
Suffolk x Texel	9/22

Table 4.11. JSRV positive ewes arranged by age and cross-breed according to the January 2004 results.
The number of JSRVpositive animals is indicated over the total number of ewes for each age and breed class of ewes.

Cross-breed	AGE 2 years	3 years	4 years	≥ 5 years
Milksheep x Suffolk	12/36	15/32	0/1	
Milks x Texel	6/6	11/11	14/14	2/2
Suffolks x Texel	4/16	4/5	1/1	

In May 2004, 106 ewes were bled; 47 (44%) were JSRV positive (Table 4.12).

12 of the 53 (23%) Milksheep x Suffolk cross-breed were JSRV positive. 35 of the 53 (66%) ewes with Texel genes were JSRV positive (Table 4.12). For one of the milksheep x Suffolk cross breed the age could not be identified. In May 2004 only 49 of the initially 2 years old ewes could be bled, 19 of which were JSRV positive, corresponding to 39%; 15 of the 43 (35%) 3 years old ewes were JSRV positive; only 13 animals that at the beginnning of the survey were ≥4 years old could be tested, all of them were JSRV positive (Table 4.13).

Table 4.12. JSRV positive ewes in the May 2004 test.
The number of positive ewes is given for each different cross-breed group.

	May 04 test
Cross breed	JSRV+/ total No. of ewes
milksheep x suffolk	12/53
milksheep x texel	24/29
Suffolk x texel	11/24

Table 4.13. JSRV positive ewes arranged by age and cross-breed according to the January 2004 results.
The number of JSRVpositive animals is indicated over the total number of ewes for each age and breed class of ewes.

	AGE			
	2 years	3 years	4 years	≥ 5 years
Cross breed				
Milksheep x Suffolk	7/23	5/29	0/0	
Milksheep x Texel	2/5	9/11	9/9	4/4
Suffolks x Texel	10/21	1/3	0/0	

In summary the ewe population after May 2003 had a percentage of positive animals of 57% and 56% in September 2003 and January 2004 respectively; in May 2004 the percentage of positive animals decreased to 44% (Table 4.14).

Table 4.14. Percentage of JSRV infected ewes examined at 3-5 months intervals, until May 2004.

Test date	Tot. No. of tested ewes	No. of JSRV+ve ewes	% of JSRV+ve ewes
September 2003	138	78	57%
January 2004	124	69	56%
May 2004	106	47	44%

In May 2003 a group of 117 lambs was chosen for the longitudinal survey. 23 of 117 (20%) lambs were JSRV positive. In September 2003, 54 of 114 (47%) lambs were positive. In January 2004, 110 lambs of the original group could be tested and the number of JSRV positive lambs increased to 65 (59%). In the following May 2004 test, 42 of the 100 lambs were tested JSRV positive. In May 2004 the percentage of positivity dropped to 42% (Table 4.15). The lambs results were subjected to further statistical analysis. The aims were to explore:

- if the positivity of the mother could influence the positivity of the lambs;
- if there were any other factors such as age and breed of the mother that could have influenced the test results in the lambs.

During the statistical survey period clinical OPA was not detected in the ewes or in the lambs.

Table 4.15. Percentage of JSRV infected lambs examined at 3-4 months intervals, from May 2003 until May 2004.

Test date	Tot. No. of tested lambs	No. of JSRV+ve lambs	% of JSRV+ve lambs
May 2003	117	23	20%
September 2003	114	54	47%
January 2004	110	65	59%
May 2004	100	42	42%

4.3.2 Statistical analysis of the ewes and lambs data

The data from ewes and lambs were collected in a working dataset as shown in Appendix XIII (Table XIII.1).

JSRV incidence was calculated for the lambs and the ewes that were present at all sampling times.

JSRV conditional incidence was calculated for the lambs and the ewes including either animals with a complete record or animals with a missing test result, where the missing test result was subsequent to a positive observation.

4.3.2.1 JSRV incidence in the ewe population

Fifty- five ewes had a record with no missing tests. Confidence intervals were calculated using the exact formula for the binomial confidence interval (CI). In December 2002, 23 of 55 ewes with a complete record were tested positive; in February 2003 the number of JSRV positive animals increased to 32. In May and

September 2003, 35 animals were JSRV positive by PCR. In January 2004, 42 ewes were JSRV positive. In May 2004, 30 animals were JSRV positive.

JSRV fractional prevalence (n. of JSRV positive animals/ total n. of animals, for each test date) and the associated 95% confidence intervals were calculated (Table 4.16) and the results are represented in Fig. 4.2. From December 2002 to January 2004 a gradual increase in the number of positive animals was noticed; in the May 2004 test a decrease of the number of JSRV positive animals was observed.

4.3.2.2 JSRV conditional incidence in the ewe population

In the list of animals for the calculations of JSRV conditional incidence 83 ewes were included. These animals had a result record either with no missing values or with missing values that were subsequent to a positive observation (Table 4.17). In this calculation a greater number of ewes could be included for each single test. The fractional prevalence of JSRV positive animals was calculated for each single bleed These prevalences, and the associated 95% confidence intervals, are presented in the Fig. 4.3.

The data expressed in Table 4.17 can be analysed further to give an idea of the population of ewes at risk of becoming JSRV positive after each sampling (Table 4.18). The animals at-risk were defined as those animals not observed as positive up to and including the previous sampling occasion; then it was considered JSRV incidence rate at the sampling occasion, this was given by the number of new JSRV positive animals. In these data the first month of the study (December 2002) was not included because it was not possible to estimate the at-risk population.

Table 4.16. JSRV incidence in the ewe population that had a complete sampling record.

The incidence of JSRV infection was calculated taking in account 55 ewes that were present at all the sampling times. The number of JSRV positive ewes for each test, the fractional prevalence and the corresponding confidence intervals were calculated. Analysis of the pattern of incidence was done using a generalised linear model, with a binomial response distribution. The incidence rate for Feb-03 was significantly higher if compared to Dec-02 ($t=5.1$, $p<0.001$).

Test Date	Ewes with a complete record	JSRV positive ewes	Fractional Prevalence
Dec-02	55	23	0.31
Feb-03	55	32	0.45
May-03	55	35	0.51
Sep-03	55	35	0.51
Jan-04	55	42	0.62
May-04	55	30	0.47

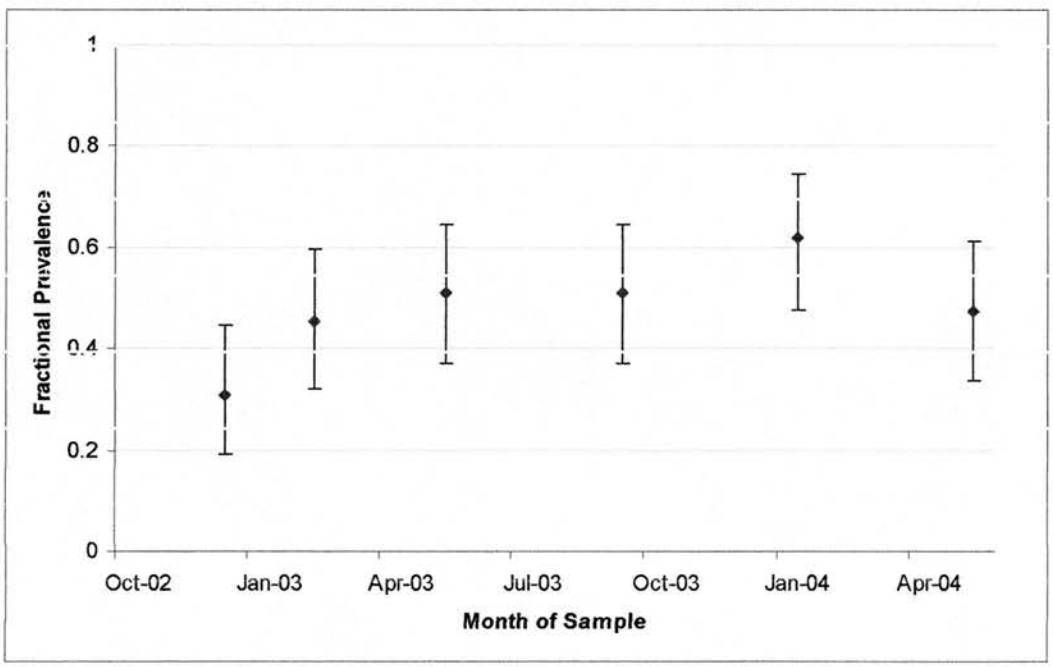


Fig. 4.2. JSRV infection in the ewe population that had a complete sampling record at different times.

JSRV incidence in each bleed was expressed as fractional prevalence. Fractional prevalence was expressed in the graph associated with 95% confidence intervals (CI).

Table 4.17. JSRV cumulative incidence in the ewe population that had either a complete sampling record or missing values subsequent to a positive observation. 83 animals included ewes with either no missing values or with missing value where missing values were subsequent to a positive observation. After each test the number of JSRV positive animals was recorded. Confidence intervals (CI) were calculated using the formula for the binomial CI.

Test Date	Ewes with complete record Or with missing value subsequent to a positive observation	JSRV(+ve) ewes	Fractional Prevalence
Dec-02	83	31	0.37
Feb-03	83	52	0.63
May-03	83	56	0.67
Sep-03	83	60	0.72
Jan-04	83	67	0.81
May-04	83	70	0.84

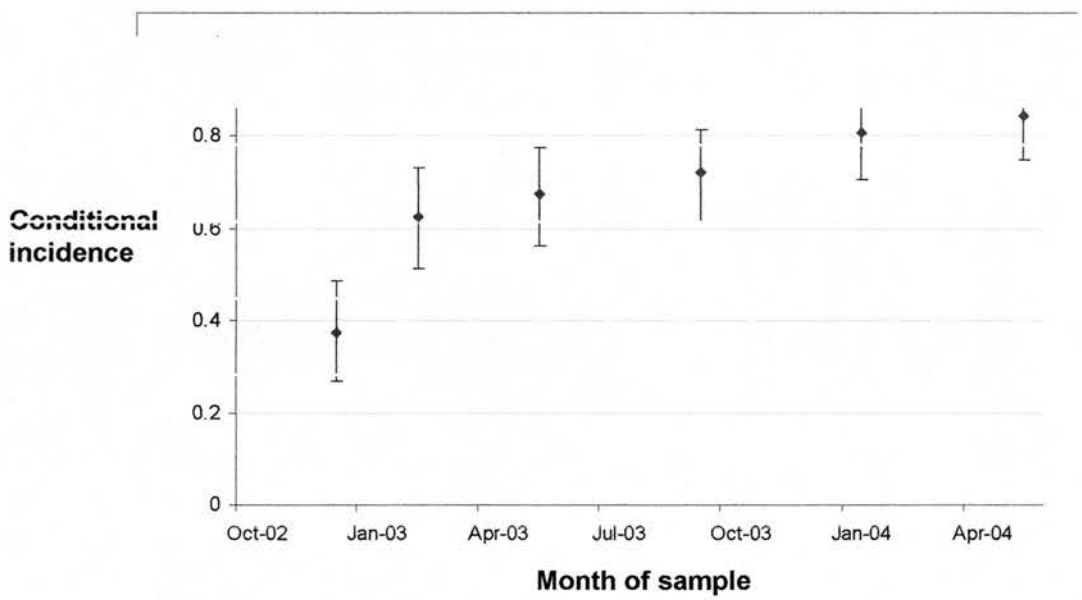


Fig 4.3. JSRV conditional prevalence in the ewe population that had either a complete sampling record or missing values subsequent to a positive observation.
83 animals included ewes with no missing values and ewes with missing value where missing values were subsequent to a positive observation. After each test the number of JSRV positive animals was recorded. Confidence intervals (CI) were calculated using the formula for the binomial CI.

Table 4.18. Incidence rates of new JSRV positive ewes after the first test (December 2002).

The animals at-risk were defined as those animals not observed as positive up to and including the previous sampling occasion; the JSRV incidence rate at the sampling occasion was given by the number of new JSRV positive animals in the following test. Data from December 2002 were excluded because was not possible to have an estimate of the at-risk animals.

Date	At-Risk Animals	Incidence of new JSRV +ve animals
Feb-03	52	21
May-03	31	4
Sep-03	27	4
Jan-04	23	7
May-04	16	3

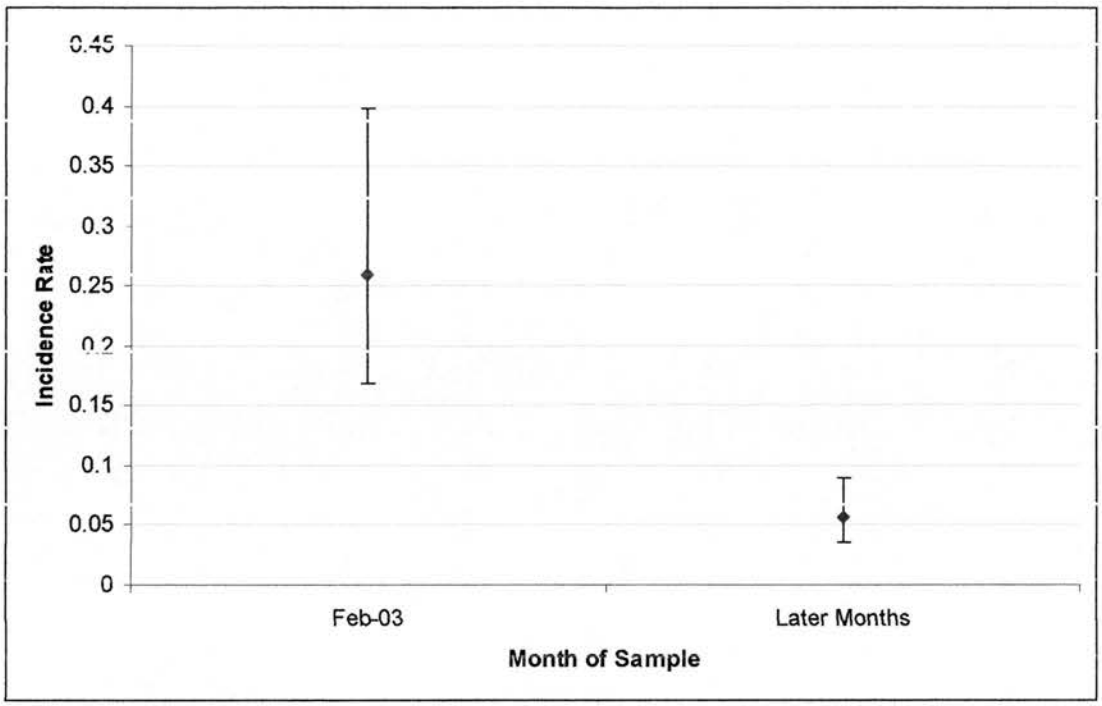


Fig. 4.4. Estimated JSRV incidence rate in the ewe population in February 2003 and in the later periods.

The incidence rates of new JSRV positive animals in February 2003 and in the later months were estimated and the 95% confidence intervals were generated.

In February 2003 the estimated incidence rate for the previous period corresponded to an incidence of 0.26 new observed JSRV positive animals per susceptible animal. In the later months, the estimated incidence rate corresponded to an incidence of 0.05 new observed JSRV positive animals per susceptible animal per month.

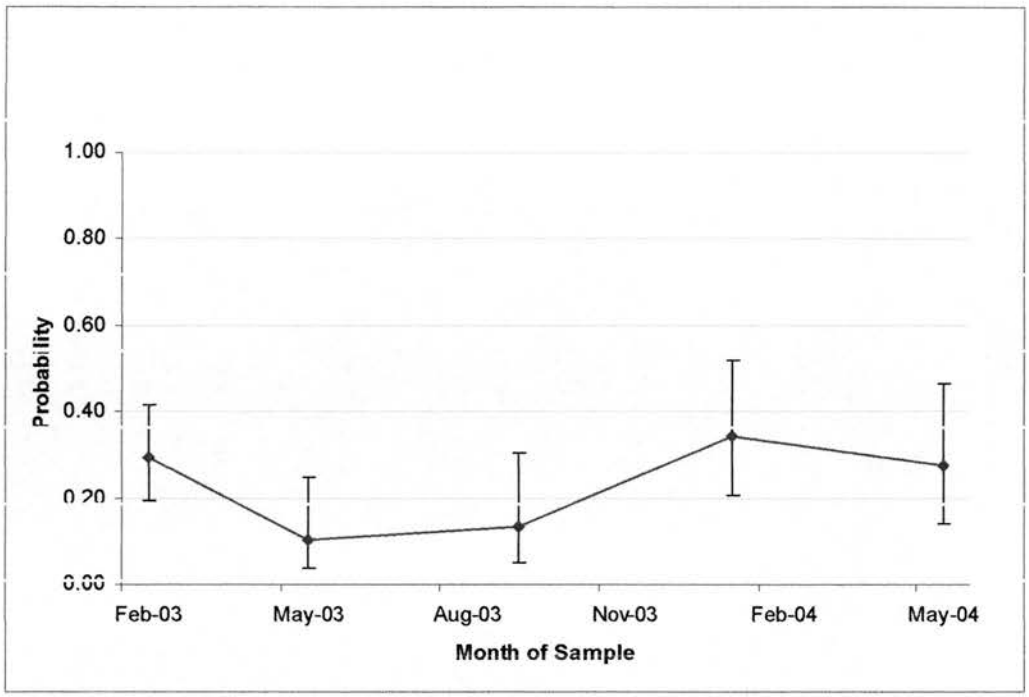


Fig 4.5. Estimation of the mean probability of observing JSRV positive cases related to the lambing period.

The results obtained from the May 2003 and 2004 bleeds were considered as belonging to the post-lambing period. The January, February and September observations were considered as pre-lambing period. The data collected in the pre-lambing and post-lambing periods were subjected to "generalised linear mixed model" analysis.
There was statistically significant evidence that the incidence rate of positive observations was significantly higher among the ewes immediately prior to lambing relative to the rates seen at other times ($p=0.04$).

The incidence rates for every month after the February 2003 were statistically significantly lower than that for February 2003 ($t=5.1$, $p<0.001$). However there was no evidence of any significant differences between the incidence rates observed in the subsequent months. In February 2003, the estimated incidence rate for the previous period corresponded to an incidence of 0.26 new observed JSRV positive animals per susceptible animal. In the later months, the estimated incidence rate corresponded to an incidence of 0.05 new observed JSRV positive animals per susceptible animal per month (Fig 4.4).

It was then explored the possibility to associate the analysis of the incidence rates of JSRV positive animals with particular physiological state of the ewe; the more obvious situation to consider was the lambing period. Hence, the May 2003 and 2004 observations were considered as belonging to the post-lambing period, and the January, February and September observations were considered as pre-lambing period. There was statistically significant evidence that the incidence rate of positive observations was significantly higher among the ewes immediately prior to lambing relative to the rates seen at other times ($p=0.04$) (Fig.4.5).

The consecutive set of results in the ewe population were evaluated to investigate the incidence of a positive observation conditional on the previous month having given rise to a negative observation.

The data across all the subsequent months were analysed together, and for each observation in the data set was also recorded whether any of the previous observations had been positive, for each ewe was taken in account the age and the cross-breed. The incidence of JSRV positive animals conditional on the previous month having given rise to a negative observation was not dependent on the effect of

the breedline, age or negative result in the previous test. These factors were not statistically significant.

Then it was investigated the incidence of observing a PCR negative result conditional on the previous month having given rise to a positive observation. As in the previous analysis all the subsequent months were analysed together and for each ewe the age and the cross-breed were considered. It was noticed that only the group of ewes which were aged 4/Texel breedline/non-Suffolk breedlines exhibited a higher mean probability ($p<0.001$) of remaining positive on consecutive samples.

4.3.2.3 JSRV incidence in the lamb population

JSRV incidence was calculated taking into account 72 lambs that registered a complete record over the study. The confidence intervals were calculated using the exact formula for the binomial CI. In May 2003, when the lambs were 20-30 days old, 21 animals out of 72 gave JSRV positive results. The number of positive lambs increased in September 2003 when 41 animals were JSRV positive. In January 2004, 44 out of the 72 lambs were JSRV positive. In May 2004, the number of JSRV positive lambs dropped to 31 out of 72 (Table 4.19). Confidence intervals were calculated using the exact formula for the binomial CI. The prevalences for each sampling date and the associated 95% confidence intervals were represented in Fig. 4.6. In Sep-03 there was a statistically significant increase of JSRV positive animals compared to May-03 ($p<0.01$). In May-04 the number of JSRV positive animals was statistically significantly lower than that for the Sep-03 period ($t=2.1$, $p=0.03$).

Table 4.19. JSRV incidence in the lamb population that had a complete sampling record.

72 lambs were present at all sampling times. The number of JSRV positive lambs for each test, the fractional JSRV prevalence and the corresponding confidence intervals were calculated. In Sep-03 there was a statistically significant increase of JSRV positive animals compared to May-03 ($p<0.01$). In May-04 the number of JSRV positive animals was statistically significantly lower than that for the Sep-03 period ($t=2.1$, $p=0.03$).

Test Date	N. of lambs with a complete record	JSRV positive lambs	JSRV fractional prevalence
May- 03	72	21	0.29
Sep-03	72	41	0.57
Jan-4	72	44	0.61
May-04	72	31	0.43

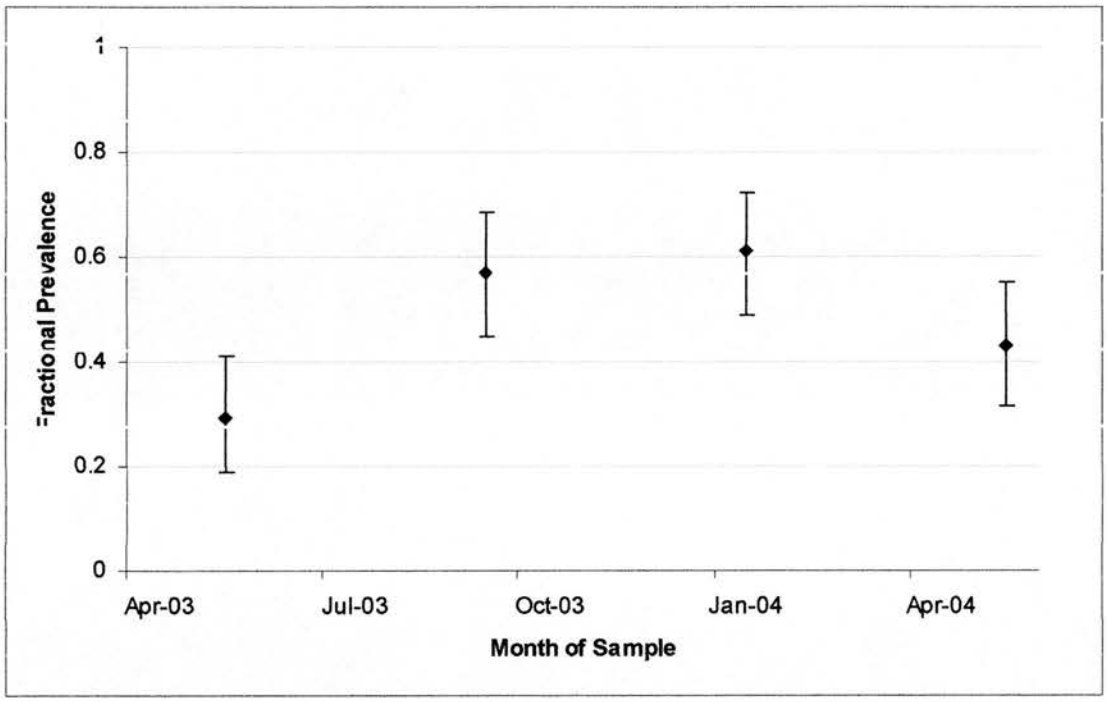


Fig 4.6. JSRV infection in the lamb population at different sampling times.

JSRV incidence for each test was expressed as JSRV fractional prevalence. The JSRV fractional prevalence was expressed in the graph associated with 95% confidence intervals.

4.3.2.4 JSRV conditional incidence in the lamb population

The lamb population was further analysed for the evaluation of JSRV conditional incidence. Eighty-two lambs had a sufficient record to be included in the analysis.

The number of JSRV positive animals was registered for each sampling time (Table 4.20) and the associated 95% confidence intervals were calculated. The JSRV fractional prevalence for each single test and the associated 95% CI were represented in Fig. 4.7.

The data in Table 4.20 were analysed and converted to provide a table of population at-risk (lambs not observed as positive up to and including the previous sampling occasion) and the incidence of new JSRV positive cases observed at the sampling occasion (Table 4.21). The estimates of new JSRV positive cases for each month were reviewed; the incidence rates for month May 2004 were statistically significantly lower than that for the initial months ($p=0.04$) (Fig.4.8). However, there was no evidence of any significant differences between the incidence rates observed in the first two months.

These estimates of new JSRV cases in the earlier months and in May 2004 were converted to incidence rates and 95% confidence intervals were generated (Fig. 4.8). In September 2003/January 2004, the estimated incidence rate corresponded to an incidence of 0.18 new observed infections per susceptible animal per month. In May 2004, the estimated incidence rate corresponded to an incidence of 0.04 new observed infections per susceptible animal per month.

The presence of a mother with Texel breedline was associated with a marginally higher chance of the lamb being observed to convert to positive but this effect was not close to statistical significance. The positivity of the mother was associated with

Table 4.20. JSRV cumulative incidence in the lamb population that had either a complete sampling record or missing values subsequent to a positive observation.

82 lambs had either a complete record or a missing value subsequent to a positive observation. The number of JSRV positive cases was reported for each sampling time; JSRV fractional prevalence and the relative 95% confidence intervals were calculated.

Test date	Tot. N. of lambs considered	JSRV +ve lambs	Fractional prevalence	95% Confidence Interval	
May-03	82	26	0.32	0.218652842	0.429156
Sep-03	82	55	0.67	0.558141419	0.770579
Jan-04	82	68	0.83	0.730163982	0.903395
May-04	82	70	0.85	0.758320584	0.922032

Table 4.21. Incidence rates of new JSRV positive lambs after the first test (May 2003).

The animals at-risk were defined as those lambs not observed as positive up to and including the previous sampling occasion; the JSRV incidence rate at the sampling occasion was given by the number of new JSRV positive animals in the following test. Data from May 2003 were excluded because was not possible to have an estimate of the at-risk animals.

Sampling Date	Lambs At-risk	Incidence of new JSRV(+ve) lambs
Sep-03	56	29
Jan-04	27	13
May-04	14	2

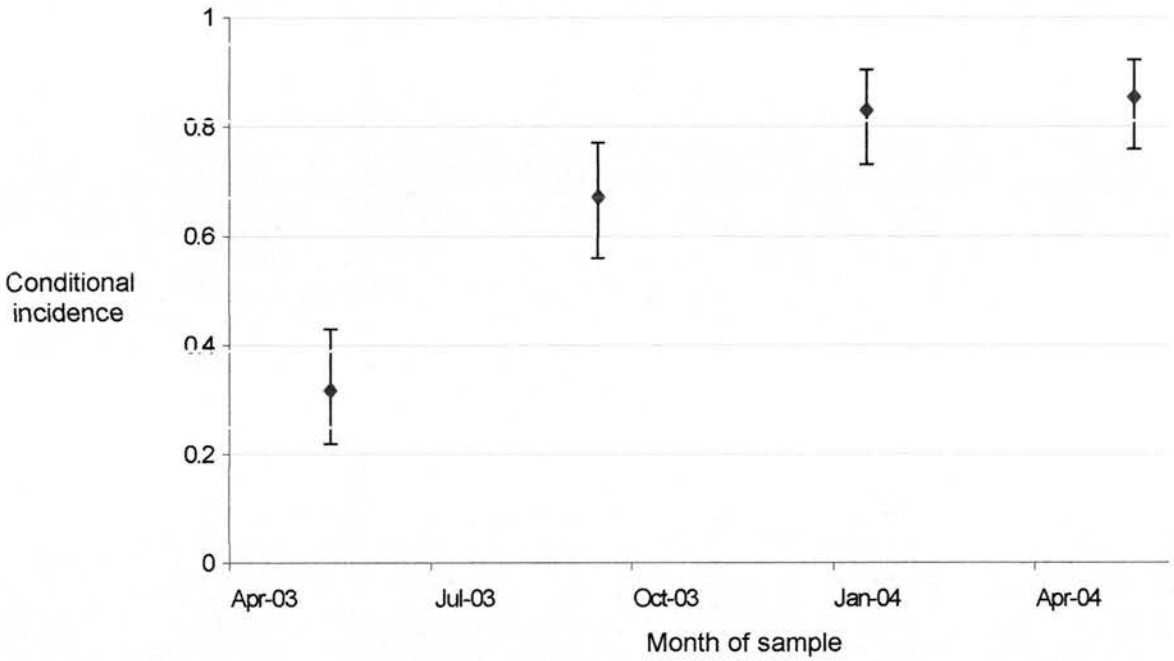


Fig 4.7. JSRV conditional incidence in the lamb population that had either a complete sampling record or missing values subsequent to a positive observation.

82 lambs were included. The 82 lambs had either a complete record or a missing values subsequent to a positive observation. The number of JSRV positive cases was reported for each sampling time; JSRV fractional prevalence and the relative 95% confidence intervals (Table 4.20) were represented.

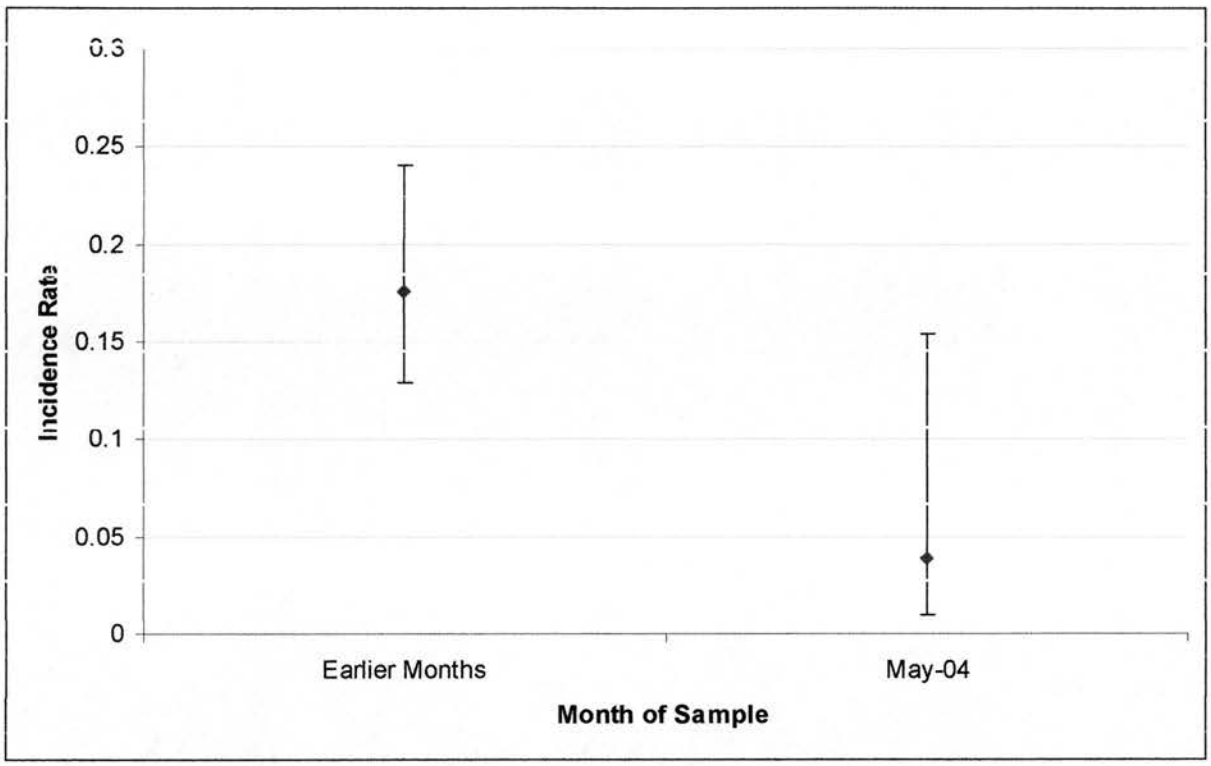


Fig. 4.8. Estimated JSRV incidence rate in the lambs population in earlier month and in the last sampling time (May 2004).

The incidence rates of new JSRV positive lambs in the earlier months (September 2003 and January 2004) and in the later month (May 2004) were estimated and the 95% confidence intervals were generated.

The incidence rates for May 2004 are statistically significantly lower than that for the initial months (p=0.04).

a higher risk of the lamb converting to positive, but none of these effects was statistically significant (p= 0.7-0.8). The results were consistent with the hypothesis that animals with Texel mothers became positive earlier in time, more quickly, than those born to non-Texel line ewes, but this result was not formally statistically significant (p=0.28).

The lamb data were elaborated analysing the incidence of negative observations conditional on previous observations being positive. There was no evidence that factors which influenced the mothers affected the lambs in the same way.

It was significant that a prior observation of a positive sample in the lamb made it more likely that subsequent samples were positive (p=0.005).

The estimates for the probabilities of remaining positive were consistently lower in the lambs than in the ewes, for each of the sampling months.

Statistically significant differences in the incidence probabilities were registered in different months (p=0.003): there was clear evidence of a down wards trend in the probability, similar to that seen in the ewes.

The positive JSRV status of the mother considered over the whole study was associated with a higher probability of the lamb remaining positive, but did not give a statistically significant value.

4.3.3 PCR analysis of the foetal tissues from OPA diseased sheep: results

In total 14 foetuses were collected (Table 4.22) from 8 ewes (A-H). It was not possible to collect blood samples from ewes A, D and H. Foetal lung tissue was

tested for all the 14 collected foetuses. From some of the fetuses (A1, E1, E2, F1, G1,H1 and H2) kidney and/or thymus tissues were collected.

The lungs from six fetuses were positive by PCR. In lambs G1, H1 and H2 when the lung tissue was positive the kidney or thymus tissues also were positive (Table 4.22).

Table 4.22. JSRV U3 PCR analysis of foetal tissues collected from OPA diseased ewes.

A,B,C,D,E,F,G,H indicates the mothers of the collected fetuses. All the ewes except E ewe were positive for OPA after clinical and histopathology examinations. Ewes B,C,E,F,G were positive for JSRV provirus by PCR from the blood. Lung tissue could be analysed for all the 14 fetuses. Thymus and kidney could be collected only from 4 and 2 fetuses, respectively. ND=not done. Fetuses were collected approximately between the second months and the last days of gestation.

Ewes OPA status	Ewes JSRV infection in the blood	Foetus n.	Foetal tissues		
			lung	thymus	Kidney
+	ND	A1	neg	neg	ND
		A2	neg		
+	+	B1	neg	ND	ND
		B2	neg	ND	ND
		B3	neg	ND	ND
+	+	C1	pos	ND	ND
+	ND	D1	pos	ND	ND
		D2	pos	ND	ND
-	+	E1	neg	neg	ND
		E2	neg	neg	ND
+	+	F1	neg	ND	neg
+	+	G1	pos	ND	pos
+	ND	H1	pos	pos	ND
		H2	pos	pos	ND

4.4 Discussion

This longitudinal survey represents the first example of a longitudinal molecular epidemiology survey for JSRV, in a flock of about 200 ewes and related offspring over 2 years of study.

Using the JSRV U3 PCR on blood, the flock tested for JSRV infection showed a high proportion of infected animals in Dec 2002. In fact at this time 60 (31%) of the 194 ewes were positive. Three months later the percentage of positive animals increased by 12% (Table 4.3). After the second test (February 2003), 80 of 187 (43%) tested ewes were positive. The high incidence of the initial December 2002 sample represents a long-term build-up of infection over the lifetime of the animals. The flock had a long history of OPA clinical cases although during the survey clinical OPA cases were not observed. The owner adopted the policy to promptly cull animals that had OPA signs. In the two years prior to the beginning of the survey, animals with OPA clinical signs were not noticed. It can be challenging doing *ante mortem* diagnosis of OPA if the tumour is not in an advanced state of development. Disease signs may be influenced by concurrent infections which may vary with different geographic areas and management practices. Longitudinal small studies in flocks with endemic OPA have shown that losses due to OPA can vary between 2% and 10% annually and at necropsy the tumour can be demonstrated in approximately 30% of the breeding sheep (Martin, unpublished data; Sharp & De las Heras, 2000). The flock under study had 60 of 194 and 80 of 187 infected ewes in December and February respectively without showing clinical signs. This situation shows that despite the high rate of infection in the flock the disease develops slowly. The fact that a high percentage of ewes and lambs in the study are JSRV viraemic

without clinical disease support the hypothesis that JSRV is a slow transforming retrovirus. The development of the clinical signs could be due to other factors such as co-infections, management practices, genetic factors and viral load such as for other small ruminant lentivirus infections (Peterhans *et al.,* 2004).

At the end of the experiment presented in this thesis, the animals will be culled. The necropsy of the ewes and of the lambs represents a crucial moment for evaluating the prognostic value of the JSRV U3 PCR in the field.

The JSRV U3 PCR showed its reliability during the survey. In fact from the results of the December 2002 and February 2003 tests, 50 animals of the ewe population had their JSRV positivity confirmed, 28 animals became JSRV positive, 100 animals stayed negative in both sampling time; seven animals were positive in December 2002 and tested negative in February 2003 (Table 4.6). The switching from positive to negative of the seven animals could be due to a variable amount of virus in the blood; the fluctuating positivity has been observed already in other studies (Garcia-Goti, 1999; Gonzalez *et al.,* 2001) and it can be due to a low viral load.

The trend of JSRV infection in the ewes showed an increase in the number of positive ewes until the May 2004 test (Table 4.14). The results collected in February 2003 showed a higher level of incidence ($p=0.04$) that can be interpreted as a residual effect arising from the use of a PCR test with less than 100% sensitivity on a population with well established infection. When this test was applied in the field situation and the target was not OPA diseased animals but JSRV infected sheep, the sensitivity and specificity can be considered below 100%. In the experimental infection of lambs of different ages the test was able consistently to detect viraemia before the onset of the clinical signs. This may be due to the high infectious dose

used in the experimental infection described in Chapter 3. The low viral load in blood is a major challenge for small ruminant lentiviruses detection. In addition, as shown in goats experimentally infected with CAEV and in sheep infected with MVV, the viral load may fluctuate over time and may differ between individual animals (Juste *et al.,* 1998; Greco *et al.,* 2002; Peterhans *et al.,* 2004).

A further step in the pathogenesis of OPA would be the investigation of viral load during natural and experimental JSRV infection. JSRV DNA and JSRV RNA in the lymphoid tissue and in the PBMC can be quantified by Real-time PCR (Niesters, 2002). Qualitative PCR provides no useful quantitative assessment of the infection level or disease progression and does not indicate pathogen replication. The ability to quantify using PCR can offer insights into the molecular pathogenesis of an infection. Quantification using Real-time PCR is rapid, accurate, with smaller risk of contaminations, and can provide an absolute determination of the number or copies of the targeted DNA. A reliable method for PCR quantification is by competitive PCR (Stöcher & Berg, 2002). This technique utilises a known amount of engineered internal standard which has the same primer binding sites as the target DNA or RNA. The internal standard is differentiated from the target on the bases of size by inclusion of a small deletion, or by including a single base mutation that allows separation after restriction enzyme digestion. Since the target DNA and internal standard are virtually identical, the efficiency of amplification should be equivalent, leading to a fair competition when co-amplified and an accurate measurement of the relative concentrations (Heid *et al.,* 1996; Gerard *et al.,* 1998). This eliminate tube-to-tube and template variability.

JSRV was detected more frequently in the animals that were 4 -7 years old (Tables 4.4, 4.9, 4.11; 4.13). In the 4-7 age group the percentage of JSRV positive ewes was 74% in December 2002 when the survey started and reached 100% in May 2004. The 4-7 year old ewes were consistently JSRV positive during the survey. This indicates that 4-7 year old animals had already established a detectable and constant level of viraemia when the survey started. For this group of animals the viral load remained over the detectable limit by PCR during the six JSRV PCR test. Natural disease has been usually observed in animals that are around 4 years of age; probably certain level of viraemia is a requisite for developing disease. For example a high HTLV-I proviral load is also associated with an increased risk of progression to disease (Barmak *et al.*, 2003).

In the 2 and 3 year old ewes the percentage of JSRV positive animal increased progressively until January 2004. In December 2002, 24% of the 2 years old 21% of the 3 years old were JSRV positive. In the second test (February 2003) the percentage of JSRV positive animals increased by 10% and 21% in the 2 and 3 years old group of animals respectively; in the following tests the increase of JSRV positivity was between 10 and 15%. In January 2004 only in the 2 years old group of ewes there was a 9% decrease of JSRV positive animals. The increased JSRV positivity from December 2003 and excluding May 2004 describe a built up of infection in the 2 and 3 years old of animals (Table 4.14). Negative and positive animals were managed together during period of this study, so it is possible that more animals were becoming JSRV positive because of an intra-flock transmission of the infection. Sheep of all ages could have acquired infection from direct and indirect contact with aerosols and lung secretions containing the virus. For example

horizontal transmission of MVV between adult sheep can be efficient (Berriatua, 2003).

In May 2004 the percentage of positive animals in the 3 years old group diminished by 28%. The same downwards trend regarding the percentage of JSRV positive animals could be observed in the lamb population (Table 4.15). The decreased JSRV positivity in the 2 and 3 years old ewe groups can be explained by the fact that the number of animals of these age groups included in the survey diminished especially in May 2004. In fact 96 of the 2 years old animals and 67 of the 3 years old animals were present in December 2002 test. In May 2004 the group of the 2 years old ewes was composed of 49 animals, and the group of 3 years old ewes was composed of 67 animals. Because the number of JSRV positive ewes and lambs in May 2004 decreased, other possible factors could have influenced the diagnostic test were investigated. After careful analysis, no difference could be found between the procedures adopted in May 2004 and those adopted in the previous tests.

JSRV was detected more frequently in animals with Texel genes. The percentage of JSRV positive animals was always higher in the animals with Texel genes than in the animals with non-Texel gene after each single test. The possibility of a Texel breed susceptibility was further analysed by statistical methods. Indeed the analysis of December 2002 and February 2003 highlights that the Texel breedline was associated with statistically significantly higher prevalences ($p<0.001$) over years 2 and 3, and a further statistically significant increase in year 4 and 5-7. It was noticeable that the statistical analysis of the consecutive test results showed that animals which were aged 4, Texel breedline, non Suffolk breedline exhibited a higher mean probability ($p<0.001$) of remaining positive on consecutive samples. In

animals of other ages and without Texel genes, the prior observation of a positive result made the animal more likely to be positive in the subsequent samples ($p<0.001$). Lambs with Texel breedline had a higher incidence of JSRV positive cases although it was not statistically significant ($p=0.67$). One explanation for the lack of statistical significance after the first two PCR tests was that the number of animals with Texel breedline was too low in the following tests.

The presence of Texel genes in the mothers influenced to a certain extent the JSRV positivity in the lambs. In fact, in May 2003 lambs with mothers that at the beginning of the survey were aged 4 -7 with Texel breedline had a higher JSRV prevalence although this result was not statistically significant ($p=0.65$). The lambs with mothers with Suffolk breedline had a lower prevalence ($p=0.56$). Lambs with Texel mothers were detected as JSRV positive earlier than those born from ewes without Texel genes. It is important to underline that the studied samples of each breedline are small and therefore may be influenced by the particular susceptibility to the disease of a certain family within the breed.

The possible involvement of a genetic factor that could influence the extent of infection and the outcome of the disease is an important finding of this study and it will deserve further future investigations. Susceptibility to infectious diseases and retroviral infections is influenced by the genetic background of the host. For example in scrapie, the fact that a genetic predisposition for this disease exists in sheep suggested that genetic selection could be helpful in the control and eradication of clinical disease within flocks (Hunter *et al.,* 1997; Baylis *et al.,*2004). The devastating visna maedi epidemic in Iceland reflected differences in the susceptibilities of Icelandic sheep and European breeds (Petursson, 1994).

Differences in breed susceptibility to MVV have been reported (Houwers *et al.,* 1989). Iceland breeds appear to be more susceptible than British breeds and Texels and Border Leicester are more susceptible to disease than Columbia sheep (Cutlip *et al.,* 1986; Joag *et al.,* 1996). Snowder *et al.,* (1990) determined significant differences in the seroprevalence of MVV between 6 breeds comprising a flock of 2,976 sheep. Nevertheless, complete breed-associated resistance has not been demonstrated and some authors suggest that apparent susceptibility may also depend on the viral strain (Houwers *et al.,* 1989).

Molecular biology genotyping techniques have become an every-day tool to solve a number of issues in the fields of detection and diagnosis of human and animal diseases (Rappuoli, 2004). Recent progress in cancer genetics has identified specific loci that are involved in cancer progression, many of which have key roles in DNA repair, cell-cycle control and cell-death pathways. Recent genetic studies and quantitative population-genetic analyses provide a framework for understanding the frequency of inherited mutations and the consequences of these mutations for increased predisposition to cancer (Frank, 2004). A larger epidemiological survey will be necessary to investigate a possible genetic predisposition to OPA. The survey should include a significant number of flocks of different breeds distributed in representative areas.

Another important finding regarding the features of JSRV infection was the statistically significant evidence that the incidence rate of JSRV positive ewes was higher among the ewes immediately prior to lambing relative to the rates seen at other times (Fig. 4.5). The immune system can play a role in the fluctuation of positive results investigated in the ewe population.

Pregnancy influences the cytokine balance even in the case of infectious diseases in the maternal periphery (Entrican, 2002a & b). For example experimental infection of pregnant mice with the protozoan parasite *Leishmania major* does not result in placental invasion by the parasite, the infection stays in the peripheral blood. However, the pregnancy influences the quality of maternal immune response and in this particular case affects the outcome of the pregnancy. In non pregnant mice the host is able to generate a Th-1 type inflammatory response mediated by IFN-γ. Pregnant mice infected with *Leishmania major* differ from non-pregnant controls in developing larger cutaneous lesions and harbouring more parasites. They also show an increased production of IL-4, IL-5 and IL-10 and a reduced production of IFN-γ when compared with infected non-pregnant controls (Krishnan *et al,* 1996).

This study for the first time proved that JSRV infection happens very early in life. In fact PCR results on blood samples from 117 lambs that were 20-30 days old (in May 2003) showed that 23 animals (20%) were JSRV positive. Nine of the 23 positive lambs were the offspring of the JSRV negative group of ewes; the remaining 14 positive lambs were the offspring of the JSRV positive group of ewes (Table 4.7a & 4.7b). Because lambs were found infected so early in their life it is possible to presume the vertical route plays an important role in the transmission of the infection. During this study 6 of the 14 analysed fetuses were JSRV positive by PCR. These data suggest that the fetuses can be exposed to JSRV infection. This is evidence in favour of intrauterine/transplacental route of transmission.

The outcome of the statistical analysis on these data supported horizontal transmission as a route of infection. Firstly, having a positive mother was not a statistically significant factor for a lamb to be positive. Secondly, the increase in

JSRV positive samples in the second test in both the lamb and ewe populations, is probably due to horizontal transmission. The fact that the positive ewes were managed together with negative could have represented a risk factor for the newborn lambs.

Horizontal transmission for OPA has been demonstrated by many studies. OPA was transmitted by cohabitation of healthy with diseased sheep already in 1929 by de Kock, 1929. This was repeated by Dungal (1946) in Iceland, who housed non diseased sheep with diseased sheep. Dungal also proved that exhaled respiratory air of an affected sheep contained the infectious agent. In one experiment a diseased sheep was made to breathe through a 20% solution of glycerine in normal saline for 30 minutes. The injection of this mixture by intra tracheal and intrapulmonary routes in lambs caused the development of the disease (Dungal, 1946).

The separation of the JSRV positive ewes from the JSRV negative ewes and the complete separated management of the two groups and their respective offspring could help to clarify if the infection of lambs from negative ewes is due to co-habitation with JSRV infected ewes.

It was investigated, taking in account the overall results, whether a ewe's current and cumulative positive JSRV infection status were associated with a higher risk of the lamb converting to positive. It was found that a lamb had a higher risk of converting to positive and staying positive if the mother was JSRV infected, but it was not statistically significant (p in the range 0.7-0.8).

The finding of JSRV positive fetuses in this study were consistent with a previous study by De las Heras *et al.* (2000) in which tissues and blood were collected from five fetuses from 2 Spanish ewes and 4 fetuses from 2 Scottish ewes. The ewes had

OPA confirmed by histopathology. PBLs and lung tissues from the fetuses were analysed. JSRV was detected by PCR in the lungs of 4/5 foetuses from one Spanish ewe and in PBMCs from two foetuses from one of the Scottish ewe.

Previous experiments showed that OPA transmission could be prevented by embrio-transfer (Parker *et al.,* 1998). Two-hundred-and-fifteen embryos recovered from 76 donor ewes from flocks endemically infected with OPA, and mated with uninfected rams, were transferred to 131 uninfected recipients under strict sanitary conditions, using International Embryo Transfer Society protocols. The recipients and their progeny were kept in a closed, isolated OPA-free flock. Thirty-eight of 51 progeny from OPA-positive donors and 55 of 74 progeny from donors in which no lesions of OPA were detected survived for at least five years after birth. No evidence of OPA was found in the recipients or their progeny by embryo transfer (Parker *et al.,* 1998). This protection offered by embryo transfer can be due to the fact the embryos are usually recovered 1-5 days after mating (Kiessling *et al.,* 1986). This can be a very short period for allowing the development of the infection in the embryo. Moreover it has been proved that the zona pellucida appears to provide an effective physic barrier protection to infection of embryos (Sinowatz *et al.,* 2001). Recently, it has been demonstrated that 8–16 cell (stage of) caprine embryos can be infected in vitro with CAEV if the zona pellucida is removed but not if the zona pellucida is intact (Lamara *et al.,* 2002). The fetuses analysed in this thesis and the fetuses in the spanish study were collected during the late stage of pregnancy.

Further experiments involving separation of newborn lambs from infected ewes immediately after birth will be necessary for establishing more evidence for vertical transmission. A certain number of the ewes involved in the epidemiological survey

described in this chapter will be culled before May 2005 and examined for gross and histological evidence of OPA lesions. It will be important to obtain foetuses from positive and negative dams. The foetuses will be carefully removed from the uterus to avoid contamination by maternal blood. PBLs from cardiac blood, lung and thymus will be obtained and analysed for JSRV. The foetal lungs will be examined for presence of tumour lesions by histopathology.

It is possible that both horizontal and intrauterine/transplacental can happen for JSRV transmission; the built up of JSRV infection in the ewe and lamb population can be due to horizontal transmission. In SRLV infection the issue of whether intrauterine infection occurs has been addressed in several studies and is still the most controversial route of transmission (Blacklaws *et al.,* 2004).

In this study 8 of the 24 rams (fathers of the May 2003 offspring) were JSRV positive. The role of JSRV infected rams for breeding to uninfected ewes requires further investigations. Transmission of infection via semen in OPA has not been studied yet.

Milk transmission has been well described and has a central role for small ruminant lentivirus infection (Sihvonen, 1980). Transmission of infection from the mother to the offspring via milk is a central point in the pathogenesis of other retroviral infections such as MLV, MMTV and HTLV-1 (Gardner et al., 1991; Acha-Orbea & MacDonald, 1995; Cann & Chen, 1996). Further studies are necessary to investigate if JSRV has tropism not only for the epithelial cells of the lung but alaso for mammary epithelial cells.

It was statistically significant in the lamb population that a prior observation of a positive sample makes the animal more likely to be positive in subsequent samples

(p=0.005). The estimates for the probabilities of remaining positive were consistently lower in the lambs than in the ewes, for each of the sampling months. This situation can be explained by the younger age of the lambs.

The JSRV U3 PCR gave consistent results along the course of the study. The presence of JSRV provirus in PBMCs represents a crucial point in JSRV pathogenesis. In experimentally infected lambs, JSRV proviral DNA could be detected in lymphoid cells as early as 7 d.p.i. when no histological signs of SPA were present (Holland *et al.,* 1999). Palmarini *et al.,* (1996b) found consistently JSRV proviral DNA and RNA in the mediastinal lymph nodes of OPA affected animal. In addition RNA was found in the spleens, in the bone marrows, in the thymuses and in the PBMC of naturally affected animals. As for other retroviral diseases the infection of lymphoid tissues can play a central role in the pathogenesis.

The role of macrophage and dendritic cells in the lung could be crucial for the establishment of the persistent and latent infection in the lymphoid tissue. For example it is now accepted that the major viral reservoir during asymptomatic HIV infection is lymphoid tissue (Pantaleo *et al.,* 1991; Pantaleo *et al.,* 1993; Embretson *et al.,* 1993; Blacklaws, 1997).

Present OPA knowledge is unable to explain the interaction between JSRV and immune system. JSRV provirus is present in PBMC after one week from JSRV experimental infection and until the animal will not present clear clinical signs of OPA. Holland *et al.,* (1999) showed that the provirus was present in macrophages.

The interaction between the macrophages and the immune system could explain the fact that the virus is fluctuant in the blood.

Macrophages are a crucial component of the innate and adaptive immune system and control several cell-mediated immune responses (Pierson *et al.,* 2000; Khati *et al.,* 2001). Macrophages are the target of HIV (Yi *et al.* 1998) and, since they are resistant to the cytopathic effects of HIV infection, they play a significant role in spreading the virus through the body contributing to AIDS pathogenesis (Gordon *et al.,* 1995). Since chronically infected macrophages allow the virus to escape detection by the immune surveillance system, they are recognised as a sanctuary of HIV and one of the main reservoirs in HIV infection (Mazzucchelli *et al.,* 2004). Macrophages that are centrally involved in both the innate and adaptive arms of the immune system are not only the main target of the human immunodeficiency virus (HIV), but also its main reservoir and vehicle of transmission. Macrophage-tropic (M-tropic) viruses are responsible for the initial infection, asymptomatic phase and persistence (Verani *et al.,* 2005). Functional impairment of HIV-infected macrophages plays a role in the immune dysregulation characteristic of acquired immunodeficiency syndrome (AIDS) (Herbein *et al.,* 2002). Efforts directed at understanding the cellular and molecular mechanisms underlying HIV-macrophage interactions remain the basis for devising novel and efficacious therapeutic strategies against HIV ant the AIDS epidemic (Khati *et al.,* 2001).

An interesting feature of OPA-affected animals is the increased numbers of macrophages in the lungs, and it is this cell type which contains the greatest proviral burden (Verwoerd *et al.,* 1985; Jassim *et al.,* 1987; Jassim, 1988). JSRV Infected macrophages could have a functional impairment and the same time could modulate the T cells present in their vicinity.

The interaction between type B and type D retroviruses and lymphoid cells have been demonstrated to be an important feature in the pathogenesis of the related diseases. B lymphocytes have a central role in the pathogenesis of MMTV infection of the mammary gland. MMTV initially infects B lymphocytes and then is delivered to the mammary gland through T cell-B cell interaction (Golovkina *et al.,* 1992; Held *et al.,* 1994). SRV-1 *in vivo* has a broad lymphoid tropism involving lymphocytes and mononuclear cells; infection leads to both T and B lymphocyte depletion although monocyte and macrophage function appears to be unaffected (Maul, *et al.,* 1988). In lambs naturally infected with MVV, viral RNA can be detected in PBMCs taken from animals less than one year of age. During MVV infection PCR tests in animals less than one year old are informative about the infection status of the lamb. In fact, during MVV infection, animals less than one year of age rarely show seropositivity and an increased seropositivity occurs with age (Johnson *et al.,* 1992; Snowder *at al.,* 1990). A number of PCR assays have been described which detect MVV DNA in infected tissues, especially bone marrow, PBMCs and pulmonary leukocytes (Brodie et al., 1993; Johnson *et al.,* 1992; Celer *et al.,* 2000). A PCR test has been used to detect infected sheep in the Dutch National MVV/CAEV control program (Wagter *et al.,* 1998).

To date JSRV U3 PCR developed in this study is the only available current option for planning OPA control strategies. Serological tools are a cheaper choice but because the lack of antibodies in OPA they cannot be considered.

In the light of current knowledge and because co-infection of flocks with JSRV and SRLV are very common (Dawson *et al.,* 1990; Gonzalez *et al.,* 1993) it would be very interesting in selected flocks where this co-infection is present to initiate JSRV

control programmes according to the roles already adopted in many EU Country for SRLV. Due to the increased role of free trade of live animals, embryos and semen in the EU, OPA should not be ignored. Control programmes for MVV have been initiated in many countries (Rowe & East, 1999). The European pioneer in the control of SRLVs was the Netherlands, where the eradication of MV in breeding flocks was initiated in the early 1980's (Houwers *et al.,* 1987). These programmes provided information on the routes of infection by SRLV sheep and goats.

The removal of serologically positive animals, as well as the separation of lambs and kids of seropositive dams immediately at birth for rearing on serologically negative or bovine colostrums, have been important features of successful MVV/CAE eradication programs (Rowe *et al.,* 1999). The separation of seronegative from seropositive flocks and the strict control of live animal trade are other important measures adopted. The prevention of contact is also necessary during transhumance. All programmes were initiated voluntarily and highlighted the crucial role played by motivated sheep and goat owners. (Peterhans *et al.,* 2004).

Vaccination for JSRV as well as for MVV/CAEV can be seen as a future goal. Vaccination for retroviral infection poses several problems. In general, retroviruses are relatively resistant to adaptive responses directed at viral antigens. This is evident from the productive infection by HIV (60 million infected people), human T cell leukemia virus (HTLV-1; 20 million infected people), and by many other animals retroviruses (Coffin *et al.*, 1997; Knipe & Howley, 2001). Most retrovirus-infected animals mount vigorous B and T cell responses to retroviral antigens (Sellom, 1993; Rimmelzwaan, *et al.*, 1994; Connor *et al.,* 1998). The ability of equine infectious anemia virus (EIAV) to persistently infect horses in the face of a profound immune

response by the host makes it an economically important disease for the horse population of the United States. Its ability to evade host immune defences by lying dormant in apparently healthy animals and by rapidly changing its antigenic determinants is proving to be a major obstacle to vaccine development (Howe *et al.*, 2002).

In the case of HIV, these responses (*i*) significantly reduce viral titers after the initial burst of viral replication early in infection (Coffin *et al.*, 1997); (*ii*) generate antibodies that can neutralize HIV *in vitro* (Sabin, 1992) and (*iii*) exert strong selective pressures that can shape the evolution of HIV genomes *in vivo* (Burns *et al.*, 1993; Burns. & Desrosiers, 1994; Goulder *et al.*, 2001; Carpenter et al., 1987). However, retroviral replication continues despite the antibody production in the vast majority of HIV-infected patients. This pattern is also observed in HTLV-1-infected patients and in a variety of animals infected by their retroviruses. EIAV, FIV, CAEV, MVV FIV (Burkard & Dean, 2003). The importance of humoral response is demonstrated by the lentiviruses that use extensive glycosylation of their envelope proteins to shield their virions from host antibodies. Retroviral resistance to adaptive immune responses is usually attributed to (*i*) the rapid and error-prone nature of retroviral replication, which allows for antigenic drift and the subsequent proliferation of escape mutants, and (*ii*) the unique problems posed by proviral insertion in host cell chromosomes (Coffin *et al.*, 1997).

Another complication in the complex relationship of the retrovirus with the host is given by the presence of endogenous retroviruses. For example a strain of avian leukosis virus (ALV) belonging to a new envelope subgroup J was isolated in the UK in 1988 from meat-type chickens. The disease caused by the members of this

subgroup has since spread very rapidly worldwide and has become one of the major problems facing the broiler meat industry. Molecular characterisation of the prototype of subgroup J, has shown that it has a structure of a typical ALV with *gag*, *pol* and *env* genes. However the *env* gene was distinct from that of other ALVs and was closely related to that of novel endogenous retroviral elements designated EAV-HP. As other regions of the genome were closely related to ALVs, it is believed that ALV-J has evolved by recombination with the *env* sequences of EAV-HP. Recent data show that ALV-J isolates evolve rapidly resulting in sequence changes within the variable regions of the *env* gene leading to antigenic variation. Eradication programmes established for other subgroups are proving to be effective in eradicating ALV-J from infected flocks (Venugopal, 1999).

It is not known how the exogenous JSRV interacts with its endogenous counterpart. enJSRVs are highly active, they are abundantly and specifically expressed in the epithelium of most of the ovine female reproductive tract. The specific spatial and temporal expression of enJSRVs supports a role in trophoblast development and differentiation as well as *conceptus* implantation. In addition, enJSRVs expression during foetal ontogeny could lead to the apparent tolerance of sheep towards the pathogenic JSRV (Palmarini *et al.,* 2004).

FeLV-induced lymphomas are a significant veterinary problem. Several commercial FeLV vaccines are now available. Some of these vaccine were based on inactivated whole virus or a mix of viral proteins, predominantly Env, or recombinant SU proteins and the overall experience for FeLV has been positive (Tartaglia *et al.,* 1993). Other experiences for Bovine leukaemia virus, Human T-cell leukaemia virus and equine infectious anemia virus, were directed towards the productions of vaccine

based on Env protein (Sugimoto *et al.,* 1994; Leroux *et al.,* 2004; Sundaram *et al.,* 2004). The first step towards vaccination for JSRV will attempt the use of recombinant envelope proteins. A vaccine for OPA can be considered as a possible future tool for the control of this disease. The optimisation of the challenge system to induce OPA in older lambs (Chapter 3) represents a central point for a JSRV vaccination project. This challenge system offers also the possibility to evaluate the viraemia as a measure of protection. The fact that JSRV genome appears to be well conserved and there is little variation in isolates from well-dispersed geographic regions is another encouraging aspect for a future OPA vaccine project (Palmarini & Fan, 2003).

Chapter 5

General Discussion

Chapter 5. General discussion

Studies directed at a further understanding of the biology and pathogenesis of JSRV infection were undertaken, with an overall aim of developing techniques to support effective control of OPA. In addition, using the newly developed techniques, features of JSRV infection were studied to highlight new aspects of the pathogenesis of OPA. Part of the work consisted of the generation of new JSRV capsid and envelope recombinant proteins to use in immunisation experiments with viraemic and non viraemic sheep. This to test the hypothesis that JSRV viraemic sheep are immuno-compromised. Production of JSRV capsid and envelope protein was achieved using a cell-free production system. This is the first time that JSRV SU has been produced successfully. The novel JSRV SU was used for producing rabbit antiserum for IHC studies.

For the first time it has been shown that not only uninfected sheep respond to immunisation with JSRV CA, but also that JSRV viraemic sheep have the ability to develop an antibody response against JSRV CA. This result indicates that JSRV infection does not directly render the immune system non-responsive to JSRV antigens at the level of B cell responsiveness. However, since the immunogen used in these studies consisted of a fusion protein, which contained non-viral protein that could act as a hapten carrier, it is not possible to draw conclusions concerning the responsiveness of T cells to the virus in infected animals. Further investigations will study the immune response towards JSRV recombinant proteins that only contain viral polypeptides.

Future research will be focused on the hypothesis that JSRV could interfere with

Antigen presenting cells. A number of viruses including HIV, vaccinia, measles and cytomegalovirus escape host immunity by interfering with dendritic cell (DC) function and the generation of effector T cells. For example, vaccinia virus evades immune responses by abortively infecting DCs, blocking their maturation and inducing apoptosis (Engelmayer *et al.,* 1999) while human cytomegalovirus (Tortorella *et al.,* 2000) and other viruses (Spriggs, 1996) can interfere with antigen processing via the MHC pathway. Retroviruses may inhibit local immune responses through immunosuppressive domains contained within the Env proteins (Cianciolo *et al.,* 1985; Manganey & Heidmann, 1998; Blaise *et al.,* 2001). There is no evidence to date to support the presence of immunomodulatory proteins encoded by JSRV although the function of the product of the JSRV *orf-x* gene remains unknown. Recent studies have revealed that T-regulatory cells generated from naïve precursors can induce peripheral tolerance, have a low proliferative capacity and can suppress both naïve and memory responses (Oldenhoven *et al.*, 2003). Examination of DC-lymphocyte interactions is required to identify the type of response induced by DCs activated by JSRV, and to determine whether T cell regulation is responsible for the loss of responses detected during vaccination and the lack of response detected in infected sheep.

Based on the findings of the current study, future vaccination programmes will aim to optimise further the combination of adjuvant and delivery route to produce stronger and more sustained responses to JSRV antigens and to determine whether these treatments can protect against JSRV infection and OPA. In particular, it will

be important to study the immune responses to the JSRV Env glycoproteins in sheep since these proteins are major immunogens in other retroviral infections.

Another important achievement obtained in this work was the development of an experimental animal model for OPA in lambs up to 6 months.

In the present study clinical disease has been reproduced, pathologically confirmed as OPA, in a high proportion of lambs inoculated intra-tracheally with infectious lung fluid at either one, three or six months of age. The incubation periods, however, were longer in the older age groups than in one week old lambs. The new experimental model for OPA will permit to test protective effects of any future vaccine to be evaluated.

JSRV experimental infection of animals at different ages confirmed that JSRV susceptibility is higher in neonatal lambs. This increased susceptibility of the neonatal animals to JSRV can be explained following the postnatal growth of the lung and evolution of major site of replication site of JSRV, the epithelial cells called pneumocytes type II (ATII) and Clara cells. High level of JSRV expression is detectable only in the transformed ATII and Clara cells of OPA affected animals (Palmarini *et al.,* 1995; Platt *et al.,* 2002). Further, the long terminal repeats of JSRV are specifically active in ATII and Clara cells (Palmarini *et al.,* 2000b).

The alveolar epithelium differentiates last in utero. Type I and II pneumocytes line the alveoli in a 1:2 ratio. In rodents and ruminants the alveoli develop for several days after birth (Kahwa *et al.,* 1997). In particular in ruminants, nonciliated bronchiolar epithelial cells retained foetal characteristics of differentiation, in calves that are 30 days old bronchiolar epithelium is well differentiated (Castleman & Lay,

1990, Kahwa *et al,* 2000). During this period, the type II pneumocyte labelling index declines from 6% at 7 days to about 1% at 3 weeks, and is 0.2% or less (Wright & Alison, 1984). Therefore the increasing OPA incubation period in 1, 3 and 6 months old lambs could be explained with the decline of the number of JSRV target cells.

IHC was performed using for the first time an anti-JSRV SU immune serum. JSRV SU protein was found widespread on the surface of tumour cells of the lung of animals infected at different ages (neonatal, 1 month, 3 months and 6 months old). The IHC results in this study gave new insight in to the histogenesis and viral pathogenesis of OPA. Widespread SU Env protein on the surface of tumour cells proves that envelope protein is consistently produced suggesting again a role in cell proliferation.

During the development of the OPA animal model, a highly specific and sensitive JSRV U3 PCR procedure was developed to provide high throughput assays of clinical samples. This to support the first extensive molecular epidemiology longitudinal survey in an OPA affected flock described in chapter 4. The one step JSRV U3 PCR test was the key technique for undertaking the epidemiology studies. This test was also used for monitoring the viraemia in lambs infected at different ages and for selecting viraemic sheep to use in immunisation trials.

The longitudinal survey was performed in a large group of dams and their offspring from a flock with natural history of OPA. The aim of this study was to investigate the pathogenesis of JSRV infection in a flock with natural history of OPA.

The characterisation of the behaviour of JSRV infection in natural condition gave, for the first time, the opportunity to investigate the routes of transmission of JSRV

and to analyse the relationship between viraemia and evolution of the disease. In particular the study i) determined the prevalence of JSRV infection, ii) compared prevalence in sheep of different ages and determined when infection first occurs, iii) investigated JSRV transmission from dam to lamb.

The group of dams of different ages tested for JSRV infection in blood, using JSRV U3 PCR, resulted heavily infected from the first test; in fact 31% of the ewes were positive. The trend of JSRV infection in the ewes showed an increase in the number of positive ewes until the May 2004 test. JSRV was detected more frequently in the animals that were 4 -7 years old. The 4-7 year old ewes were consistently JSRV positive during the survey. Despite the high number of infected ewes and lambs followed over a period of nearly 3 years, it was remarkable that animals with clinical signs of OPA could not be detected. This situation shows that despite the high rate of infection in the flock the disease develops slowly.

Post-mortem examination of these animals in the future part of the study will be very useful to correlate viraemia and tumour development.

This study for the first time proved that JSRV infection happens very early in life. In fact PCR results on blood samples from 117 lambs that were 20-30 days old (in May 2003) showed that 23 animals (20%) were JSRV positive. The percentage of JSRV positive lambs increased with time but none of the lambs showed signs of disease during the observation time. Foetuses from OPA affected mothers were found to be JSRV infected, indicating the possibility of a transplacental/intrauterine transmission of the virus. Further studies will be necessary to clarify the importance of each route of transmission. Future work that will involve the separation of newborn lambs from

infected ewes immediately after birth will be necessary for establishing more evidence for vertical transmission. The possibility of the presence of JSRV in the milk will be investigated.

This first extensive epidemiological survey for natural JSRV infection represents a breakthrough for future JSRV control and management studies.

References

Acha-Orbea, H. & MacDonald, H.R. (1995). Superantigens of mouse mammary tumor virus. *Annu Rev Immunol* 13, 459-486.

Alberti, A., Murgia, C., Liu, S.L., Mura, M., Cousens, C., Sharp, J.M., Miller, A.D., Palmarini, M. (2002). Envelope-induced cell transformation by ovine betaretroviruses. *J Virol* 76, 5387-5394.

Alcorn, J.F. & Wright J.R. (2004). Surfactant protein A inhibits alveolar macrophage cytokine production by CD14 pathway. *Am J Physiol Lung Cell Mol Physiol* 286, 129-36.

Alian, A., Sela-Donenfeld, D., Panet, A., Eldor, A. (2000). Avian hemangioma retrovirus induces cell proliferation via the envelope (env) gene. *Virology* 276, 161-168.

Alimov, A.P., Khmelnitsky, A.Y., Simonenko, P.N., Spirin A.S, and Chetverin, A.B. (2000). Cell-free synthesis and affinity isolation of proteins on a nanomole scale. *BioTechniques* 28, 338–344.

Alkhatib, G., Broder, C.C., Berger, E.A. (1996). Cell type-specific fusion cofactors determine human immunodeficiency virus type 1 tropism for T-cell lines versus primary macrophages. *J Virol* 70, 5487-5494.

Allen, T.E., Sherrill, K.J., Crispell, S.M., Perrott, M.R., Carlson, J.O., DeMartini J.C. (2002). The jaagsiekte sheep retrovirus envelope gene induces transformation of the avian fibroblast cell line DF-1 but does not require a conserved SH2 binding domain. *J Gen Virol 83*, 2733-2742.

Al-Zubaidy, A. & Sokkar S.M. (1979). Studies on ovine pulmonary adenomatosis in Iraq. *Indian Vet J* 56, 360-362.

Andresson, O.S., Elser, J.E., Tobin, G.J., Greenwood, J.D., Gonda, M.A., Georgsson, G., Andresdottir, V., Benediktsdottir, E., Carlsdottir, H.M., Mantyla, E.O. (1993). Nucleotide sequence and biological properties of a pathogenic proviral molecular clone of neurovirulent visna virus. *Virology* 193, 89-105.

Armitage, P. & Berry,G. (1987). Statistical methods in medical research, Section 4.7., 2nd Edition, Blackwell, Oxford.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl,K. (1994). *Current protocols in Molecular Biology* Vol. 1. John Wiley and Sons, New York.

Aynaud, M. (1926). Origin verminouse du cancer pulmonaire de la brebis. *Comp Rend Sci Soc Biol* 95, 1540-1542.

Azran, I., Schavinsky-Khrapunsky, Y., Aboud, M. (2004). Role of Tax protein in human T-cell leukemia virus type-I leukemogenicity. *Retrovirology* 13, 1-20.

Bai, J., Bishop, J.V., Carlson, J.O., DeMartini, J.C. (1999). Sequence comparison of JSRV with endogenous proviruses: envelope genotypes and a novel ORF with similarity to a G-protein-coupled receptor. *Virology* 258, 333-343.

Bai, J., Payne, L.N., Skinner, M.A. (1995). HPRS-103 (exogenous avian leukosis virus, subgroup J) has an *env* gene related to those of endogenous elements EAV-0 and E-51 and an E element found previously only in sarcoma viruses. *J Virol* 69, 779-784.

Bai, J., Zhu, R.-Y., Stedman, K., Cousens, C., Carlson, J., Sharp, J.M., DeMartini, J.C. (1996). Unique long terminal repeat U3 sequences distinguish exogenous jaagsiekte sheep retroviruses associated with ovine pulmonary carcinoma from endogenous loci in sheep genome. *J Virol* 70, 3159-3168.

Baneyx, F. (1999). Recombinant protein expression in *Escherichia coli. Curr Opin Biotech* 10, 411-421.

Baranov,V.I., Morozov,I. Y., Ortlepp, S.A., Spirin, A.S. (1989).Gene expression in a cell-free system on the preparative scale. *Gene* 84, 463-466.

Barmak, K., Harhaj, E.W., Grant, C., Alefantis, T., Wigdahl, B. (2003). Human T cell leukemia virus type I-induced disease: pathways to cancer and neurodegeneration. *Virology* 308, 1-12.

Barnett, A.L., Wensel, D.L., Li, W., Fass, D., Cunningham, J.M. (2003). Structure and mechanism of a coreceptor for infection by a pathogenic feline retrovirus. *J Virol* 77, 2717-2729.

Basharov, M.A. (2003). Protein folding. *J Cell Mol Med* 7, 223-237.

Baxter, S. I., Pow, I., Bridgen, A. & Reid, H.W. (1993). PCR detection of the sheep-associated agent of malignant catarrhal fever. *Archives of Virology* 132, 145-159.

Baylis, M., Chihota, C., Stevenson, E., Goldmann, W., Smith, A., Sivam, K., Tongue, S., Gravenor, M.B. (2004). Risk of scrapie in British sheep of different prion protein genotype. *J Gen Virol* 85, 2735-2740.

Beemon, K.L.,. Faras, A.J Haase, A.T. Duesberg, P.H., Maisel. J.E. (1976). Genomic complexities of murine leukemia and sarcoma, reticuloendotheliosis, and visna viruses. *J Virol* 17, 525-537.

Bellacosa, A., Testa, J.R., Staal, S.P., Tsichlis, P.N. (1991). A retroviral oncogene, akt, encoding a serined-threonine kinase containing an SH2-like region. *Science* 254, 274-277.

Belshaw, R., Pereira, V., Katzourakis, A., Talbot, G., Paces, J., Burt, A., Tristem, M. (2004). Long-term reinfection of the human genome by endogenous retroviruses. *Proc Natl Acad Sci U S A* 6, 4894-4899.

Benjamin, T, & Vogt, P.K. (1990). Cell transformation by viruses. In: Fields BN, Knipe DM, Howley PM, editors. Virology. 2nd ed. New York (NY): Raven Press; p. 317–67.

Benson, S.J., Ruis, B.L., Fadly, A.M., Conklin, K.F. (1998). The unique envelope gene of the subgroup J avian leukosis virus derives from ev/J proviruses, a novel family of avian endogenous viruses. *J Virol* 72, 10157-10164.

Bergeron, D., Houde, J., Poliquin, L., Barbeau, B., Rassart, E. (1993). Expression and DNA rearrangement of proto-oncogenes in Cas-Br-E-induced non-T-, non-B-cell leukemias. *Leukemia* 7, 954–962.

Berriatua, E., Alvarez, V., Extramiana, B., Gonzalez, L., Daltabuit, M., Juste, R. (2003). Transmission and control implications of seroconversion to Maedi-Visna virus in Basque dairy-sheep flocks. *Prev Vet Med* 60, 265-279.

Best, S., Le Tissier, P., Towers, G., Stoye, J.P. (1996). Positional cloning of the mouse retrovirus restriction gene Fv1. *Nature* 382, 826-829.

Betton, J.M. (2000). Production of a soluble and active form of a defective protein with the rapid translation system. *Biochemica* 4, 7-10.

Billeter, M.A., Parsons, J.T., Coffin. J.M. (1974). The nucleotide sequence complexity of avian tumor virus RNA. *Proc Natl Acad Sci 71*, 3560-3564.

Birnboim, H.C. & Doly, J. (1979). A rapid alkaline lysis procedure for screening recombinat plasmid DNA. *Nucl Acids Res* 7, 1513-1522.

Bishop. J.M. (1983). Cellular oncogenes and retroviruses. *Annu Rev Biochem* 52, 301-354.

Bishop, J.M. & Varmus, H.E. (1984). Functions and origins of retroviral transforming genes. In: Weiss, R., Teich, N., Varmus, H., Coffin, J., editors. RNA tumour viruses. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory press; p. 475-585.

Blacklaws, B.A. (1997). Quantification of the reservoir of HIV-1. *Trends Microbiol* 5, 215-216.

Blacklaws, B.A., Berriatua, E., Torsteinsdottir, S., Watt, N.J., de Andres, D., Klein, D., Harkiss, G.D. (2004). Transmission of small ruminant lentiviruses. *Vet Microbiol* 101, 199-208.

Blackwood, E.M., Luscher, B., Eisenman, R.N. (1992a). Myc and Max associate in vivo. *Genes Dev* 6, 71-80.

Blackwood, E.M., Kretzner, L., Eisenman, R,N. (1992b). Myc and Max function as a nucleoprotein complex. *Curr Opin Genet Dev* 2, 227-235.

Blaise, S., Mangeney, M., Heidmann, T. (2001). The envelope of Mason-Pfizer monkey virus has immunosuppressive properties. *J Gen Virol* 82, 1597-600.

Blaschke, U.K., Silberstein, J., Muir, T.W. (2000). Protein engineering by expressed protein ligation. *Methods In Enzymology* 328 ,478-496.

Blond, J. L., Lavillette, D., Cheynet, V., Bouton, O., Oriol, G., Chapel-Fernandes, S., Mandrand, B., Mallet, F. & Cosset, F. L. (2000). An envelope glycoprotein of the human endogenous retrovirus HERV-W is expressed in the human placenta and fuses cells expressing the type D mammalian retrovirus receptor. *J Virol* 74, 3321–3329.

Boeke, J.D. & Stoye, J.P. (1997). Retrotrasposons, endogenous retroviruses and the evolution of retroelements. In J.M. Coffin, S.H. Hughes, and H.E. Varmus (ed.), Retroviruses, p.343-436. Cold Spring Harbor Laboratory Press, Plainview, N.Y.

Bonne, C. (1939). Morphological resemblance of pulmonary adenomatosis (Jaagsiekte) in sheep and certain cases of cancer of the lung in man. *Am J Cancer* 35, 491-501.

Borron, P.J., Mostaghel, E.A., Doyle, C., Walsh, E.S., McHeyzer-Williams, M.G., Wright, J.R. (2002).Pulmonary surfactant proteins A and D directly suppress CD3+/CD4+ cell function: evidence for two shared mechanisms. *J Immunol* 169, 5844-5850.

Boyd, M. T., Bax, C. M., Bax, B. E., Bloxam, D. L. & Weiss, R. A. (1993). The human endogenous retrovirus ERV-3 is upregulated in differentiating placental trophoblast cells. *Virology* 196, 905–909.

Bradac, J., & Hunter, E. (1984). Polypeptides of Mason-Pfizer monkey virus. I. Synthesis and processing of the gag-gene products. *Virology* 138, 260-275.

Bridgen, A. & Reid, H. W. (1991). Derivation of a DNA clone corresponding to the viral agent of sheep-associated malignant catarrhal fever. *Research in Veterinary Science* 50, 38-44.

Brodie, S.J., Pearson, L.D., Snowder, G.D., DeMartini, J.C. (1993). Host-virus interaction as defined by amplification of viral DNA and serology in lentivirus-infected sheep. *Arch Virol* 130, 413-28.

Brophy, P.M. & Pritchard, P.I. (1994). Parasitic helminth glutathione S-transferase: an update on their potential as targets for immuno- and chemotheraphy. *Exp Parasitol* 79, 89-96.

Brown, H. & Prescott, R. (1999). Applied mixed models in medicine. John Wileyed. Chichester.

Brown, P.O. (1997). Integration. In: Coffin JM, Hughes SH, Varmus HE, editors. Retroviruses. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; p. 161–203.

Bucciarelli, E. (1973). Pulmonary adenomatosis of sheep (Jaagsiekte). Ultrastructural study. *Lav Ist Anat Istol Patol. Universita' degli Studi di Perugia.* 33, 99-117.

Burkhard, M.J. & Dean, G.A. (2003). Transmission and immunopathogenesis of FIV in cats as a model for HIV. *Curr HIV Res* 1, 115-129.

Burns, D. P., Collignon, C., Desrosiers, R.C. (1993). Simian immunodeficiency virus mutants resistant to serum neutralization arise during persistent infection of rhesus monkeys. *J Virol* 67, 4104–4113.

Burns, D.P. & Desrosiers, R.C. (1994). Envelope sequence variation, neutralizing antibodies, and primate lentivirus persistence. *Curr Top Microbiol Immunol* 188, 185-219.

Cameron, P., Pope, M., Granelli-Piperno, A., Steinman, R.M. (1996). Dendritic cells and the replication of HIV-1. *J Leukoc Biol* 59, 158-171.

Cann, A.J. & Chen, I.S.Y. (1996). Human T-cell leukemia virus type I and II. In *Fields virology* (ed. B.N. Fields *et al.*), p.1849–1880. Lippincott-Raven, New York.

Carpenter, S., Evans, L. H., Sevoian, M., Chesebro, B. (1987) Role of the host immune response in selection of equine infectious anemia virus variants. *J Virol* 61, 3783–3789.

Casares, N., Lasarte, J.J., de Cerio, A.L., Sarobe, P., Ruiz, M., Melero, I., Prieto, J., Borras-Cuesta, F. (2001). Immunization with a tumor-associated CTL epitope plus a tumor-related or unrelated Th1 helper peptide elicits protective CTL immunity. *Eur J Immunol* 31, 1780-1789.

Castleman, W.L. & Lay, J.C. (1990). Morphometric and ultrastructural study of postnatal lung growth and development in calves. *Am J Vet Res* 51, 789-795.

Celer, V. Jr, Celer, V., Nejedla, E., Bertoni, G., Peterhans, E., Zanoni, R.G. (2000).The detection of proviral DNA by semi-nested polymerase chain reaction and phylogenetic analysis of Czech Maedi-Visna isolates based on gag gene sequences. *J Vet Med B Infect Dis Vet Public Health* 47, 203-215.
Ceretto, F. & Deiana , S. (1967). Adenomatosi polmonare enzootica degli ovini (jaagsiekte) in Sardegna. *Atti della Societa' Italiana di Scienze Veterinarie* 21, 606-611.

Chan, S.S., McConnell, I., Blacklaws, B.A. (2002). Generation and characterization of ovine dendritic cells derived from peripheral blood monocytes. *Immunology* 107, 366-372.

Chang, H.W., Aoki, M., Fruman, D., Auger, K.R., Bellacosa, A., Tsichlis, P.N., Cantley, L.C., Roberts, T.M., Vogt, P.K. (1997). Transformation of chicken cells by the gene encoding the catalytic subunit of PI-3 kinase. *Science* 276, 1848-1850.

Charray, J., Aman, N., Tanoh, K.G. (1985). Note on enzootic of adenocarcinoma of the pituitary nasal mucosa in Djalonke ewes. (1985). *Rev Elev Med Vet Pays Trop* 38, 406-410.

Chesebro, B., Portis, J.L., Wehrly, K., Nishio, J. (1983). Effect of murine host genotype on MCF virus expression, latency, and leukemia cell type of leukemias induced by Friend murine leukemia helper virus. *Virology* 128, 221–233.

Chiu, I.M., Yaniv, A., Dahlberg, J.E., Gazit, A., Skunntz, S.F., Tronick, S.R., Aaronson, S.A. (1985). Nucleotide sequence evidence for relationship of AIDS retrovirus to lentiviruses. *Nature* 317, 366-368.

Cho, H.S., Pelton, J.G., Wang, W., Yokota, H., Wemmer, D.E. (2001). In-vitro protein production for structure determination with the rapid translation system (RTS). *Biochemica* 4, 27-29.

Choe, H., Farzan, M., Sun., Y., Sullivan, N., Rollins, B., Ponath, P.D., Wu, L., Mackay, C.R., LaRosa, G., Newman, W., Gerard, N., Gerard, C., Sodroski, J. (1996). The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates.
Cell 85, 1135-1148.

Chow, Yen-HungJ., Alberti, A., Mura, M., Pretto, C., Murcia, P., Albritton, L.M., Palmarini, M. (2003). Transformation of Rodent fibroblasts by the jaagsiekte sheep retrovirus envelope is receptor independent and does not require the surface domain. *J Virol* 77, 6341-6350.

Chrunyk, B.A., Evans, J., Lillquist, J., Young P., Wetzel, R. (1993).Inclusion body formation and protein stability in sequence variants of interleukin-1beta. *Journal of Biological Chemistry* 268, 18053-18061.

Cianciolo, G.J., Copeland, T.D., Oroszlan, S., Snyderman, R. (1985). Inhibition of lymphocyte proliferation by a synthetic peptide homologous to retroviral envelope proteins. *Science* 230, 453-455.

Cianciolo, G.J., Matthews, T. J., Bolognesi, D.P., Snyderman, R. (1980). Macrophage accumulation in mice is inhibited by low molecular weight products from murine leukemia viruses. *J Immunol* 124, 2900-2905.

Clark, J.M. (1988). Novel non-template nucleotide addition-reactions catalyzed by procaryotic and eukariotic DNA-polymerases. *Nucleic Acid Research* 16, 9677-9686.

Clevidence, D.E., Overdier, D.G., Peterson, R. S., Porcella, A., Ye, H., Paulson, K.E., Costa, R.H. (1994). Members of the HNF-3/forkhead family of transcription factors exhibit distinct cellular expression patterns in lung and regulate the surfactant protein B promoter. *Dev Biol* 166, 195–209.

Coffin. M. (1979). Structure, replication, and recombination of retrovirus genomes: Some unifying hypotheses. *J Gen Virol* 42, 1-26.

Coffin. J.M. (1992a). Structure and classification of retroviruses. In *The retroviridae* (ed. J.A. Levy), pp. 19-49. Plenum Press, New York.

Coffin. J.M. (1992b). Genetic diversity and evolution of retroviruses. *Curr Top Microbiol Immunol* 176, 143-164.

Coffin, J.M., Hughes, S.H., Varmus, H.E. (1997). In *Retroviruses* (Cold Spring Harbor Lab. Press, Plainview, NY), p. 843.

Cohrs, P. (1953). Infectiöse Adenopapillome der Riechschleimhautbeim Schaf. *Berliner und Münchener Tierärztlicher Wochenschrift* 66, 225-228.

Coil, D.A., Strickler, J.H., Sharath, K.R., Miller, A.D. (2001). Jaagsiekte sheep retrovirus Env protein stabilizes retrovirus vectors against inactivation by lung surfactant, centrifugation, and freeze-thaw cycling. *J Virol* 75, 8864-8867.

Connor, R.I., Korber, B.T., Graham, B. S., Hahn, B.H., Ho, D.D., Walker, B.D., Neumann, A.U., Vermund, S.H., Mestecky, J., Jackson, S., *et al.* (1998). Immunological and Virological Analyses of Persons Infected by Human Immunodeficiency Virus Type 1 while Participating in Trials of Recombinant gp120 Subunit Vaccines. *J Virol* 72, 1552–1576.

Conrad, B., Weissmahr, R.N., Boni, J., Arcari, R., Schupbach, J., Mach B. (1997). A human endogenous retroviral superantigen as candidate autoimmune gene in type I diabetes. *Cell* 90, 303-313.

Costa, R.H., Kalinichenko, V.V., Lim, L. (2001). Transcription factors in mouse lung development and function. *Am J Physiol Lung Cell Mol Physiol* 280, 823–838.

Cousens, C. (1993). Membrane Fusion and the Transmembrane Envelope Glycoprotein of Maedi-Visna Virus. Ph.D. Thesis. Edinburgh University.

Cousens, C., Bishop, J.V., Philbey, A.W., Gill, C.A., Palmarini, M., Carlson, J.O., DeMartini, J.C., Sharp, J.M. (2004). Analysis of integration sites of jaagsiekte sheep retrovirus in ovine pulmonary adenocarcinoma. *J Virol* 78, 8506-8512.

Cousens, C., Minguijon, E., Garcia, M., Ferrer, L.M., Dalziel, R.G., Palmarini, M., De las Heras, M., Sharp, J.M. (1996). PCR-based detection and partial characterization of a retrovirus associated with contagious intranasal tumors of sheep and goats. *J Virol* 70, 7580-7583.

Cowdry, E.V. (1925). Studies on the etiology of Jaagsiekte. I. The primary lesions. *J Exp Med* 42, 323.

Cox, W.I., Tartaglia, J., Paoletti, E. (1993). Induction of cytotoxic T lymphocytes by recombinant canarypox (ALVAC) and attenuated vaccinia (NYVAC) viruses expressing the HIV-1 envelope glycoprotein. *Virology* 195, 845-850.

Cremer, K. & Gruber, J. (1992). Animal models for retrovirus-associated malignancies. *Vet Pathol* 29, 572-578.

Csoka, A.B., Scherer, S.W., Stern, R. (1999). Expression analysis of six paralogous human hyaluronidase genes clustered on chromosomes 3p21 and 7q31. *Genomics* 60, 356-361.

Cuba-Caparo, A., De la Vega, E., Copaira, H. (1961). Pulmonary adenomatosis of sheep: metastasizing bronchiolar tumors. *Am J Vet Res* 22, 673-682.

Cullen, B. (1998). HIV-1 auxiliary proteins: making connections in a dying cell. *Cell* 93, 685-692.

Cutlip, R.C., Lehmkuh, H.D., Brogden, K.A., Sacks, J.M. (1986). Breed susceptibility to ovine progressive pneumonia (maedi/visna) virus. *Vet Microbiol* 12, 283-288.

Cutlip, R.C. & Young, S. (1982). Sheep pulmonary adenomatosis (jaagsiekte) in the United States. *Am J Vet Res* 43, 2108-2113.

Cuypers, H.T., Selten, G., Quint, W., Zijlstra, M., Maandag, E.R., Boelens, W., *et al.* (1984). Murine leukemia virus-induced T-cell lymphomagenesis: integration of proviruses in a distinct chromosomal region. *Cell* 37, 141–150.

Dalefield, R.R. & Alley, M.R. (1988). An ovine pulmonary tumour of alveolar epithelial type II cells. *N Z Vet J* 36, 25-27.

D'Andrea, A.D., Moreau, J.F., Showers, M.O. (1992). Molecular mimicry of erythropoietin by the spleen focus-forming virus gp55 glycoprotein: The first state of Friend virus-induced erythroleukemia. *Biochim Biophys Acta* 1114, 31-41.

Danilkovitch-Miagkova, A., Duh, F.M., Kuzmin, I., Angeloni, D., Liu, S.L., Miller, A.D., Lerman, M.I. (2003). Hyaluronidase 2 negatively regulates RON receptor tyrosine kinase and mediates transformation of epithelial cells by jaagsiekte sheep retrovirus. *Proc Natl Acad Sci U S A* 100, 4580-4585.

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

de Kock, G. (1929). Further observations on the etiology of jaagsiekte in sheep. *15th Ann Rept Dir Vet Services, S Africa* p. 611-641.

De las Heras, M., Barsky, S.H., Hasleton, P., Wagner, M., Larson, E., Egan., J., Ortin, A., Gimenez-Mas, J.A., Palmarini, M., Sharp,J.M. (2000). Evidence for a protein related immunologically to the jaagsiekte sheep retrovirus in some human lung tumours. *Eur Respir J* 16, 330-332.

De las Heras, M., Calafat, J.J., Jaime, J.M., García de Jalon, J.A., Ferrer, L.M., García-Goti, M., Minguijón, E. (1992). Sheep pulmonary adenomatosis (jaagsiekte) in slaughtered sheep. Variation in pathological characteristics. *Medicina Veterinaria* 9, 52-53.

De las Heras, M., García de Jalon, J.A., Minguijon, E., Gray, E,W., Dewar, P., Sharp. J.M. (1995). Experimental transmission of enzootic intranasal tumors of goats. *Vet Pathol* 32, 19-23.

De las Heras, M., García de Jalon, J.A., Sharp, J.M. (1991). Pathology of enzootic intranasal tumor in thirty-eight goats. *Vet Pathol* 28, 474-481.

De las Heras M, García Goti M, Ortín A, Pascual Z, Minguijón E, Ferrer LM, García de Jalón JA, Dewar P, Sharp JM y Gonzalez L. (2000).Presencia del retrovirus asociado a la adenomatosis pulmonar ovina en fetos y en una neoplasia pulmonar de un cordero de tres días. *Pequeños Rumiantes* 1, 36-37.

De las Heras, M., González, L., Sharp, J.M. (2003). Pathology of ovine pulmonary adenocarcinoma. *Curr Top Mibrobiol Immunol* 275, 25-54.

De las Heras, M., Minguijón, E., Ferrer, L.M., Ortin, A., Dewar, P., Cebrian, L.M., Pascual, Z., García de Jalon, J.A., Sharp, J.M. (1998). Naturally occurring enzootic nasal tumour of sheep in Spain: pathology and associated retrovirus. *European Journal of Veterinary Pathology* 4, 11-15.

De las Heras, M., Minguijón, E., Ferrer, L.M., Pérez, V., Bolea, R. (1995). Adenomatosis pulmonar ovina (jaagsiekte): células que infiltran el tumor y modificaciones en ganglios linfáticos regionales. *Medicina Veterinaria* 12, 32-36.

De las Heras, M., Ortin, A., Perez de Villareal, M., Salvatori, D., Dewar, P., Cousens, C., Sharp, J.M. (2003). Immunohistochemical evaluation of the mitogen activated protein kinases (MAPKS) and protein kinase B (AKT) signalling pathways in contagious respiratory tumours of sheep using activation state antibodies. 15th International Workshop on retroviral pathogenesis, 17-21st September, Glasgow.

De las Heras, M., Sharp, J.M., Garcia de Jalon, J.A., Dewar, P. (1991). Enzootic nasal tumour of goats: demonstration of a type D-related retrovirus in nasal fluids and tumours. *J Gen Virol* 72, 2533-2535.

DeMartini, J.C., Bishop, J.V., Allen, T.E., Jassim, F.A., Sharp, J.M., De las Heras, M., Voelker, D.R., Carlson, J.O., (2001). Jaagsiekte sheep retrovirus proviral clone JSRVjs7, derived from the JS7 lung tumour cell line, induces ovine pulmonary carcinoma and is integrated into the surfactant protein A gene. *J Virol* 75, 4239-4246.

DeMartini, J.C., Carlson, J.O., Leroux, C., Spencer, T., Palmarini, M. (2003). Endogenous retroviruses related to Jaagsiekte sheep retrovirus. *Curr Top Microbiol Immunol* 275, 117-137.

DeMartini, J.C., Rosadio, R.H, Lairmore, M.D., (1988). The etiology and pathogenesis of ovine pulmonary carcinoma (sheep pulmonary adenomatosis). *Veterinary Microbiology* 17, 219-236.

DeMartini, J.C., Rosadio, R.H., Sharp, J.M., Russell, H.I., Lairmore, M.D. (1987). Experimental coinduction of type D retrovirus-associated pulmonary carcinoma and lentivirus-associated lymphoid interstitial pneumonia. *J Natl Cancer Inst* 79, 167-177.

DeMartini, J.C. & York, D.F. (1997). Retrovirus-associated neoplasms of the respiratory system of sheep and goats. Ovine pulmonary carcinoma and enzootic nasal tumor. *Vet Clin North Am Food Anim Pract* 13, 55-70.

Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R.E., Hill, C.M., Davis, C.B., Peiper, S.C., Schall, T.J., Littman, D.R., Landau, N.R. (1996). Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 381, 661-666.

Dent, P. (1972). Immunodepression by oncogenic viruses. *Prog Med Virol* 14, 1-35.

De Villiers, E.M., Els, H.J., Verwoerd, D.W. (1975). Characteristics of an ovine herpesvirus associated with pulmonary adenomatosis (jaagsiekte) in sheep. *South African Journal of Medical Science* 40, 165-175.

DeVries, J.K., Zubay, G. (1967). DNA-directed peptide synthesis, II. The synthesis of the α-fragment of the enzyme β-galactosidase. *Proc Natl Acad Sci USA* 57, 1010.

Dickson, C., Smith, R., Brookes, S., Peters, G. (1984).Tumorigenesis by mouse mammary tumor virus: proviral activation of a cellular gene in the common integration region int-2. *Cell* 37, 529–536.

Diehl, L., den Boer, A.T., Schoenberger, S.P., van der Voort, E.I., Schumacher, T. N., Melief, C.J., Offringa, R., Toes. R.E. (1999). CD40 activation in vivo overcomes peptide-induced peripheral cytotoxic T-lymphocyte tolerance and augments anti-tumor vaccine efficacy. *Nat Med* 5, 774.

Dion, A.S., Williams, C.J., Pomenti, A.A. (1977). The major structural proteins of murine mammary tumor virus: techniques for isolation. *Anal Biochem* 82, 18-28.

Dirks, C., Duh, F.M., Rai, S.K., Lerman, M.I., Miller, A.D. (2002) Mechanism of cell entry and transformation by enzootic nasal tumor virus. *J Virol* 76(5), 2141-2149.

Doolittle, R.F., Feng, D.F., Johnson, M.S., McClure, M.A. (1989). Origins and evolutionary relationships of retroviruses. *Quart Rev Biol* 64, 1-30.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., *et al.* (1984). Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. *Nature* 307, 521–527.

Dragic, T., Litwin, V., Allaway, G.P., Martin, S.R., Huang, Y., Nagashima, K.A., Cayanan, C., Maddon, P.J., Koup, R.A., Moore, J.P., Paxton, W.A. (1996). HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* 381, 667-673.

Dualde-Perez, D. (1966). Estudios sobre la adenomatosis pulmonar ovina en Espanã. Ph.D Thesis, Universidad de Zaragoza. Instituto de Estudios Turolenses-CSIC, Ed.Teruel.

Duesberg, P.H. & Vogt, P.K. (1970). Differences between the ribonucleic acids of transforming and nontransforming avian tumor viruses. *Proc Natl Acad Sci U S A* 67, 1673–1680.

Duncan, J.R., Tyler, D.E., Van Der Maaten, M.J., Andersen, J.R. (1967). Enzootic nasal adenocarcinoma in sheep. *J Am Vet Med Assoc* 151, 732-734.

Dungal, N. (1946). Experiments with jaagsiekte. *Am J Pathol* 22, 737-759.

Dungal, N., Gislason, G., Taylor, E.L. (1938). Epizootic adenomatosis in the lungs of sheep. Comparison with jaagsiekte, verminous pneumonia and progressive pneumonia. *Journal of Comparative Pathology and Therapeutics* 51, 46-68.

Eber, A., (1899). Uber multiple Adenombildung in den Lungen der Shafe. *Zeitschrift fuer Tiermedizin* 3, 161-172.

Edwards, C.W. (1984). Alveolar carcinoma: a review. *Thorax* 39, 166-174.

Embretson, J., Zupancic, M., Ribas, J.L., Burke, A., Racz, P., Tenner-Racz, K., Haase, A.T. (1993). Massive covert infection of helper T lymphocytes and macrophages by HIV during the incubation period of AIDS. *Nature* 362, 359-362.

Emerman, M., & Malim. M. H. (1998). HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology. *Science* 280, 1880-1884.

Endo, Y., Otsuzuki, S., Kimio Ito, K., Miura, K.-I. (1992). Production of an enzymatic active protein using a continuous flow cell-free translation system. *J Biotechnol* 25, 221–230.

Endo, Y. & Sawasaki, T. (2003). High-throughput, genome-scale protein production method based on the wheat germ cell-free expression system. *Biotechnology Advances* 21, 695-713.

Engelmayer, J., Larsson, M., Subklewe, M. *et al.* (1999). Vaccinia virus inhibits the maturation of human dendritic cells: a novel mechanism of immune evasion. *J Immunol* 163(12), 6762-6768.

Entrican, G. (2002a). Immune Regulation during Pregnancy and Host–Pathogen Interactions in Infectious Abortion. *J Comp Path* 126, 79-94.

Entrican, G. (2002b). New technologies for studying immunoregulation in ruminants.*Vet Immunol immunopathol* 87, 485-490.

Evans, L.H. & Cloyd, M.W. (1985). Friend and Moloney murine leukemia viruses specifically recombine with different endogenous retroviral sequences to generate mink cell focus-forming viruses. *Proc Natl Acad Sci USA* 82 , 459–463.

Fan, H. (1994). Retroviruses and their role in cancer. In A. Levy (ed.), The Retroviridae, vol. 3. Plenum Press, New York.

Fan, H. (1997). Leukemogenesis by Moloney murine leukemia virus: a multistep process. *Trends Microbiol* 5, 74–82.

Federspiel, M.J. & Hughes, S.H. (1997). Retroviral gene delivery. *Methods in cell biology* 52, 179-214.

Felder, M.-P., Eychene, A., Barnier, J.V., Calogeraki, I., Calothy, G., Marx, M. (1991). Common mechanism of retrovirus activation and transduction of c-*mil* and c-R*mil* in chicken neuroretina cells infected with Rousassociated virus type 1. *J Virol* 65, 3633-3640.

Felder, M.-P., Laugier, D., Eychene, A., Calothy, G., Marx, M. (1993). Occurrence of alternatively spliced leader-Δ*onc*-poly(a) transcripts in chicken neuroretina cells infected with Rousassociated virus type 1: Implication in transduction of the c-*mil*/c-*raf* and c-R*mil*/B-*raf* oncogenes. *J Virol* 67, 6853-6856.

Feuer, G., & Chen, I.S. (1992). Mechanisms of human T-cell leukemia virus-induced leukemogenesis. *Biochim Biophys Acta* 1114, 223-233.

Fischinger, P.J., Nomura, S., Bolognesi, D.P. (1975). A novel murine oncornavirus with dual eco- and xenotropic properties. *Proc Natl Acad Sci USA* 72, 5150–5155.

Flynn, J.N., Hanlon, L., Jarrett, O. (2000). Feline leukaemia virus: protective immunity is mediated by virus-specific cytotoxic T lymphocytes. *Immunology* 101, 120-125.

Frank, S.A. (2004). Genetic predisposition to cancer-insights from piopulation genetics. *Nature Reviews Genetics* 5, 764-772.

Frendo, J. L., Olivier, D., Cheynet, V., Blond, J.-L., Bouton, O., Vidaud, M., Rabreau, M., Evain-Brion, D., Mallet, F. (2003). Direct involvment of HERV-W glycoprotein in human trophoblast cell fusion and differentiation. *Mol Cell Biol* 23, 3566–3574.

Fukushima, M., Kawaguchi, R. (2001). Cell-free expression of human interleukin 2(IL-2) with the RTS 500 E. coli circular template kit. *Biochemica* 4, 30-32.

Fultz, P.N., McClure, H.M., Anderson, D.C., Switzer, W.M. (1989). Identification and biologic characterisation of an acutely lethal variant of simian immunodeficiency viirus from sooty mangabeys (SIV/SMM). *AIDS Res Hum Retroviruses* 5, 397-409.

Fung, Y.K., Lewis, W.G., Crittenden, L.B., Kung, H.J. (1983). Activation of the cellular oncogene c-erbB by LTR insertion: molecular basis for induction of erythroblastosis by avian leukosis virus. *Cell* 33, 357–368.

Gardner, M.B., Kozak, C.A., O'Brien, S.J. (1991). The Lake Casitas wild mouse: Evolving genetic resistance to retroviral disease. *Trends Genet* 7, 22-27.

Gardner, M.B., Luciw, P., Lerche, N., Marx, P. (1988). Non-human primate retrovirus isolates and AIDS. In *Immunodeficiency Disorders and Retroviruses,* p. 171-226. Edited by Perk. New York: Academic Press.

García-Goti, M. (1999). Estudios sobre la patogenia y diagnóstico de la adenomatosis pulmonary ovina. *PhD Thesis*. University of Zaragoza, Spain.

García-Goti, M., González, L., Cousens, C., Cortabarría, N., Extramiana, A.B., Minguijon, E., Ortin, A., De las Heras, M., Sharp, J.M. (2000). Sheep pulmonary adenomatosis: characterization of two pathological forms associated with jaagsiekte retrovirus. *J Comp Pathol* 122, 55-65.

Gazdar, A.F. & Linnoila, R.I. (1988). The pathology of lung cancer-changing concepts and newer diagnostic techniques. *Seminars in Oncology* 15, 215-225.

Gazquez, A., Roncero,V., Redondo, E., Duran, E., Masot, J., Gomez, L. (1992). Adenocarcinoma of the ethmoid olfactory mucosa: a histopathological and ultrastructural study with evidence of virus-like particles. *Zentralbl Veterinarmed A* 39, 609-615.

Geddes, D.M. (1987). Bronchioloalveolar carcinoma. *Br Med J (Clin Res Ed)* 294 (6563), 3.

Gerard, C.J., Olsson, K., Ramanathan, R., Reading, C., Hanania, E.G. (1998). Improved quantitation of minimal residual disease in multiple myeloma using real-time polymerase chain reaction and plasmid-DNA complementarity determining region III standards. *Cancer Res* 58, 3957-3964.

Gherardi, M.M. & Esteban, M. (1999), Mucosal and systemic immune responses induced after oral delivery of vaccinia virus recombinants. *Vaccine* 17,1074–1083.

Gibbs, C.J., Gajdusek, D.C., Amyx, H. (1979). Strain variation in the viruses of Creutzfeldt-Jacob disease and kuru. In Pathogenesis, immunobiology, virology, and molecular biology of the spongiform encephalopathies /edited by S.B. Prusiner and W.J. Hadlow, New York : Academic Press, 1979. Slow transmissible diseases of the nervous system ; vol.2.

Goff, S.A. &Goldberg, A.L. (1987). An increased content of protease La, the lon gene product, increases protein degradation and blocks growth in Escherichia coli. *BIOL CHEM* 262, 4508-4515.

Goldfarb, M.P. & Weinberg ,R.A. (1981). Generation of novel biologically active Harvey sarcoma viruses via apparent illegitimate recombination. *J Virol* 38, 136-150.

Golemis, E., Li, Y. Fredrickson, T. N., Hartley, J. W., Hopkins, N. (1989). Distinct segments within the enhancer region collaborate to specify the type of leukemia induced by nondefective Friend and Moloney viruses. *J Virol* 63, 328–337.

Golovkina, T.V., Chervonsky, A., Dudley, J.P., Ross, S.R. (1992). Transgenic mouse mammary tumour virus superantigen expression prevents viral infection. *Cell* 69, 637-645.

Golovkina, T.V., Jaffe, A.B., Ross, S.R. (1994). Coexpression of exogenous and endogenous mouse mammary tumour virus RNA *in vivo* results in viral recombination and broadens the virus host range. *J Virol* 68, 5019-5026.

Golovkina, T.V., Piazzon, I., Nepomnaschy, I., Buggiano, V., de Olano Vela, M., Ross, S.R. (1997). Generation of a tumorigenic milk-born mouse mammary tumor

virus by recombination between endogenous and exogenous viruses. *J Virol* 71, 3895-3903.

Golovkina, T.V., Prakash, O., Ross, S.R. (1996). Endogenous mouse mammary tumor virus Mtv-17 is involved in Mtv-2-induced tumorigenesis in GR mice. *Virology* 218, 14-22.

González, L. (1990). El maedi o neumonía progresiva en el conjunto de las enfermedades respiratorias crónicas del ganado ovino en la Comunidad autónoma Vasca. Ph.D. Thesis. Universidad de Zaragoza. Servicio central de Publicaciones del Gobierno Vasco, Ed., Vitoria. D.L. VI-546-1990.

González L, García-Goti, M., Cousens, C., Dewar, P., Cortabarría, N., Extramiana, B., Ortin, A., De las Heras, M., Sharp, J.M. (2001). Jaagsiekte sheep retrovirus can be detected in the peripheral blood during the preclinical period of sheep pulmonary adenomatosis. *J Gen Virol* 82, 1355-1358.

González L., Juste, R.A., Cuervo, L.A., Idigoras, I., Saez de Ocariz, C. (1993). Pathological and epidemiological aspects of the coexistence of maedi-visna and sheep pulmonary adenomatosis. *Res Vet Sci* 54, 140-146.

Gordon, S., Clarke, S., Greaves, D., Doyle, A. (1995). Molecular immunobiology of macrophages: recent progress. *Curr Opin Immunol* 7, 24–33.

Gotzinger, N., Sauter, M., Roemer, K., Mueller-Lantzsch, N. (1996). Regulation of human endogenous retrovirus-K Gag expression in teratocarcinoma cell lines and human tumours. *J Gen Virol* 77, 2983-90.

Goulder, P.J., Brander, C., Tang, Y., Tremblay, C., Colbert, R.A., Addo, M.M., Rosenberg, E.S., Nguyen, T., Allen, R., Trocha, A., Altfeld, M., He, S., Bunce, M., Funkhouser, R., Pelton, S.I., Burchett, S.K., McIntosh, K., Korber, B.T., Walker, B.D. (2001).Evolution and transmission of stable CTL escape mutations in HIV infection. *Nature* 412, 334-338.

Greco, E., Profiti, M., Giammaroli, M., Giannino, L., Rutili, D., Woodall, C., Rosati, S. (2002). Genetic Heterogeneity of small ruminant lentiviruses involves immunodominant epitope of capsid antigen and affects sensitivity of single-train-based immunoassay. *Clin Diagn Lab Immunol* 9, 828-832.

Greco, R.J., Steiner, R.M., Goldman, S., Cotler, H., Patchefsky, A., Cohn, H.E. (1986). Bronchioloalveolar cell carcinoma of the lung. *Ann Thorac Surg* 41, 652-656.

Green, P.L., Kaehler, D.A., Risser, R. (1987). Cell transformation and tumor induction by Abelson murine leukemia virus in the absence of helper virus. *Proc Natl Acad Sci U S A* 84, 5932–5936.

Greenlee, R.T., Murray, T., Bolden, S., Wingo, P.A. (2000). Cancer statistics. *CA Cancer J Clin* 50, 7-33.

Grittiths, D.J., Venables, P.J., Weiss, R.A., Boyd, M.T. (1997). A novel exogenous retrovirus sequence identified in humans. *J Virol* 71, 2866-2872.

Gross, L. (1970). Oncogenic viruses. Pergamon Press, Elmsford, New York.

Gross-Bellard, M. Oudet P. Chambon, P. (1973). Isolation of high molecular weight DNA from mammalian cells. *Eur J Biochem* 36 ,32–38.

Grossman, D.A., Hiti, A.L., McNiel, E.A., Ye, Y., Alpaugh, M.L., Barsky, S.H. (2002a). Comparative oncological studies of feline bronchioloalveolar lung carcinoma, its derived cell line and xenograft. *Cancer Res* 62, 3826-3833.

Grossman, D.A., McNie, E.A., Hackett, T.B., Barsky, S.H. (2002b). Establishment of an immortalized cell line and transplantable xenograft from a bronchioloalveolar lung carcinoma of a cat. Am J Vet Res *63*, 1745-1753.

Grumbach. A. (1926). Etude cancer. *Bull A Franc* 15, 213.

Haase, A.T., Stowring, L., Narayan, O., Griffin, D., Price, D. (1977). Slow persistent infection caused by Visna virus: role of host restriction. *Science* 195, 175-177.

Hahn, F.F., Muggenburg, B.A., Griffith, W.C. (1996). Primary lung neoplasia in a beagle colony. *Veterinary Pathology* 33, 633-638.

Hallberg, B., Schmidt, J., Luz, A., Pederson F.S., Grundstrom, T. (1991). SL3–3 enhancer factor 1 transcriptional activators are required for tumor formation by SL3–3 murine leukemia virus. *J Virol* 65, 4177–4181.

Hamaguchi, K. & Geiduschek, E.P. (1962). The effect of electrolytes on the stability of deoxyribonucleate helix. *J Am Chem Soc* 84, 1329-1337.

Hanecak, P., Pattengale, K., Fan, H. (1991). Deletion of a GC-rich region flanking the enhancer element within the long terminal repeat sequence alters the disease specificity of Moloney murine leukemia virus. *J Virol* 65, 5357–5363.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* 166, 557.

Hanahan, D. (1985). In: DNA cloning, Volume 1, (ed. D.Glover) IRL Press Ltd., p. 109-135.

Haraguchi, S., Good, R.A., Cianciolo, G.J., Engelman, R.W., Day, N.K. (1997). Immunosuppressive retroviral peptides; immunopathological implications for immunosuppressive influences of retroviral infections. *J Leukoc Biol* 61, 654-666.

Haraguchi, S., Good, R.A., James-Yarish, M., Cianciolo, G.J., Day, N.K. (1995). Differential modulation of Th1- and Th2-related cytokine mRNA expression by synthetic peptide homologous to a conserved domain within retroviral envelope protein. *Proc Natl Acad Sci USA.* 92, 3611-3615.

Harris, D.T., Cianciolo, G.J., Snyderman, R., Argov, S., Koren, H.S. (1987). Inhibition of human natural killer cell activity by a synthetic peptide homologous conserved region in the retroviral protein, p15E. *J Immunol* 138, 889-894.

Hartley, J.W. & Rowe, W.P. (1976). Naturally occurring murine leukemia viruses in wild mice: characterization of a new "amphotropic" class. *J. Virol.* 19, 19–25.

Hartley, J.W., Wolford, N.K., Old, L.J., Rowe, W.P. (1977). A new class of murine leukemia virus associated with development of spontaneous lymphomas. *Proc Natl Acad Sci USA* 74, 789-792.

Hawkins, T.L., O'Connor-Morin, T., Roy, A., Santillan, C. (1994). DNA purification and isolation using a solid-phase. *Nucl Acid Res* 22, 4543–4544.

Hayashi, H., Matsubara, H., Yokota, T., Kuwabara, I., Kanno, M., Koseki, H., Isono, K., Asano, T., Taniguchi, M. (1992). Molecular cloning and characterization of the gene encoding mouse melanoma antigen by cDNA library transfection. *J Immunol* 149, 1223-1229.

Hayward, W.S., Neel, B.G., Astrin, S.M. (1981). Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis. *Nature* 290, 475–80.

Hecht, S.J., Carlson, J.O., DeMartini, J.C. (1994). Analysis of a type D retroviral capsid gene expressed in ovine pulmonary carcinoma and present in both affected and unaffected sheep genomes. *Virology* 202, 480-484.

Hecht, S.J., Stedman, K.E., Carlson, J.O., DeMartini, J.C. (1996). Distribution of endogenous type B and D retroviral sequences in ungulates and other mammals. *Proc Natl Acad Sci* 93, 3297-3302.

Heid, C.A., Stevens, J., Livak, K.J., Williams, P.M. (1996). Real-time quantitative PCR. *Genome Res* 6, 986-994.

Held, W., Acha-Orbea, H., MacDonald, H.R., Waanders, G.A. (1994). Superantigens and retroviral infection: insights from mouse mammary tumour virus. *Immunology Today* 15, 184-190.

Held, W., Waanders, G.A., Shakkhov, A.N., Scarpellino, L., Acha-Orbea, H., MacDonald, H.R. (1993). Superantigen-induced immune stimulation amplifies mouse mammary tumor virus infection and allows virus transmission. *Cell* 74, 529-540.

Henrich, B., Lubitz, W., Plapp, R. (1982) Lysis of Escherichia coli by induction of cloned phi X174 genes. *Molecular & General Genetics* 185, 493-497.

Herbein, G., Coaquette, A., Perez-Bercoff, D., Pancino, G. (2002). Macrophage activation and HIV infection: can the Trojan horse turn into a fortress? *Curr Mol Med* 2, 723-738.

Herbst, H., Santer, M., Müller-Lantzsch, N. (1996). Expression of human endogenous K elements in germ cell and trophoblastic tumours. *Am J Pathol* 149, 1727-1735.

Herring, A.J., Sharp, J.M., Scott, F.M.M, Angus, K.W. (1983). Further evidence for a retrovirus as the etiological agent of sheep pulmonary adenomatosis (Jaagsiekte). *Veterinary Microbiology* 8, 237-249.

Hiendleder, S., Mainz, K., Plante, Y., Lewalski, H. (1998). Analysis of mitochondrial DNA indicates that domestic sheep are derived from two different ancestral maternal sources: no evidence for contributions from urial and argali sheep. *Journal of Heredity* 89, 113-120.

Himly, M., Foster, D.N., Bottoli, I., Iacovoni, J.S., Vogt, P.K (1998). The DF-1 chicken fibroblast cell line: transformation induced by diverse oncogenes and cell death resulting from infection by avian leukosis viruses. *Virology* 248, 295-304.

Hizi, A., L. E. Henderson, T. D. Copeland, R. C. Sowder, C. V. Hixson, and S. Oroszlan. (1987). Characterization of mouse mammary tumor virus gag-pro gene products and the ribosomal frameshift site by protein sequencing. *Proc Natl Acad Sci USA* 84, 7041-7045.

Hizi, A., L. E. Henderson, T. D. Copeland, R. C. Sowder, H. C. Krutzsch, and S. Oroszlan. (1989). Analysis of gag proteins from mouse mammary tumor virus. *J Virol* 63, 2543-2549.

Hod, I., Herz, A., Zimber, A. (1977). Pulmonary carcinoma (jaagsiekte) of sheep. Ultrastructural study of early and advanced tumours lesions. *Am J Pathol* 86, 545-557.

Hofacre, A. & Fan, H. (2004). Multiple domains of the Jaagsiekte sheep retrovirus envelope protein are required for transformation of rodent fibroblasts. *J Virol* 78, 10479-10489.

Holland, M., Palmarini, M., Garcia-Goti, M., Gonzales, L., McKendrick, L., De las Heras, M., Sharp, J.M. (1999). Jaagsiekte retrovirus is widely distributed both in T and B lymphocytes and in mononuclear phagocytes of sheep with naturally and experimentally acquired pulmonary adenomatosis. *J Virol* 73, 4004-4008.

Hooper, P.T. & Ellard, K.A. (1995). A pulmonary adenoma in an Australian sheep. *Aust Vet J* 72, 477.

Hoover, E.A., Olsen, R. G., Hardy, W. D. Jr, Schaller, J. P., Mathes, L. E. (1976). Feline leukemia virus infection: age-related variation in response of cats to experimental infection. *J Natl Cancer Inst* 57, 365-9.

Houwers D.J., König C.D.W., Bakker J., de Boer M.J., Pekelder J.J., Sol J., Vellema P., de Vries G. (1987). Maedi-visna control in sheep. III. Results and evaluation of a voluntary control program in the Netherlands over a period of four years, Vet Q 9, 29-36.

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

Howe, L., Leroux, C., Issel, C.J., Montelaro, R.C. (2002). Equine infectious anemia virus envelope evolution in vivo during persistent infection progressively increases resistance to in vitro serum antibody neutralization as a dominant phenotype. *J Virol* 76, 10588-10597.

Hunter, A.R. & Munro, R. (1983). The diagnosis, occurrence and distribution of sheep pulmonary adenomatosis in Scotland 1975 to 1981. *Br Vet J* 139, 153-164.

Hunter, E., Casey, J., Hahn, B., Hayami, M., Korber, B., Kurth, R., *et al.* (2000). The Retroviridae. In: van Regenmortel, M.H., Fauquet, C.M., Bishopo, D.H., Carstens, E.B., Estes, M.K., Lemon, S.M., *et al.* Editors. Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses. San Diego (CA): Academic Press; 2000, p. 369-387.

Hunter, N., Goldmann, W., Foster, J. D., Cairns, D. & Smith, G. (1997). Natural scrapie and PrP genotype: case–control studies in British sheep. *Vet Rec* 141, 137–140.

Imai, S., Tsubura, J., Hilgers, J., Michalides, R. (1983). A new locus (Mtv-4) for for endogenous mammary tumor virus expression and early mammary tumor development in the SHN mouse strain. *J Natl Cancer Inst* 71, 517-521.

Irving, S.G., Perk, K., Hod, I., Gazit, A., Yaniv, A., Zimber, A., Tal, M. (1984). Isolation and characterization of a novel retrovirus from sheep affected by pulmonary carcinoma. *Virology* 134, 244 -248.

Isfort, R., Jones, D., Kost, R., Witter, R., Kung, H.J., (1992). Retrovirus insertion into herpesvirus in vitro and in vivo. *Proc Natl Acad Sci USA* 89, 991–995.

Ishimoto, A., Takimoto, M., Adachi, A., Kakuyama, M., Kato, S., Kakimi, K., Fukuoka, K., Ogiu T., Matsuyama, M. (1987). Sequences responsible for erythroid and lymphoid leukemia in the long terminal repeats of Friend mink cell focus-forming and Moloney murine leukemia virus. *J Virol* 61, 1861–1866.

Jacks, T., Townsley, K., Varmus, H.E., Majors, J. (1987). Two efficient ribosomal frame shifting events are required for synthesis of MMTV *gag*-related polyproteins. *PNAS USA* 84, 4298-4302.

Jarrett, O. (2001). Feline leukemia virus. Encyclopedia of life sciences. *Nature publishing group*/www.els.net, 1-6.

Jassim, F.A. (1988). Isolation and characterisation of transformed cells in jaagsiekte, a contagious lung tumour of sheep. University of Edinburgh, Edinburgh, United Kingdom.

Jassim. F.A., Sharp, J.M., Marinello, P.D. (1987). Three-step procedure for isolation of epithelial cells from the lungs of sheep with jaagsiekte. *Res Vet Sci* 43, 407-409.

Jeang, K.T., Giam, C.Z., Majone, F., Aboud, M. (2004). Life, death and Tax: role of HTLV-I oncoprotein in genetic instability and cellular transformation. *J Biol Chem* 279, 31991-31994.

Joag, S.V., Stephen, E.B., Narayan, O. (1996). Lentiviruses in Fields Virology, third edition ed. BN Fields, DM Kapie, PM Howley *et al.,* Lippincott-Raven Publishers, Philadelphia, p.1977-1999.

Johnson, J.L. & Craig, E.A., (1997). Protein folding *in vivo*: unraveling complex pathways. *Cell* 90, 201-204.

Johnson, L.K., Meyer, A.L., Zink, M.C. (1992). Detection of ovine lentivirus in seronegative sheep by in situ hybridization, PCR, and cocultivation with susceptible cells. *Clin Immunol Immunopathol* 65, 254-260.

Johnson, P. & Benchimol, S. (1992). Friend virus induced murine erythroleukaemia: The p53 locus. *Cancer Sur* 12, 137-151.

Johnson, W.E & Coffin, J.M., (1999).Constructing primate phylogenies from ancient retrovirus sequences. *Proc Natl Acad Sci USA* 96,10254-10260.

Juste, R.A., Kwang, J., de la Concha-Bermejillo, A. (1998). Dynamics of cell-associated viremia and antibody response during the early phase of lentivirus infection in sheep. *Am J Vet Res* 59, 563-568.

Kahwa, C.K., Atwal, O.S., Purton, M. (1997). Transmission electron microscopy of the epithelium of distal airways and pulmonary parenchyma of the goat lung. *Res Vet Sci* 63, 49-56.

Kahwa, C.K., Balemba, O.B., Assey, R.J. (2000). The pattern of ciliation and the development of the epithelial lining of the respiratory tract in the neonatal kid: a scanning electron microscopic study. *Small Rumin Res* 37, 27-34.

Karapetian, O., Shakhov, A.N., Kraehenbuhl, J.P., Acha-Orbea, H. (1994). Retroviral infection of neonatal Peyer's patch lymphocytes: the mouse mammary tumor virus model. *J Exp Med* 180, 1511-1516.

Katz, R.A. & Skalka, A.M. (1990). Generation of diversity in retroviruses. *Annu Rev Genet* 24, 409–445.

Kawarasaki, Y., Kawai, T., Nakano, H., Yamane, T. (1995). A long-lived batch reaction system of cell-free protein synthesis. *Anal Biochem* 226, 320–324.

Kensil, C.R., Mo, A.X., Truneh, A. (2004). Current vaccine adjuvants: an overview of a diverse class. *Front Biosci* 9, 2972-2988.

Kershaw, M.H., Hsu, C., Mondesire, W., Parker, L.L., Wang, G., Overwijk, W. W., Lapointe, R., Yang, J.C., Wang, R.F., Restifo, N.P. *et al.* (2001). Immunization against endogenous retroviral tumor-associated antigens. *Cancer Res* 61, 7920-7924.

Khati, M., James, W., Gordon, S. (2001). HIV-macrophage interactions at the cellular and molecular level. *Arch Immunol Ther Exp (Warsz)* 49, 367–378.

Kiessling, A.A., Hughes, W.H., Blankevoort, M.R. (1986). Superovulation and embryo transfer in the dairy goat. *J Am Vet Med Assoc* 188, 829-832.

Kigawa, T., & Yokoyama, S.A (1991). A continuous cell-free protein synthesis system for coupled transcription–translation. *J Biochem* 110, 166–168.

Kigawa T., Yabuki, T., Yoshida, Y., Tsutsui, M., Ito, Y., Takehiko Shibata, T., Yokoyama, S. (1999). Cell-free production and stable-isotope labeling of milligram quantities of proteins. *FEBS Lett* 442, 15–19.

Kim D.-M., Kigawa, T., Choi, C.-Y., Yokoyama, S. (1996). A highly efficient cell-free protein synthesis system from *Escherichia coli*. *Eur J Biochem* 239, 881–886.

Kim, D-Y, Mitchell, M.A., De las Heras, M., Taylor, H.W., Cho, D-Y. (2002). Spontaneous squamous cell carcinoma of the tongue and multiple bronchioloalveolar carcinomas in a Virginia opossum (Didelphis virginiana). *J Comp Pathol* 126, 226-230.

Kim, F.J., Battini, J.L., Manel, N., Sitbon, M. (2004). Emergence of vertebrate retroviruses and envelope capture. *Virology* 318, 183-191.

Kleinerman, E.S., Lachman, L.B., Knowles, R.D., Snyderman, R., Cianciolo, G.J. (1987). A synthetic peptide homologous to the envelope proteins of retroviruses inhibits monocyte-mediated killing by inctivating interleukin 1. *J Immunol* 139, 2329-2327.

Knipe, D.M. & Howley, P.M. (2001) *Fields Virology* (Lippincott Williams & Wilkins, Philadelphia).

Kowalski, P.E., Freeman, J.D., Mager, D.L. (1999). Intergenic splicing between a HERV-H endogenous retrovirus and two adjacent human genes. *Genomics* 57, 371-379.

Kozak, C.A., Gromet, N.J., Ikeda, H., Buckler, C.E. (1984). A unique sequence related to the ecotropic murine leukemia virus is associated with the Fv-4 resistance gene. *Proc Natl Acad SciUSA* 81, 834-837.

Krishnan, L., Guilbert, L.J., Russell, A.S., Wegmann, T.G., Mosmann, T.R., Belosevic, M. (1996). Pregnancy impairs resistance of C57BL/6 mice to Leishmania major infection and causes decreased antigen-specific IFN-γ responses and increased production of T helper 2 cytokines. *J Immunol* 156, 644–652.

Kung, H.-J. , Bailey, J.M., Davidson, N., Nicolson, M.O., McAllister, R.M. (1975). Structure, subunit composition, and molecular weight of RD-114 RNA. *J Virol* 16, 397-411.

Kung, H.J., Boerkoel, C., Carter, T.H. (1991). Retroviral mutagenesis of cellular oncogenes: a review with insights into the mechanisms of insertional activation. *Curr Top Microbiol Immunol* 171, 1–25.

Kung , H.J. & Vogt. P. (1991). Retroviral insertion and oncogene activation. *Curr Top Microbiol Immunol* 171, 1-25.

Kurland, G.C. (1982). Translational accuracy in vitro. *Cell* 18, 201-202.

Kurts, C., Carbone, F.R., Barnden, M., Blanas, E., Allison, J., Heath, W.R., Miller, J.F. (1997). $CD4^+$ T cell help impairs $CD8^+$ T cell deletion induced by cross-presentation of self-antigens and favors autoimmunity. *J Exp Med* 186, 2057.

Kwang, J., Keen, J., Rosati, S., Tolari, F. (1995). Development and application of an antibody ELISA for the marker protein of ovine pulmonary carcinoma. *Vet Immunol Immunopathol* 47, 323-331.

Lairmore, M.D., Rosadio, R.H., DeMartini, J.C. (1986). Ovine lentivirus lymphoid interstitial pneumonia. Rapid induction in neonatal lambs. *Am J Pathol* 125, 173-181.

Lam, N-Y, Rainer, T-H, Chiu, R-W, Lo, Y-M. (2004). EDTA is a better anticoagulant than heparin or citrate for delayed blood processing for plasma DNA analysis. *Clin Chem* 50, 256-257.

Lamara, A., Fieni, F., Mselli-Lakhal, L., Chatagnon, G., Bruyas, J.F., Tainturier, D., Battut, I., Fornazero, C., Cheboune, Y. (2002). Early embryonic cells from in vivo-produced goat embryos transmit the caprine arthritis–encephalitis virus (CAEV). *Theriogenology* 58,1153–1163.

Lavignon, M., Richardson, J., Evans, L.H. (1997). A small region of the ecotropic murine leukemia virus (MuLV) gag gene profoundly influences the types of polytropic MuLVs generated in mice. *J Virol* 71, 8923–8927.

Lazo, P.A. & Tsichlis, P.N. (1990). Biology and pathogenesis of retroviruses. *Semin Oncol* 17, 269-294.

Lee, C.R., Cervi, D., Truong, A.H., Li, Y.J., Sarkar, A., Ben-David, Y. (2003). Friend virus-induced erythroleukemias: a unique and well-defined mouse model for the development of leukemia. *Anticancer Res* 23, 2159-2166.

Lee, D., Graham, B.S., Chiu, Y.L., Gilbert, P.B., McElrath, M.J., Belshe, R.B., Buchbinder, S.P., Sheppard, H.W., Koblin, B.A., Mayer, K.H., Keefer, M.C., Mulligan, M.J., Celum, C.L. (2004). Breakthrough infections during phase 1 and 2 prime-boost HIV-1 vaccine trials with canarypox vectors (ALVAC) and booster dose of recombinant gp120 or gp160. *J Infect Dis* 190, 903-907.

LeGrand, E.K., Donovan, R.M., Marx, P.A., Moulton, J.E., Cheung, A.T.W., Lewis, A.E., Gardner, M.B. (1985). Monocyte function in rhesus monkeys with simian acquired immune deficiency syndrome. *Vet Immunol Immunopathol* 10, 131-146.

Leis, J., Baltimore, D., Bishop, J.M., Coffin, J.M., Fleissner, E., Goff, S.P., Roszlan, S., Robinson, H., Skalka, A.M., Temin, H.M., Vogt, V. (1988). Standardized and simplified nomenclature for proteins common to all retroviruses. *J Virol* 62, 1808-1809.

Lepperdinger, G., Strobl, B., Kreil, G. (1998). *HYAL2*, a Human Gene Expressed in Many Cells, Encodes a Lysosomal Hyaluronidase with a Novel Type of Specificity. *J Biol Chem* 273, 22466-22470.

Leroux, C., Cadore, J.L., Montelaro, R.C. (2004). Equine Infectious Anemia Virus (EIAV): what has HIV's country cousin got to tell us? *Vet Res* 35, 485-512.

LeVine, A.M. & Whitsett, J.A. (2001). Pulmonary collectins and innate host defense of the lung. *Microbes Infect* 3, 161-166.

Li, E., Golemis, J., Hartley, W., Hopkins, N. (1987). Disease specificity of nondefective Friend and Moloney murine leukemia viruses is controlled by a small number of nucleotides. *J Virol* 61, 693–700.

Li, J.K. (1987). Chromatographic purification and immunological analysis of viral polypeptides of mouse mammary tumor virus. *J Virol Methods* 17, 299-310.

Li, J.P., D'Andrea, A.D., Lodish, H.F., Baltimore, D. (1990). Activation of cell growth by binding of Friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. *Nature* 343, 762-764.

Liebow, A. (1960). Bronchiolar-alveolar carcinoma. *Advances in Internal Medicine* 10, 329-358.

Linial, M. & Blair, D. (1982). Genetics of retroviruses. In *Molecular biology of tumor viruses,* 2nd edition: *RNA tumor viruses* (ed. R. Weiss et al.), pp. 649-683. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Liu, S.L., Duh, F.M., Lerman, M.I., Miller, A.D. (2003). Role of virus receptor Hyal2 in oncogenic transformation of rodent fibroblasts by sheep betaretrovirus env proteins. *J Virol* 77, 2850-2858.

Loftus, B. & Rogers, M. (1997). Characterization of a prion protein (PrP) gene from rabbit; a species with apparent resistance to infection by prions. *Gene* 184, 215-219.

Low, M.G. (1989). Glycosyl-phosphatidylinositol: a versatile anchor for cell surface proteins. *FASEB J* 3, 1600-1608.

Löwer, R. (1999). The pathogenicpotential of endogenous retroviruses: facts and fantasies. *Trends Microbiol* 7, 350-356.

Löwer, R., Löwer, J., Kurth, R. (1996). The viruses in all of us:characteristics and biological significance of human endogenous retrovirus sequences. Proc *Natl Acad Sci USA* 93, 5177-5184.

Lublin, D.M. (1992). Glycosyl-phosphatidylinositol anchoring of membrane proteins. *Curr Top Microbiol Immunol* 178, 141-162 .

Mackay, J.M.K. (1969a). Tissue culture studies of sheep pulmonary adenomatosis (jaagsiekte). I. Direct cultures of affected lungs. *J Comp Pathol* 79, 141-146.

Mackay, J.M.K. (1969b). Tissue culture studies of sheep pulmonary adenomatosis (jaagsiekte). II. Transmission of cytophatic effects to normal cultures. *J Comp Pathol* 79, 147-154.

Madisen, L., Hoar, D.I., Holroyd, C.D., Crisp, M., Hodes, M.E. (1987). DNA banking: The effects of storage of blood and isolated DNA on the integrity of DNA. *Am J Med Genet* 27, 379–390.

Maeda, N., Palmarini, M., Murgia C., Fan, H. (2001). Direct transformation of rodent fibroblasts by jaagsiekte sheep retrovirus DNA. *Proc Natl Acad Sci USA* 98, 4449-4454.

Malik, H.S., Henikoff, S., Eickbush, T.H., (2000). Poised for contagion: evolutionary origins of the infectious abilities of invertebrate retroviruses. *Genome Res* 10, 1307–1318.

Malmquist, W.A., Krauss, H.H., Moulton, J.E., Wandera, J.G. (1972). Morphologic study of virus infected lung cell cultures from sheep pulmonary adenomatosis (jaagsiekte). *Laboratory Investigation* 26, 628-533.

Mangeney, M. & Heidmann, T. (1998). Tumor cells expressing a retroviral envelope escape immune rejection *in vivo*. *Proc Natl Acad Sci USA* 95, 14920-14925.

Marchetti, A., Robbins, J., Campbell, G., Buttitta, F., Squartini, F., Bistocchi, M., Callahan, R. (1991). Host genetic background effect on the frequency of mouse mammary tumor virus-induced rearrangements of the int-1 and int-2 loci in mouse mammary tumors. *J Virol* 65, 4550-4554.

Markson, L.M., Spence, J.B., Dawson, M. (1983). Investigations of a flock heavily infected with maedi-visna virus. *Vet Rec* 112, 267-271.

Martin, G.A., Kawaguchi, R., Lam, Y., DeGiovanni, A., Fukushima, M., Mutter, W. (2001). High-yield, in vitro protein expression using a continuous-exchange, coupled transcription/ translation system. *Biotechniques* 31, 948-953.

Martin, W.B., Angus, K.W., Robinson, G.W., Scott, F.M. (1979). The herpesvirus of sheep pulmonary adenomatosis. CIMID 2, 313-325.

Martin, W.B., Scott, F.M., Sharp, J.M., Angus, K.W., Norval, M. (1976). Experimental production of sheep pulmonary adenomatosis. *Nature* 264, 183-184.

Maruo, K., Sugimoto, T., Suzuki, K., Shirota, K., Ejima, H., Nomura, T. (1995). Xenotransplantation and high tumorigenicity of feline tumors in SCID mice. *J Vet Med Sci* 57, 967-969.

Mason, R.J. & Shannon, J.M. (1997). Alveolar type II pneumocytes. In: Crystal R.G., West, J.B., Weibel, E.R., Barnes, P.J., editors. The lung: scientific foundations. Vol.1. Philadelphia (PA): Lippincott-Raven. p.543-555.

Massaro, D. (1989). Non ciliated bronchiolar ephitelial (Clara) cells. In: Massaro D., editor. Lung cell biology. New York (NY): Marcel Dekker. p.81-114.

Mathis, D. & Benoist, C. (2004). Back to Central Tolerance. *Immunity* 20, May 509-516.

Matsuzawa, A., Nakano, H., Yoshimoto, T., Sayama, K. (1995). Biology of mouse mammary tumour virus (MMTV). *Cancer Letters* 90, 3-11.

Maul, D.H., Zaiss, C.P., MacKenzie, M.R., Shiigi, S.M., Marx, P.A., Gardner, M.B. (1988). simian retrovirus D serogroup 1 has a broad cellular tropism for lymphoid and nonlymphoid cells. *J Virol* 62, 1768-1773.

Mazzucchelli, R., Amadio, M., Curreli, S., Denaro, F., Bemis, K., Reid, W., Bryant, J., Riva, A., Galli, M., Zella, D. (2004).Establishment of an ex vivo model of monocytes-derived macrophages differentiated from peripheral blood mononuclear cells (PBMCs) from HIV-1 transgenic rats. *Mol Immunol* 41, 979-984.

McClure, M.A., Johnson, M.S., Feng, D.F., Doolittle, R.F. (1988). Sequence comparisons of retroviral proteins: relative rates of change and general phylogeny. *Proc Natl Acad Sci USA.* 85, 2469-2473.

McDougall, A.S., Terry, A., Tzavaras,T., Cheney, C., Rojko, J., Neil, J.C. (1994). Defective endogenous proviruses are expressed in feline lymphoid cells: evidence for a role in natural resistance to subgroup feline leukemia viruses. *J Virol* 68, 2151-2160.

McFadyean, J. (1888). Lung disease in sheep, caused by *Strongylus rufescens*. *J Comp Pathol Ther* 1, 39.

McFadyean, J. (1894). Verminous pneumonia in the sheep. *J Comp Pathol Ther* 7, 31-42.

McFadyean, J. (1920). Transformation of the alveolar epithelium in verminous pneumonia in the sheep. *J Comp Pathol Ther* 33, 1-10.

McGee-Estrada K., Palmarini, M., Fan, H. (2002). HFN-3beta is a critical factor for the expression of jaagsiekte sheep retrovirus long terminal repeat in type II pneumocytes but not in Clara cells. *Virology* 292, 87-97.

Medzhitov, R. & Janeway Jr., C.A. (2002). Decoding the patterns of self and nonself by the innate immune system. *Science* 296, 298-300.

Mi, S. Li, X., Veldman, G.M., Finnerty, H., Racie, L., La Vallie, E., Tang, X.Y., Edouard, P., Howes, S., Keith, J.C.Jr, McCoy, J.M. (2000). Syncitin is a captive retroviral envelope protein involved in human placental morphogenesis. *Nature* 403, 715-717.

Miller, S.A., Dykes D.D., Polesky, H.F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucl Acid Res* 16 ,1255.

Miller, R.J., Cairns, J.S., Bridges, S., Sarver, N. (2000). Human immunodeficiency virus and AIDS: insights from animal lentiviruses. *J Virol* 74, 7187-7195.

Monlux, W., Bennett, P., Kingrey, B. (1955). Pulmonary adenomatosis in Iowa Cattle. *Iowa Vet* 26, 11-13.

Monlux, W., Fitte, J., Kendrick, G., Dubuisson, H. (1953). Progressive Pulmonary adenomatosis in cattle. *Southwest Vet* 6, 267-269.

Morabia, A. & Wynder, E.L. (1992). Relation of bronchioloalveolar carcinoma to tobacco. *B M J* 304, 541-543.

Morozov, V.A., Lagaye, S., Lower, J., Lower, R. (2004). Detection and characterization of betaretroviral sequences, related to sheep Jaagsiekte virus, in Africans from Nigeria and Cameroon. *Virology* 327, 162-168.

Mura M., Murcia, P., Caporale, M., Spencer, T.E., Nagashima, K., Rein, A., Palmarini, M. (2004). Late viral interference induced by transdominant Gag of an endogenous retrovirus. *Proc Natl Acad Sci U S A* 101, 11117-11122.

Murayama, M., Sun, X., Takashima, A. (2001). Synthesis of the His-6-tagged recombinat proteins APPC99-His and His-PS1 using the RTS 500 instrument. *Biochemica* 4, 24-26.

Murphy, F.A., Fauquet, C.M., Bishop, D.H.L., Ghabrial, S.A. , Jarvis, A.W., Martelli, G.P., Mayo, M.A. , Summers, M.D. (1994). *Virus taxonomy: The classification and nomenclature of viruses, Retroviridae*. Springer-Verlag, Vienna.

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

Nakagawa, K. & Harrison, L. (1996). The potential roles of endogenous retroviruses in autoimmunity. *Immunol Rev* 152, 193-236.

Neil, J.C., Fulton, R., Rigby, M., Stewart, M. (1991). Feline leukaemia virus: generation of pathogenic and oncogenic variants. *Curr Top Microbiol Immunol* 171, 67-93.

Nevjestic, A., Forsek, Z., Cvjetanovic, V., Rukavina, L. (1971). Sheep pulmonary adenomatosis (SPA) in Crna Gora. Isolation of viral agent from the lungs of sheep affected affected with sheep pulmonary adenomatosis. *Veterinaria* 20, 31-35.

Nieddu, A.M., Coda, S., Loi, P. (1987). L'adenomatosi polmonare del muflone (Ovis musimon). *Atti della Societa' Italiana di Scienze Veterinarie* 41, 892-894.

Nisbet, D.I., Mackay, J.M., Smith, W., Gray, E.W. (1971). Ultrastructure of sheep pulmonary adenomatosis (Jaagsiekte). *J Pathol* 103, 157-162.

Nobel, T.A., (1958). Pulmonary adenomatosis (jaagsiekte) in sheep with special reference to its occurrence in Israel. *Refuah Veterinarith* 15, 98-101.

Nobel, T.A., Neumann, F., Klopfer, U. (1969). Histological patterns of the metastases in pulmonary adenomatosis of sheep (jaagsiekte). *J Comp Pathol* 79, 537-540.

Nobel, T.A. & Perk, K. (1978). Bronchiolo-alveolar cell carcinoma. Animal model: pulmonary adenomatosis of sheep, pulmonary carcinoma of sheep (Jaagsiekte). *Am J Pathol* 90, 783-786.

Nusse, R. & Varmus, H.E. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cell* 31, 99–109.

Ohashi, P.S. (2003). Negative selection and autoimmunity. *Curr Opin Immunol* 15, 668–676.

Okada, Y., Ochiai, K., Osaki, K., Kimura, T., Itakura, C. (1998). Bronchiolar-alveolar carcinoma in a cow. *J Comp Pathol* 118, 69-74.

Oldenhoven, G., de Heusch, M., Urbain-Vansante, G., Urbain, J., Maliszewski, C., Leo, O., Moser, M. (2003). CD4+ CD25+ regulatory T cells control T helper cell type 1 responses to foreign antigens induced by mature dendritic cells in vivo. *J Exp Med 2003* 198, 259-126.

Ortín, A., Cousens, C., Minguijon. E., Pascual, Z., Perez de Villarreal, M., Sharp, J.M., De las Heras, M. (2003). Characterization of enzootic nasal tumour virus of goats: complete sequence and tissue distribution. *J Gen Virol* 84, 2245-2252.

Ortín, A., Minguijon. E., Dewar, P., Garcia, M., Ferrer, L.M., Palmarini, M., Gonzales, L., Sharp, J.M., De las Heras, M. (1998). Lack of a specific immune response against a recombinant capsid protein of Jaagsiekte sheep retrovirus in sheep and goats naturally affected by enzootic nasal tumour or sheep pulmonary adenomatosis. *Vet Immunol Immunopathol* 61, 229-37.

Otto, W.R. (1997). Lung stem cells. *Int J Exp Pathol* 78, 291-310.

Overbaugh, J., Miller, A.D., Eiden, M.V. (2001). Receptors and entry cofactors for retroviruses include single and multiple transmembrane-spanning proteins as well as newly described glycophosphatidylinositol-anchored and secreted proteins. *Microbiol Mol Biol Rev* 65, 371-389.

Palmarini, M., Cousens, C., Dalziel, R.G., Bai, J., Stedman, K., DeMartini, J.C., Sharp, J.M. (1996a). The exogenous form of Jaagsiekte retrovirus is specifically associated with a contagious lung cancer of sheep. *J Virol* 70, 1618-1623.

Palmarini, M., Datta, S., Omid, R., Murgia, C., Fan, H. (2000b). The long terminal repeat of jaagsiekte sheep retrovirus is preferentially active in differentiated epithelial cells of the lungs. *J Virol* 74, 5776-5787.

Palmarini, M., Dewar, P., De las Heras, M., Inglis, N.F., Dalziel, R.G., Sharp, J.M. (1995). Epithelial tumour cells in the lungs of sheep with pulmonary

adenomatosis are major sites for replication of Jaagsiekte retrovirus. *J Gen Virol* 76, 2731-2737.

Palmarini, M. & Fan, H. (2001c). Retrovirus-induced ovine pulmonary adenocarcinoma, an animal model for lung cancer. *J Natl Cancer Inst* 93, 1603-1614.

Palmarini, M. & Fan, H. (2003). Molecular biology of jaagsiekte sheep retrovirus. *Curr Top Microbiol Immunol* 275, 81-115.

Palmarini, M., Fan, H., Sharp, J.M. (1997). Sheep pulmonary adenomatosis: a unique model of a retrovirus-associated lung cancer. *Trends in Microbiology* 5, 478-483.

Palmarini, M., Gray, C.A., Carpenter, K., Fan, H., Bazer, F.W., Spencer, T.E. (2001b). Expression of endogenous betaretroviruses in the ovine uterus: effects of neonatal age, estrous cycle, pregnancy, and progesterone. *J Virol* 75, 11319-11327.

Palmarini, M., Hallwirth, C., York, D., Murgia, M., de Oliveira, T., Spencer, T., Fan, H. (2000a). Molecular cloning and functional analysis of three type D endogenous retroviruses of sheep reveal a different cell tropism from from that of the highly related exogenous jaagsiekte sheep retrovirus. *J Virol* 74, 8065-8076.

Palmarini, M., Holland, M.J., Cousens, C., Dalziel, R.G., Sharp, J.M. (1996b). Jaagsiekte retrovirus (JSRV) establishes a disseminated infection of the lymphoid tissues in sheep affected by pulmonary adenomatosis. *J Gen Virol* 77, 2991-2998.

Palmarini, M., Maeda, N., Murgia, C., De-Fraja, C., Hofacre, A., Fan, H. (2001a). A phosphatidylinositol-3-kinase (PI3-K) docking site in the cytoplasmatic tail of the jaagsiekte sheep retrovirus transmembrane protein is essential for envelope-induced transformation of NIH 3T3 cells. *J Virol* 75, 11002-11009.

Palmarini, M., Mughetti, L., Tollis, M., Alunni, L., Vitellozzi, G. (1994). Il tumore intranasale enzootico della capra. *Summa* 4, 49-53.

Palmarini, M., Mura, M., Spencer, T.E. (2004). Endogenous betaretroviruses of sheep teaching new lessons in retroviral interference and adaptation. *J Gen Virol* 85, 1-13.

Palmarini, M., Murgia, C., Fan, H. (2002). Spliced and prematurely polyadenylated Jaagsiekte sheep retrovirus-specific RNAs from infected or transfected cells. *Virology* 294, 180-188.

Palmarini, M., Sharp, J.M, De las Heras, M., Fan, H. (1999b). Jaagsiekte sheep retrovirus is necessary and sufficient to induce a contagious lung cancer in sheep. *J Virol* 73, 6964-6972.

Palmarini, M., Sharp, J.M, Lee, C., Fan, H. (1999a). *In vitro* infection of ovine cells lines by Jaagsiekte sheep retrovirus. *J Virol* 73, 10070-10078.

Pantaleo, G., Graziosi, C., Butini, L, Pizzo, P.A., Schnittman, S.M., Kotler, D.P., Fauci, A.S. (1991). Lymphoid organs function as major reservoirs for human immunodeficiency virus. *Proc Natl Acad Sci U S A* 88, 9838-9842.

Pantaleo, G., Graziosi, C., Demarest, J.F., Butini, L., Montroni, M., Fox, C.H., Orenstein, J.M., Kotler, D.P., Fauci, A.S. (1993). HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease. *Nature* 362, 355-358.

Papoulis, A. (1984). Conditional Probabilities and Independent Sets. Paragraphs 2-3 in Probablility, Random Variables, and Stochastic processes 2nd ed. New York: McGraw-Hill, p. 33-45.

Parker, B.N.J., Wrathall, A.E., Saunders, R.W., Dawson, M., Done, S.H., Francis, P.G., Dexter, I., Bradley, R. (1998). Prevention of transmission of sheep pulmonary adenomatosis by embryo transfer. *Vet Rec* 142, 687-689.

Parkin, D.M. (1998). The global burden of cancer. *Semin Cancer Biol* 8, 219-235.

Pastoret, P.-P. & Vanderplasschen, A. (2003).Poxviruses as vaccine vectors. *CIMID* 26, 343-355.

Pastoret, P.P. & Brochier, B. (1996). The development and use of vaccinia-rabies recombinant oral vaccine for the control of wildlife rabies: a link between Jenner and Pasteur. *Epidemiol Infect* 116, 235–240.

Patience, C., Wilkinson, D.A., Weiss, R.A. (1997). Our retroviral heritage. *Trends Genet* 13, 116-120.

Pavlov, M.Y. & Ehrenberg, M. (1996). Rate of Translation of Natural mRNAs in an Optimized *in Vitro* System. *Arch Biochem Biophys* 328, 9-16.

Payne, A.L., & Verwoerd, D.W. (1984). A scanning and transmission electron microscopy of jaagsiekte lesions. *Onderstepoort J Vet Res* 51, 1-13.

Payne, A.L., Verwoerd, D.W., Garnett, H.M. (1983). The morphology and morphogenesis of jaagsiekte. *Onderstepoort J Vet Res* 50, 317-322.

Payne, A.L., York, D.F., deVilliers, E.M., Verwoerd, D.W., Querat, G., Barban, V., Sauze, N., Vigne, R. (1986).Isolation and identification of a South African lentivirus from jaagsiekte tumour tissue and cell lines. *Onderstepoort J Vet Res* 53, 55-62.

Payne, G.S., Bishop J.M., Varmus, H.E. (1982). Multiple arrangements of viral DNA and an activated host oncogene in bursal lymphomas. *Nature* 295, 209–214.

Payne, L.N. & Pani, P.K. (1971). A dominant epistatic gene which inhibits cellular susceptibility to RVS (RAV-0). *J Gen Virol* 13, 455-462.
Payne, R.W. (ed.). (2000). The guide to Genstat, VSN Internationational Ltd., Oxford.

Pearson, M.N. & Rohrmann, G.F. (2002). Transfer, incorporation, and substitution of envelope fusion proteins among members of the Baculoviridae, Orthomyxoviridae, and Metaviridae (Insect Retrovirus) families. *J. Virol.* 76, 5301–5304.

Perk, K., De Villiers, E.M., Dawson, M., Herring, H.J., Sharp, J.M., De Martini, J.C. (1985). Comparison by Western blotting of the retroviruses associated with sheep adenomatosis (jaagsiekte). *In:* Slow Virus Diseases in Sheep, Goats and Cattle. Sharp J.M. & Hoff-Jorgensen R., eds. CEC Report EUR 8076 EN, Luxembourg, p. 349–351.

Perk, K. & Hod, I. (1982). Sheep lung carcinoma: an endemic analogue of a sporadic human neoplasm. *J Natl Cancer Inst* 69, 747-749.

Perk, K., Hod, I., Nobel T.A. (1971). Pulmonary adenomatosis of sheep (jaagsiekte). I. Ultrastructure of the tumour. *J Natl Cancer Inst* 46, 525-537.

Perk, K., Michalides, R., Spiegelman, S., Sholn, J. (1974). Biochemical and morphological evidence for the precence of an RNA tumour virus in pulmonary carcinoma of sheep (Jaagsiekte). *J Natl Cancer Inst* 53, 131-135.

Perkus, M.E., Piccini, A., Lipinskas, B.R., Paoletti, E. (1985). Recombinant vaccinia virus: immunization against multiple pathogens. *Science* 229, 981–984.

Perron, H., Garson, J.A., Bedin, F. et al. 10 authors (1997). Molecular identification of a novel retrovirus repeatedly isolated from patients with multiple sclerosis. *Proc Natl Acad Aci USA* 94, 7583-7588.

Peterhans, E., Greenland, T., Badiola, J., Harkiss, G., Bertoni, G., Amorena, B., Eliaszewicz, M., Juste, R.A., Krassnig, R., Lafont, J., Lenihan, P., Petursson, G., Pritchard, G., Thorley, J., Vitu, C., Mornex, J.F., Pepin, M. (2004). Routes of transmission and consequences of small ruminant lentiviruses (SRLVs) infection and eradication schemes. *Vet Res* 35, 257-274.

Petursson, G. (1994). Experience with visna virus in Iceland. *Ann N Y Acad Sci* 724, 43-49.

Pierson, T., McArthur, J., Siliciano, R.F. (2000). Reservoirs for HIV-1: mechanisms for viral persistence in the presence of antiviral immune responses and antiretroviral therapy. *Ann Rev Immunol* 18, 665–708.

Pizer, E.S., Baba,T.W., Humphries, E.H. (1992). Activation of the c-myb locus is

insufficient for the rapid induction of disseminated avian B-cell lymphoma. *J Virol* 66, 512-523.

Platt, J.A., Kraipowich, N., Villafane, F., DeMartini, J.C. (2002). Alveolar type II cells expressing jaagsiekte sheep retrovirus capsid protein and surfactant proteins are the predominant neoplastic cell type in ovine pulmonary adenocarcinoma. *Vet Pathol* 39, 341-352.

Plopper, C.G., Dallas, M.H., Buckpitt, A.R. (1997). Clara cells. In: Crystal R.G., West, J.B., Weibel, E.R., Barnes, P.J., editors. The lung: scientific foundations. Vol.1. Philadelphia (PA): Lippincott-Raven. p. 517-533.

Poiesz, B.J., Ruscetti, F.W., Gazdar, A.F., Bunn, P.A., Minna, J.D., Gallo, R.C. (1980). Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci U S A* 77, 7415-7419.

Poirier, Y. & Jolicoeur, P. (1989). Distinct helper virus requirements for Abelson murine leukemia virus-induced pre-B- and T-cell lymphomas. *J Virol* 63, 2088-2098.

Polakova, H. Kadasi L., Zelinkova, M. (1989). The yield and quality of DNA extracted from blood samples stored under various conditions. *Bratisl Lek Listy* 90, 844–847.

Pritchard, G.C. & Done, S.H. (1990). Concurrent maedi-visna virus infection and pulmonary adenomatosis in a commercial breeding flock in East Anglia. *Vet Rec* 127, 197-200.

Querat, G., Barban, V., Sauze, N., Vigne, R., Payne, A.L., York, D.F., De Villiers, E.M., Verwoerd, D.W. (1987). Characteristics of a novel lentivirus derived from South African sheep with pulmonary adenomatosis (jaagsiekte). *Virology* 158, 158–167.

Rabson, A.B. & Graves, B.J. (1997). Synthesis and processing of viral RNA, In: Retroviruses (JM Coffin, SH Hughes, H Varmus, eds, p.205-261. Cold Spring Harbor Laboratory press, Cold Spring Harbor (NY).

Rai, S.K., DeMartini, J.C., Miller, A.D. (2000). Retrovirus vectors bearing jaagsiekte sheep retrovirus Env transduce human cells by using a new receptor localized to chromosome 3p21.3. *J Virol* 74, 4698-4704.

Rai, S.K., Duh, F.M., Vigdorovich, V., Danilkovitch-Miagkova, A., Lerman, M.I., Miller, A.D. (2001). Candidate tumor suppressor HYAL2 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor for jaagsiekte sheep retrovirus, the envelope protein of which mediates oncogenic transformation. *Proc Natl Acad Sci USA* 98, 4443-4448.

Raines, M.A. , Maihle, N.J., Moscovici, C., Crittenden, L., Kung. H.-J. (1988). Mechanism of c-*erbB* transduction: Newly released transducing viruses retain poly(A) tracts of *erbB* transcripts and encode C-terminally intact *erbB* proteins. *J Virol* 62, 2437-2443.

Rajya, B.S. & Singh, C.M. (1964). The pathology of pneumonia and associated respiratory disease of sheep and goats. I.Occurrence of jaagsiekte and maedi in sheep and goats in India. *Am J Vet Res* 25, 61-67.

Rappuoli, R. (2004). From Pasteur to genomics: progress and challenges in infectious diseases. *Nature Medicine* 10, 1117-1185.

Rasheed, S. (1995). Retroviruses and oncogenes. In: Levy JA, editor. *The Retroviridae.* Vol 4. New York (NY): Plenum Press; p. 293–408.

Rassa, J.C., Meyers, J.L., Zhang, Y., Kudaravalli, R., Ross, S.R. (2002). Murine retroviruses acitivate B cells via interaction with toll-like receptor 4. *Proc Natl Acad Sci USA* 99, 2281-2286.

Rimmelzwaan, G.F., Siebelink, K.H., Broos, H., Drost, G.A., Weijer, K., van Herwijnen, R., Osterhaus, A.D. (1994). gag- and env-specific serum antibodies in cats after natural and experimental infection with feline immunodeficiency virus. *Vet Microbiol* 39, 153-165.

Roberts, B.E. & Paterson, B.M. (1973). Efficient translation of tobacco mosaic virus RNA and rabbit globin 9S RNA in a cell free system from commercial wheat germ. *Proc Nat Acad Sci USA* 70, 2330-2334.

Robertson, W.M. (1904). Jagziekte or chronic catarral pneumonia (sheep). *Journal of Comparative Pathology and Therapeutics* 17, 221-224.

Roizman, B., Desrosiers, R.C., Fleckenstein, B., Lopez, C., Minson, A.C., Studdert, M.J. (1992). The family herpesviridae: an update. *Arch Virol* 123, 425-449.

Rosadio, R.H., Lairmore, M.D., Russell, H.I., DeMartini, J.C. (1988). Retrovirus-associated ovine pulmonary carcinoma (sheep pulmonary adenomatosis) and lymphoid interstitial pneumonia. Lesion development and age susceptibility. *Vet Pathol* 25, 475-483.

Rosadio, R. & Sharp, J.M. (1992). Leukocyte frequency alterations in sheep with naturally and experimentally-induced lung cancer. *Veterinaria Medicina* 9, 49-51.

Rosadio, R.H., Sharp, J.M., Lairmore, M.D., Dahlberg, J.E., DeMartini, J.C. (1988). Lesions and retroviruses associated with naturally occurring ovine pulmonary carcinoma (sheep pulmonary adenomatosis). *Vet Pathol* 25, 58-66.

Rosati, S., Pittau, M., Alberti, S., Pozzi, D., York, D.F., Sharp, J.M., Palmarini, M. (2000). An accessory open reading frame (orf-x) of jaagsiekte sheep retrovirus is conserved between different virus isolates. *Virus Research* 66, 109-116.

Rosenberg, N. (2001). New transformation tricks from a barnyard retrovirus: implications for human lung cancer. *Proc Natl Acad Sci USA* 98, 4285-4287.

Rosenberg, N. & Jolicoeur, P. (1997). Retroviral Pathogenesis. In: *Retroviruses,* p. 475-585. Ed. Coffin, J. M., Hughes, S.H., Varmus, H.E. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.

Rosenberg, S.A. (2001). Progress in human tumour immunology and immunotherapy. *Nature* 411, *380.*

Rous, P. (1911). A sarcoma of the fowl transmissible by an agent separable from the tumor cells. *J Exp Med* 13, 397-411.

Rowe J.D. & East N.E. (1999). Risk factors for transmission and methods for control of caprine arthritis-encephalitis virus infection. *Vet Clin North Am Food Anim Pract* 13, 35-53.

Rubin, H., Cornelius, A., Fanshier, L. (1961). The pattern of congenital transmission of an avian leukosis virus. *Proc Natl Acad Sci* 47, 1058-1069.

Rubin, H., Fanshier, L., Cornelius, A., Hughes, W.F. (1962). Tolerance and immunity in chickens after congenital and contact infection with an avian leukosis virus. *Virology* 17, 143-156.

Ruis, B.L., Benson, S.J., Conklin, K.F. (1999). Genome structure and expression of the ev/J family of avian endogenous viruses. *J Virol* 73, 5345-5355.

Rundall, B.K., Denlinger, C.E., Jones, D.R. (2004). Combined Histone deacetylase and NF-kappaB inhibition sensitizes non-small cell lung cancer to cell death. *Surgery* 136, 416-425.

Ruscetti, S., Davis, L., Field, J., Oliff, A. (1981). Friend murine leukemia is associated with the formation of mink cell focus-inducing viruses and is blocked in mice expressing endogenous mink cell focus-inducing xenotropic viral envelope genes. *J Exp Med* 154, 907-920.

Ruscetti, S.K. (1999). Deregulation of erythropoiesis by the Friend spleen focus-forming virus. *Int J Biochem Cell Biol* 31, 1089-1109.

Ruscetti, S.K., Janesch, N.J., Chakraborti, A., Sawyer, S.T., Hankins, W.D. (1990). Friend spleen focus-forming virus induces factor independence in an erythropoietin-dependent erythroleukemia cell line. *J Virol* 64, 1057-1062.

Ryan, S., Tiley, L., McConnell, I., Blacklaws, B. (2000).Infection of dendritic cells by the Maedi-Visna lentivirus. *J Virol* 74, 10096-10103.

Sabin, A.B. (1992). Improbability of Effective Vaccination Against Human Immunodeficiency Virus Because of Its Intracellular Transmission and Rectal Portal of Entry. *Proc Natl Acad Sci USA* 89, 8852–8855.

Saleh, H.A., Haapaniemi, J., Khatib, G., Sakr, W. (1998). Bronchioloalveolar carcinoma: diagnostic pitfalls and immunocytochemical contribution. *Diagn Cytopathol* 18, 301-306.

Sambrook, J., Fritsh, E. F., Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual*, 2nd edn. New York: Cold Spring Harbor Laboratory.

Sanger, F., Nicklen, S., Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci U S A* 74, 5463-5467.

Sanna, M.P., Sanna, E., De las Heras M., Leoni, A., Nieddu, A.M., Pirino, S., Sharp, J.M., Palmarini, M. (2001). Jaagsiekte sheep retrovirus is associated with ovine pulmonary carcinoma in Sardinian moufflons. *J Comp Path* 125, 145-152.

Sarkar, N.H. (1995). Clonal variations among multiple primary mammary tumors and within a tumor of individual mice: insertions mutations of int oncogenes. *Virology* 212, 490-499.

Sauter, M., Schommer, S., Kremmer, E., Remberger, K., Dölken, G., Müller-Lantzsch, N. (1995). Human endogenous retrovirus K-10: expression of gag protein and detection of antibodies in patients with seminomas. *J Virol* 69, 414-421.

Schaefer-Klein, J., Givol, I., Barsov, E.V., Whitcomb, J.M., Van Brocklin, M., Foster, D.N., Federspiel, M.J., Hughes, S.H. (1998). The EV-O-derived cell line DF-1 supports the efficient replication of avian leukosis-sarcoma viruses and vectors. *Virology* 248, 305-311.

Schiavetti, F., Thonnard, J., Colau, D., Boon, T., Coulie, P.G. (2002). A human endogenous retroviral sequence encoding an antigen recognized on melanoma by cytolytic T lymphocytes. *Cancer Res* 62, 5510.

Schmidt, D.M. & Snyderman, R. (1988). Retroviral protein P15E and tumorigenesis. Expression is neither required nor sufficient for tumor development. *J Immunol* 140, 4035-4041.

Schraufnagel, D., Peloquin, A., Pare, J.A., Wang, N.S. (1982). Differentiating bronchioloalveolar carcinoma from adenocarcinoma. *Am Rev Respir Dis* 125, 74-79.

Schulte, A.M., Lai, S., Kurtz, A., Czubayko, F., Riegel, A.T., Wellstein, A. (1996). Human trophoblast and choriocarcinoma expression of the growth factor

pleiotrophin attributable to germ-line insertion of an endogenous retrovirus. *Proc Natl Acad Sci USA* 93, 14759-14764.

Schunemann, H.J., Stanulla, M., Trevisan, M., Aplan, P.D., Freudenheim, J.L., Muti, P. (2000). Short-term storage of blood samples and DNA isolation in serum separator tubes for application in epidemiological studies and clinical research. *Ann Epidemiol* 10, 538-544.

Scott, F.M., Sharp, J.M., Angus, K.W, Gray, E.W. (1984). Infection of specific-pathogen free lambs with a herpesvirus isolated from pulmonary adenomatosis. *Arch Virol* 80, 147-162.

Sekido, Y., Bader, S., Latif, F., Chen, J.Y., Duh, F.M., Wei, M.H., Albanesi, J.P., Lee, C.C., Lerman, M.I., Minna, J.D. (1996). Human semaphorins A(V) and IV reside in the 3p21.3 small cell lung cancer deletion region and demonstrate distinct expression patterns. *Proc Natl Acad Sci U S A* 93, 4120-4125.

Sellon DC. (1993). Equine infectious anemia. *Vet Clin North Am Equine Pract* 9, 321-36.

Sharma, D.N., Rajya, B.S., Dwivedi, J.N. (1975). Experimental transmission of Jaagsiekte and Maedi in sheep and goats. *Indian Journal of Animal Science* 45, 275-281.

Sharp, J.M. (1981). Slow virus infections of the respiratory tract of sheep. *Vet Rec* 108, 391-393.

Sharp, J.M. (1987). Sheep pulmonary adenomatosis: a contagious tumour and its cause. *Cancer Surv* 6, 73-83.

Sharp, J.M. (1991).Chronic respiratory virus infections of the respiratory tract of sheep. In: *Diseases of Sheep.* Martin, W.B. & Aitken, I.D., (eds), 2nd Ed., Blackwell Scientific Publications, Oxford, p. 143-150.

Sharp, J.M. (2002). Ovine pulmonary adenomatosis. *Proceedings 16th International Seminar W.A.V.M.I.* p.35-41.

Sharp, J.M. & Angus, K.W. (1985). Jaagsiekte (Sheep Pulmonary adenomatosis). *Sheep Veterinary Society Proceedings of meetings* 9, 123.

Sharp, J.M. & Angus, K.W. (1990). Sheep pulmonary adenomatosis: clinical, pathological and epidemiological aspects. In : *Development in Veterinary Virology: Maedi-visna and related diseases.* Pètursson G. and Hoff-Jorgensen R.Eds.: Kluver Academic Publishers Boston; p. 177-185.

Sharp, J.M., Angus, K.W., Gray, E.W., Scott, F.M. (1983). Rapid transmission of sheep pulomonary adenomatosis (Jaagsiekte) in young lambs. *Arch Virol* 78, 89-95.

Sharp, J.M., Angus, K.W., Jassim, F.A. *et al.* (1986). Experimental transmission of sheep pulmonary adenomatosis to a goat. *Vet Rec* 19, 245.

Sharp, J.M. & De las Heras, M. (2000). Contagious respiratory tumours. In *Diseases of Sheep,* third edition. Ed. Martin and Aitken.p.181-186.

Sharp, J.M. & DeMartini, J.C. (2003). Natural History of JSRV in sheep. *Curr Top Mibrobiol Immunol* 275, 55-79.

Sharp, J.M. & Herring, A.J. (1983). Sheep pulmonary adenomatosis: demonstration of a protein that cross-reacts with the major core protein of mason-Pfizer monkey virus and mouse mammary tumour virus. *J Gen Vir*ol 64, 2323-2327.

Sherwood, B.F., Rowlands, D.t., Hackel, D.B. (1969). Pulmonary adenomatosis in opossums (*Didelphis virginiana*). *Journal of the American Veterinary Medical Association* 155, 1102-1107.

Shih, C., & Weinberg, R.A. (1982). Isolation of a transforming sequence from a human bladder carcinoma cell line. *Cell* 29, 161-169.

Shirlaw, J.F. (1959). Studies on jaagsiekte in Kenya. *Bull Epizootic Dis Afr* 7, 287-302.

Shopland D.R. (1995). Tobacco use and its contribution to early cancer mortality with a special emphasis on cigarette smoking. *Envion Health Perspect* 103, 131-142.

Sigurdsson, B. (1958). Adenomatosis of sheep's lung; experimental transmission. *Arch Gesamte Virusforsch* 8, 51-58.

Sigurdsson, B. (1954). Rida, a chronic encephalitis of sheep: with general remarks on infections which develop slowly and some of their special characteristics. *Br Vet J* 110, 287-302.

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

Silverman, L.R., Phipps, A.J., Montgomery, A., Ratner, L., Lairmore, M.D. (2004). Human T-cell lymphotropic virus type 1 open reading frame II-encoded p30II is required for in vivo replication: evidence of in vivo reversion. *J Virol* 78, 3837-3845.

Sinowatz, F., Kölle, S., Töpfer-Petersen, E. (2001). Biosynthesis and Expression of Zona pellucida Glycoproteins in Mammals. *Cells Tissues Organs* 168, 24-35.

Sitbon, M., Nishio, J., Wehrly, K., Chesebro, B. (1985). Pseudotyping of dual-tropic recombinant viruses generated by infection of mice with different ecotropic murine leukemia viruses. *Virology* 140, 144–151.

Smith, D.B. & Jhonson, K.S. (1988). Single step purification of polipeptides expressed in *E. coli* with glutanione S-transferase. *Gene* 67, 31-40.

Smith, G.L., Mackett, M., Moss, B. (1983a). Infectious vaccinia virus recombinants that express hepatitis B virus surface antigen. *Nature* 302, 490–495.

Smith, G.L., Murphy B.R., Moss, B. (1983b). Construction and characterisation of an infectious vaccinia virus recombinant that expresses the influenza hemagglutinin gene and induces resistance to influenza virus infection in hamsters. *Proc Natl Acad Sci USA* 80, 7155–7159.

Snowder, G.D., Gates, N.L., Glimp, H.A., Gorham, J.R. (1990). Prevalence and effect of subclinical ovine progressive pneumonia virus infection on ewe wool and lamb production. *J Am Vet Med Assoc* 197, 475-479.

Snyder, S.P., DeMartini, J.C., Ameghino, E. (1983). Coexistence of pulmonary adenomatosis and progressive pneumonia in sheep in the Central Sierra of Peru. *Am J Vet Res* 44, 1334-1338.

Sorensen, J.B., Hirsch, F.R., Gazdar, A., Olsen J.E. (1993). Interobserver variability in histopathologic subtyping and grading of pulmonary adenocarcinoma. *Cancer* 71, 2971-2976.

Sotomayor, E.M., Borrello, I., Tubb, E., Rattis, F.M., Bien, H., Lu, Z., Fein, S., Schoenberger, S., Levitsky, H.I. (1999). Conversion of tumor-specific $CD4^+$ T-cell tolerance to T-cell priming through in vivo ligation of CD40. *Nat Med* 5, 780-787.

Sourvinos, G., Tsatsanis, C., Spandidos, D.A. (2000). Mechanism of retrovirus-induced oncogenesis. *Folia Biologica (Praha)* 46, 226-232.

Speck, N.A., Renjifo, B., Golemis, E., Fredrickson. T.N., Hartley , J.W., Hopkins, N. (1990). Mutation of the core or adjacent LVb elements of the Moloney murine leukemia virus enhancer alters disease specificity. *Genes Dev* 4, 233–242.

Spencer, T.E., Mura, M., Gray, C.A., Griebel, P.J., Palmarini, M. (2003). Receptor usage and fetal expression of ovine endogenous betaretroviruses: implications for coevolution of endogenous and exogenous retroviruses. *J Virol* 77, 749-753.

Spencer, T.E., Stagg, A.G., Joyce, M.M., Jenster, G., Wood, C.G., Bazer, F.W., Wiley, A.A., Bartol, F.F. (1999). Discovery and characterization of endometrial epithelial messenger ribonucleic acids using the ovine uterine gland knockout model. *Endocrinology* 140, 4070-4080.

Spirin, A.S., Baranov, V.I., Ryabova, L.A., Ovodov, S.Y., Alakhov, Y.B. (1988). A continous cell-free translation system capable of producing polypetides in high yield. *Science* 42, 1162-1164.

Spriggs, M.K. (1996). One step ahead of the game: viral immunomodulatory molecules. *Annu Rev Immunol* 14, 101-130.

Sriraman, P.K., Pao, P.R., Naidu, N.R.G. (1982). Goat mortality in Andhra Pradesh. *Indian Veterinary Journal* 59, 96-99.

Stedman, N.L., Brown, T.P., Brown, C.C. (2001). Localization of avian leukosis virus subgroup J in naturally infected chickens by RNA in situ hybridization. *Vet Pathol* 38, 649-656.

Steffen, D. & Weinberg, R.A. (1978). The integrated genome of murine leukemia virus. *Cell* 15, 1003–1010.

Stehelin, D., Guntaka, R.V., Varmus, H.E., Bishop, J.M. (1976).Purification of DNA complementary to nucleotide sequences required for neoplastic transformation of fibroblasts by avian sarcoma viruses. *J Mol Biol* 101, 349–65.

Steinberg, K.K., Sanderlin, K.C., Ou, C.-Y., Hannon, W.H., McQuillan, G.M. Sampson, E.J. (1997). DNA banking in epidemiologic studies. *Epidemiol Rev* 19,156–162.

Stevenson, R.G., Rehmtulla, A.J., Thomson, R.G. (1980). Pulmonary adenomatosis (jaagsiekte) in sheep in Canada. *Can Vet J* 21, 267-268.

Stewart, M.A., Warnock, M., Wheeler, A., Wilkie, N., Mullins, J.I., Onions, D.E., Neil, J.C. (1986). Nucleotide sequences of a feline leukemia virus subgroup A envelope gene and long terminal repeat and evidence for the recombinational origin of subgroup B viruses. *J Virol* 58, 825–834.

Stocher, M. & Berg, J. (2002). Normalized quantification of human cytomegalovirus DNA by competitive real-time PCR on the LightCycler instrument. *J Clin Microbiol* 40, 4547-4553.

Stoye, J.P. & Coffin, J.M. (1987). The four classes of endogenous murine leukemia virus: structural relationships and potential for recombination. *J Virol* 61, 2659-2669.

Stoye, J., Morini, C., Coffin, J.M. (1991).Virological events leading to spontaneous AKR thymomas. *J Virol* 65, 1273-1285.

Stubbs, A.C., Martin, K.S., Coeshott, C., Skaates, S.V., Kuritzkes, D.R., Bellgrau, D., Franzusoff, A., Duke, R.C., Wilson, C.C. (2001). Whole recombinant yeast vaccine activates dendritic cells and elicits protective cell-mediated immunity. *Nat Med* 7, 625-629.

Sugimoto, M., Ohishi, K., Ikawa, Y. (1994). Role of cell-mediated immunity in bovine leukemia virus (BLV) infection in ruminants: its implication for the vaccination strategy against retroviruses. *Ther Immunol* 1, 297-301.

Summers, C., Neill, W., Dewar, P., Gonzales, L., van der Molen, R., Norval, M.,

Sharp, J.M. (2002). Systemic immune responses following infection with Jaagsiekte sheep retrovirus and in the terminal stages of ovine pulmonary adenocarcinoma. *J Gen Virol* 83, 1753-1757.

Sundaram, R., Lynch, M.P., Rawale, S.V., Sun, Y., Kazanji, M., Kaumaya, P.T. (2004). De novo design of peptide immunogens that mimic the coiled coil region of human T-cell leukemia virus type-1 glycoprotein 21 transmembrane subunit for induction of native protein reactive neutralizing antibodies. *J Biol Chem* 279, 24141-24151.

Swain, A. & Coffin, J.M. (1992). Mechanism of transduction by Retroviruses. *Science* 255, 841-845.

Swanstrom, R., Parker, C., Varmus, H.E, Bishop. J.M. (1983). Transduction of a cellular oncogene: The genesis of Rous sarcoma virus. *Proc Natl Acad Sci* 80, 2519-2523.

Tartaglia, J., Jarrett, O., Neil, J.C., Desmettre, P., Paoletti, E. (1993). Protection of cats against feline leukemia virus by vaccination with a canarypox virus recombinant, ALVAC-FL. *J Virol* 67, 2370-2375.

Teich, N., Wyke, J., Mak, T., Bernstein, A., Hardy, W. (1984).Pathogenesis of retrovirus-induced disease. In: Weiss RA, Teich N, Varmus H, Coffin JM, editors. RNA tumor viruses. 2nd ed. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; p. 25–208.

Temin, H.M. (1964). Nature of the provirus of Rous sarcoma. *Natl Cancer Inst Monogr* 17, 557-570.

Theiler. A. (1918). Jaagsiekte in Horses. 7th and 8th Ann. Report of the Director of veterinary Education and Research, Union of South Africa, p.57-104.

Ting, C.N., Rosenberg, M.P., Snow, C.M., Samuelson, L.C., Meisler, M.H. (1992). Endogenous retroviral sequences are required for tissue-specific expression of a human salivary amylase gene. *Genes Dev* 6,1457-1465.

Tonjes, R.R., Löwer, Boller, K., Denner, J., Hasenmaier, B., Kirsch, H. *et al.* (1996). HERV-K; the biologically most active human endogenous retrovirus family. *J Acquir Immune Defic Syndr Hum Retrovirol* 13 (Suppl.1), 261-267.

Tontis, A., Bestetti, G., Konig, H., Luginbuhl, H. (1979). Enzootic occurrence of pulmonary adenomatosis in 13 sheep near Bern. *Schweiz Arch Tierheilkd* 121, 251-262.

Tortorella, D., Gewurz, B., Schust, D., Furman, M., Ploegh, H. (2000). Down-regulation of MHC class I antigen presentation by HCMV; lessons for tumor immunology. *Immunol Inves*t 29, 97-100.

Toumazos, P. (1989). First report of sheep pulmonary adenomatosis in Cyprus. *Br Vet J* 145, 289-290.

Travis, W.D., Colby, T.V., Borrin, B., Shimosato, Y., Brambilla, E. (1999). Histological typing of lung and pleural tumours. *International histological classification of tumours. Third ed. Geneva (Switzerland): World Health Organization.*

Tsubura, A., Inaba, M., Imai, S., Murakami, M., Oyaizu, N., Yasumizu, R., Ohnishi, Y., Tanaka, H., Morii, S., Ikehara, S. (1988). Inervention of T-cells in transportation of mouse mammary tumour virus (milk factor) to mammary cells *in vivo. Cancer Research* 48, 6555-6559.

Tustin, R.C. (1969). Ovine jaagsiekte. *J S Af Vet Med Assoc* 40, 3-23.

Tustin, R.C., Williamson, A., York, D.F. *et al.* (1977). Experimental transmission of jaagsiekte (ovine pulmonary adenomatosis) to goats. *Onderstepoort J Vet Res* 55, 27-32.

Tyzzer, E.E. (1907). J med Res 17, 155; cited by Laipply, T.C. & Fisher, C.I. (1949). Primary alveolar cell Tumours of the lung. *Arch Path* 48, 107-118.

van Lohuizen, M., & Berns., A. (1990). Tumorigenesis by slow-transforming retroviruses: An update. *Biochim Biophys Acta* 1032, 213-215.

Van Nie, R. & Verstraeten, A.A. (1975). Studies of genetic transmission of mammary tumour virus by GR mice. *Int J Cancer* 16, 922-931.

Van Nie, R., Verstraeten, A.A., De Moes, J. (1977). Genetic transmission of mammary tumour virus by GR mice. *Int J Cancer* 19, 383-390.

van Regenmortel, M.H., Fauquet C.M., Bishop, D.H.L. (2000). Virus Taxonomy. Reports of the International Committee on Taxonomy of Viruses. Academic Press, p. 369-387.

Varmus, H.E. (1984). The molecular genetics of cellular oncogenes. *Annu Rev Genet* 18, 553-612.

Venables, P. J., Brookes, S. M., Griffiths, D., Weiss, R. A. & Boyd, M. T. (1995). Abundance of an endogenous retroviral envelope protein in placental trophoblasts suggests a biological function. *Virology* 211, 589–592.

Venanzi, E.S., Benoist, C., Mathis, D. (2004). Good riddance: thymocyte clonal deletion prevents autoimmunity. *Curr Opin Immunol* 16, 197–202.

Venugopal, K. (1999). Avian leukosis virus subgroup J: a rapidly evolving group of oncogenic retroviruses. *Res Vet Sci* 67, 113-119.

Verani, A., Gras, G., Pancino, G. (2005). Macrophages and HIV-1: dangerous liaisons. *Mol Immunol* 42, 195-212.

Verstraeten, A.A. &Van Nie, R. (1978). Genetic transmission of mammary tumour virus in the DBAf mouse strain. *Int J Cancer* 21, 473-475.

Verwoerd, D.W. (1990). Jaagsiekte (ovine pulmonary adenomatosis)virus. In: Dento, Z., Mosein, B. (Eds.), Virus Infections of Ruminants. Elsevier, p. 453-462.

Verwoerd, D.W., Mayer-Scharrer, E., Du Plessis, J.L. (1977). Transplantation of cultured jaagsiekte (sheep pulmonary adenomatosis) cells into athymic nude mice. *Onderstepoort J Vet Res* 44, 271-274.

Verwoerd, D.W., Payne, A.N., York, D.F., Myer, M.S. (1983). Isolation and preliminary characterization of the jaagsiekte retrovirus (JSRV). *Onderstepoort J Vet Res* 50, 309-316.

Verwoerd, D.W., Tustin, R.C., Payne, A.L. (1985). Jaagsiekte: An infectious pulmonary adenomatosis of sheep. In: Olsen, R.G., Krakowaka, S., Blackslee, J.R., editors. *Comparative Pathobiology of Viral Diseases* Boca Raton (FL): CRC Press. p.53-76.

Verwoerd, D. W., Williamson, A-L., De Villiers, E-M. (1980). Aetiology of Jaagsiekte: transmission by means of sucellular fractions and evidence for the involvement of a retrovirus. *Onderstepoort J Vet Res* 47, 275-280.

Vitellozzi , G., Mughetti, L., Palmarini, M., Mandara, M.T., Mechelli, L., Sharp, J.M., Manocchio, I. (1993). Enzootic intranasal tumour of goats in Italy. *Zentralbl Veterimarmed A* 40, 459-468.

Vivanco, I. & Sawyers, C.L. (2002). The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer* 2, 489-501.

Voelker, D.R. & Mason, R.J. (1989). Alveolar type II ephitelial cells. In: Lung cell biology. Massaro D. (Ed.) Marcel Dekker (New York), p. 487-538.

Vogelstein, B. & Gillespie, D. (1979). preparative and analytical purification of DNA from agarose. *Pro Natl Acad Sci USA* 76, 615-619.

Vogt, P.K. (1997). Historical introduction to the general properties of retroviruses. In: Coffin JM editor. Retroviruses. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; p. 1-25.

Wagter, L.H., Jansen, A., Bleumink-Pluym, N.M., Lenstra, J.A., Houwers, D.J. (1998). PCR detection of lentiviral GAG segment DNA in the white blood cells of sheep and goats. *Vet Res Commun* 22, 355-362.

Walker, L.S. & Abbas, A.K. (2002). The enemy within: keeping self-reactive T cells at bay in the periphery. *Nat. Rev. Immunol.* 2, 1–19.

Wandera, J.G. (1967). Pneumonia of sheep in Kenya. Part I. Bacterial and Parasite Pneumonia. *Bullettin of Epizootic Diseases of Africa* 15, 245-258.

Wandera, J.G. (1968). Experimental transmission of sheep pulmonary adenomatosis (jaagsiekte). *Vet Rec* 83, 478-482.

Wandera, J.G. (1970). Clinical pulmonary adenomatosis of sheep produced experimentally. Br *Vet J* 126, 185-193.

Wang, J.T., Wang, T.H., Sheu, J.C., Lin, J.T., Chen, D.S. (1992).Effects of anticoagulants and storage of blood samples on efficacy of the polimerase chain reaction assay for hepatitis C virus . *J Clin Microbiol* 30, 750-753.

Weibel, E.R. & Taylor, C.R. (1996). Functional anatomy of the human lung. In: *Oxford Textbook of Medicine*, p. 2593-2609. Ed. D.J. Weath-erall, J.G.G. Ledingham, & D.A. Warrell. Oxford University Press. Oxford.

Weiss. R.A. (1993). Cellular receptors and viral glycoproteins. In *The retroviridae* (ed. J.A. Levy), vol. 3, p.1-108. Plenum Press, New York.

Weissman, D., Rabin, R.L., Arthos, J., Rubbert, A., Dybul, M., Swofford, R., Venkatesan, S., Farber, J.M., Fauci, A.S. (1997). Macrophage-tropic HIV and SIV envelope proteins induce a signal through the CCR5 chemokine receptor. *Nature* 389, 981-985.

Wright, J.R. (1997). Immunomodulatory functions of surfactant. *Physiol Rev* 77, 931-954.

Wright, N.A. & Alison , M.A. (1984). Cell proliferation in respiratory epithelia. In: *The biology of epithelial cell populations*. Vol. 2, p.1068-1078. Ed. Hardcover. Oxford University Press. Oxford.

Wright, J.R., Borron, P., Brinker, K.G., Folz, R.J. (2001). Surfactant protein A. Regulation of innate and adaptive immune responses in lung inflammation. *Am J Respir Med Biol* 24, 513-517

Wright, P.F., Mestecky, J., McElrath, M.J., Keefer, M.C., Gorse, G.J., Goepfert, P.A., Moldoveanu, Z., Schwartz, D., Spearman, P.W., El Habib, R., Spring, M.D., Zhu, Y., Smith, C., Flores, J., Weinhold, K.J. (2004). Comparison of systemic and mucosal delivery of 2 canarypox virus vaccines expressing either HIV-1 genes or the gene for rabies virus G protein. *J Infect Dis* 189, 1221-1231.

Wurm, F.M. (2004). Production of recombinant protein therapeutics in cultivated mammalian cells. *Nature Biotechnology 22, 1393 – 1398.*

Yi, J.-M., Kim, H.-M., Kim, H.-S. (2004). Expression of the human endogenous retrovirus HERV-W family in various human tissues and cancer cells. *JGV* 85, 1203-1210.

Yi, J.-M., Kim, H.-M., Lee, W.-H., Kim, H.-S. (2002). Molecular cloning and phylogenetic analysis of new human endogenous retrovirus HERV-W family in cancer cells. *Curr Microbiol* 44, 216-220.

Yi, Y., Rana, S., Turner, J.D., Gaddis, N., Collman, R.G. (1998). CXCR-4 is expressed by primary macrophages and supports CCR5-independent infection by dual-tropic but not T-tropic isolates of human immunodeficiency virus type 1. *J Virol* 72, 772–777.

York, D.F., Vigne, R., Verwoerd, D.W., Querat, G. (1991). Isolation, identification, and partial cDNA cloning of genomic RNA of Jaagsiekte retrovirus,the etiological agent of sheep pulmonary adenomatosis. *J Virol* 65, 5061-5067.

York, D.F., Vigne, R., Verwoerd, D.W., Querat, G. (1992). Nucleotide sequence of Jaagsiekte retrovirus, an exogenous and endogenous type D and B retrovirus of sheep and goats. *J Virol* 66, 4930-4939.

Yousem. S.A., Finkelstein, S. D., Swalsky, P.A., Bakker, A., Ohori, N.P. (2001). Absence of Jaagsiekte sheep retrovirus DNA and RNA in bronchioloalveolar and conventional human pulmonary adenocarcinoma by PCR and RT-PCR analysis. *Human Pathology* 32, 1039-1042.

Yu, H., Babiuk, L.A., van Drunen Littel-van den Hurk, S. (2004). Priming with CpG-enriched plasmid and boosting with protein formulated with CpG oligodeoxynucleotides and Quil A induces strong cellular and humoral immune responses to hepatitis C virus NS3. *J Gen Virol* 85, 1533-1543.

Zavala, G., Pretto, C., Chow, Yen-Hung J., Jones, L., Alberti, A., Greco, E., De las Heras, M., Palmarini, M. (2003). Relevance of Akt phosphorylation in cell transformation induced by Jaagsiekte sheep retrovirus. *Virology* 312, 95-105.

Zimber, A., Perk, K., Hod, I., Irving, S., Yegana, Y. (1984). Heterotransplantation of experimentally induced sheep lung adenomatosis into nude mice. *Res Vet Sci* 36, 122-124.

Appendix I: Bacterial culture medium and transformation of *E.coli*

I.1 Transformation of competent cells JM109 (Promega)

Promega's JM109 competent cells were prepared according to a modified Hanahan (1983), procedure. All cells were supplied were supplied in 0.2 ml aliquots and 3ng of pGEM-3Z plasmid DNA was provided as a test control vector. Typically, 100µl of competent cells are required for standard transformation. Competent cells were stored at -70°C and they were thawed in an ice-bath. The cells were gently mixed by flicking the tube and removed into a sterile pre-chilled (on ice) polypropylene tube (Eppendorf).

1-50 ng of cloned DNA was added per 100µl of competent cells, in a volume that was never greater than 10µl. Immediately the tube was placed on ice for 10 minutes. The cells were heat-shocked by transferring the tube in a water bath at exactly 42°C for 45-50 seconds, without shaking the tube at all. Immediately the tube was placed on ice for 2 minutes. 450µl of L-broth kept at room temperature was added and the suspension was incubated for 60 minutes at 37°C with shaking at approximately 225 rpm. 100µl aliquots were spread on selective agar plates (LB/Amp or LB/Amp/X-Gal/IPTG) agar plates which were then incubated overnight at 37°C (Hanahan, 1985).

I.2 L-Broth /Ampicillin (LB/Amp) agar plates

15 g of agarose was added per litre of L-broth. The agar was placed in a steamer until it had dissolved then it was autoclaved. The agar was cooled to 55°C before addition

of Ampicillin to 150μg/ml. About 20ml of agar was used for each plate. LB/Amp plates were stored at 4 °C for up to weeks.

I.3 LB/Amp/X-Gal/IPTG plates

Base agar was made by dissolving 15 g of bacteriological agar per litre of L-broth. The agar was dissolved by placing in a steamer before autoclaving. It was then cooled to 55°C. Ampicillin was added to 150μg/ml. 80mg of X-gal was dissolved in 1ml of DMF and 120mg of IPTG were dissolved in distilled water. X-gal and IPTG were dissolved in 1 litre of LB/Amp agar.

I.4 Storage of bacterial stocks

An overnight culture was grown up in L-broth (plus 150μg/ml Ampicillin if required). The bacteria were spun down by centrifugation at 6,000 rpm in a bench centrifuge. The media was removed and the cells were resuspended in L-broth/15% glycerol. 1ml aliquots were transferred to Eppendorf tubes and snap frozen on dry ice. Stocks were stored at -70°C.

APPENDIX II: Preparation and electrophoresis of DNA

II.1 Restriction endonuclease digestion

The optimum conditions required for digestion vary between each restriction enzyme. The informations were supplied by the manufacturer. Generally 10x concentrated digestion buffers supplied with each enzyme were used to create optimum buffer conditions. One tenth volume of 10x digestion buffer was added to a DNA solution in distilled water. Restriction enzyme was then added depending on the estimated amount of DNA to be digested. The digestion reactions were incubated in water bath at 37°C usually overnight.

II.2 Agarose gel electrophoresis

Solutions.

50xTAE: 242g Tris, 57.1 glacial acetic acid, 100ml 0.5 EDTA pH (8.0), and distilled water to 1000ml.

Loading buffer: 100nm EDTA. 30% Ficoll, 0.025 bromophenol blue, 0.025 Xylene cyanol.

Method.

Agarose was dissolved by boiling in TAE buffer then allowed to cool a little before that Ethidium Bromide was added to a concentration of 0.6μg/ml. The gel was poured and left to set. TAE running buffer was made up and enough was added to the tank to just cover the gel. 0.2 volumes of loading buffer was added to each sample. Samples were loaded and the gel was run at 70-80Volts. Nucleic acid in the gels was visualised on a UV transilluminator.

II.3 Recovery of DNA from agarose

Extraction and purification of DNA from standard or low-melting agarose gels in TAE or TBE buffer

QIAquick gel extraction kit protocol (Qiagen).

The protocol is designed to extract and purify DNA of 70bp to 10kb from standard or low- melt agarose gels in TAE or TBE buffer. The system combines the spin-column technology with the selective properties of a silica-gel membrane. For processing the spin column a bench microcentrifuge was used. Buffers are provided with each kit. DNA adsorbs to the silica-membrane in the presence of high salt while contaminants pass through the column. Impurities are washed away, and DNA is eluted with Tris buffer or water. All the reagents were used according to the manufacturer 's instructions.

For gel extraction of DNA the QIAquick gel extraction Kit (QIagen) protocol was used:

1) DNA fragment was excised from the agarose gel with a clean scalpel.
2) The gel fragment was weighed and 3 volumes of Buffer QG to 1 volume of gel in 1.5 ml Eppendorf tube.
3) The gel slice was incubated at 50°C until dissolved; every 2-3 minutes the gel slice was mixed by vortexing to help to dissolve.
4) After that the slice was dissolved completely the pH of the mixture was checked and according to the manufacturer's protocol if the mixture was orange or violet instead of yellow (it means pH ≥ 7.5) 10µl of 3M sodium acetate, pH 5.0 were added.

5) After the slice was dissolved completely 1 volume of isopropanol was added to the sample and mixed by vortexing. The binding and solubilization buffer QG has been specially optimized for use with the QIAquick silica-gel membrane.
6) A QIAquick spin column was placed in a provided 2 ml collection tube.
7) To bind DNA to the silica gel membrane in the spin column, the sample was simply applied to the spin column and it was centrifugated for 1 minute.
8) The flow-through was discarded and the spin column was placed back in the same collection tube.
9) The spin column was washed with 0.75ml of buffer PE and it was centrifugated for 1 minute at about 17,900x g.
10) The flow-through was completed discarded and the spin column was centrifugated for additional 1 min at about 17,900x g. Salts are quantitatively washed away by the ethanol-containing buffer PE. Any residual buffer PE, which could interfere with subsequent enzymatic reactions, was removed by an additional centrifugation step.
11) The spin column was placed into a clean 1.5 microcentrifuge tube.
For eluting DNA 50µl of Buffer EB (10mM TrisCl, pH 8.5) were added to the center of the spin column and the column was centrifugated for 1 minute (Vogelstein & Gillespie, 1979; Hamaguchi & Geiduschek, 1962).

II.4 Minipreps for the isolation of plasmid DNA

For the purification of plasmid DNA was used the QIAprep Miniprep kit (Qiagen).Trasformants grown on selective agarose plates were inoculated into L-broth/Amp tubes and grown up overnight at 37°C with shaking. 3ml of overnight

culture was pelletted by microcentrifugation and then the protocol by Qiagen was followed.

The procedure is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica in the presence of high salt (Vogelstein & Gillespie, 1979). The purification consists of three basic steps:

1) preparation and clearing of a bacterial lysate;

2) adsorption of DNA on to the Qiaprep membrane;

3) washing and elution of plasmid DNA.

Bacteria were lysed under alkaline conditions, and the lysate was subsequently neutralized and adjusted to high-salt binding conditions in one step, ready for purification on the Qiaprep silica-gel membrane (Birnboim & Doly, 1979). Qiaprep columns use a silica-gel membrane for selective adsorption of plasmid DNA in high salt buffer and elution in low salt buffer.

Appendix III: Quantification of DNA

Nucleid acid was quantified by the use of a spectrophotometer. DNA and RNA have a peak of absorbance at260nm, while contaminating proteins have their peak at 280 nm (Sambrook *et al.,* 1989). The absorbance at 260 nm reflects the amount of nucleic acid present in the sample. An absorbance of 1 OD_{260} corresponds to 50μg ml^{-1} of double stranded DNA and 40μg ml^{-1} for RNA. The ratio OD_{260} /OD_{280} should be between 1.8 and 2.0 for a clean nucleic acid preparation. Samples were diluted to give an OD_{260} between 0.1 and 0.5. An automatic spectrophotometer (Genequant, Amersham Pharmacia) was used in this study and software automatically calculates the concentration of nucleic acid and the OD_{260} /OD_{280} ratio.

APPENDIX IV: SDS-PAGE and Western blotting

IV.1 SDS- Page protein gels

The Biorad Mini protean II slab gel apparatus was used. Either 10% or 5-20% gradient separating gels were used, using a 30% acrylamide/0.8% bis stock solution and a final concentration of 0.38M tris-Cl pH8.8, 2mM EDTA, 0.1% SDS, 0.08% APS, 0.05% TEMED. The stacking gel was 3.48% acrylamide/0.093% bis acrylamide,

0.145M Tris-Cl pH6.8, 0.12% SDS, 0.12% APS, 0.17% TEMED.

Running buffer contained 0.05M Tris, 0.37M glycine, 19.5μM EDTA, 0.1% SDS. Samples were boiled for 2-3min with an equal volume of reducing buffer (0.1M Tris pH6.8, 2% 2-mercaptoethanol, 30% glycerol, 1% SDS, 0.001% bromophenol blue) before being loaded into the wells. Molecular weight markers were run in at least one well of each gel. Electrophoresis was performed at 150V for approximately 45 min. or until the dye front had reached the bottom of the gel.

IV.2 Coomassie staining

The PAGE gel was placed in a tray of 0.2% Coomassie stain (0.2% Coomassie Blue R250 (Sigma, UK), 20% methanol, 5% acetic acid), for a minimum of 4 hours to overnight , followed by extensive destaining with a solution of 5% acetic acid and 20% ethanol. The gel was dried under vacuum at 80°C for 1 hour.

IV.3 Western blotting

The polypeptides separated by SDS-Page were transferred from the gel onto nitrocellulose (Hybond-C, Amersham, UK), using a blotting apparatus (Biorad), in a

solution made with 25mM Tris, 20% methanol, 123mM glycine buffer. A current of 120mA was applied across the blotter for 1 hour.

IV.4 Developing a blot

After transfer, power supply was disconnected. The sandwich was disassembled and the nitrocellulose membrane was marked to retain orientation. The filter was rinsed several time with PBS. Then the primary antibody solution. Use 10 ml per 15x15-cm blot. All the antibody dilutions were done in PBS.The blot was incubated with antibody for 1 hour at room temperature with agitation. The blot was washed with four changes of PBS for 5 minutes each. Horseradish peroxidase conjugated serum was used as secondary antibody. 3,3'- diaminobenzidine (DAB) was the substrate used for the final stage in the blot development.

It was then incubate with suitable chemiluminescence reagents according to manufacturers protocol (DAB, Sigma).

Appendix V: Purification of recombinant proteins

V.1 Purification of recombinant proteins under denaturing conditions

Denaturing Buffer Preparation

Stock solution A (10X):

200 mM monobasic sodium phosphate (NaH_2PO_4), 5M NaCl. It was prepared dissolving 27.6 g of monobasic sodium phosphate and 292.9g of NaCl in 1000 ml of deionized water. The urea/phosphate buffers were made up to 100 ml.

Stock solution B (10X):

200mM dibasic sodium phosphate (NaH_2PO_4), 5M NaCl. It was prepared by dissolving 28.4 g of dibasic sodium phosphate and 292.9g of NaCl in 1000 ml of deionized water.

Mixing different predetermined amounts of stock solution A and B was the method for making phosohate buffers of differing pH.

Denaturing binding buffer pH 7.8:

8M Urea, 20mM Sodium Phosphate, 500mM Sodium Chloride.

For 100 ml: 0.58ml of stock solution A (10x) were combined with 9.42 ml of stock solution B (10x). It was added 48.1g of urea.

Denaturing wash buffer pH 6.0:

8M Urea, 20mM Sodium Phosphate, 500mM sodium Chloride.

For 100 ml: 7.38ml of stock solution A (10x) were combined with 2.62 ml of stock solution B (10x). It was added 48.1g of urea.

Denaturing wash buffer pH 5.3:

8M Urea, 20mM sodium Phosphate. 500mM Sodium Chloride.

For 100 ml: 9.17ml of stock solution A (10x) were combined with 0.83ml of stock solution B (10x). It was added 48.1g of urea.

Denaturing elution Buffer pH 4.00:

8M Urea, 20mM sodium Phosphate, 500mM Sodium Chloride.

For 100 ml: 10ml of stock solution A (10x) were combined with 48.1g of urea. The solutions were stirred with gentle heating (50-60°C), until they were completely dissolved. The pH was adjusted using 1N NaOH or 1N HCl. The solutions were filtered using a 0.45μm filters.

V.2 Purification of recombinant proteins under native conditions

Preparation of the Native Purification Buffers

Stock solution A (10X) (see Appendix V.1)

Stock solution B (10X) (see Appendix V.1)

3M Imidazole Stock buffer 10X

500mM NaCl, 20mM NaPO4 buffer, pH 6.0. For preparing 100 ml of this solution 20.6g of imidazole, 8.77 ml of Stock Solution A, 1.23 ml of Stock Solution B were combined to 90 ml of distilled water. The pH was adjusted to 6.0 with HCl or NaOH as necessary.

Native Binding Buffer

20 mM Sodium Phosphate, 500 mM Sodium Chloride, pH 7.2 to 7.6.

For 50 ml: 2.9ml of 1x Stock solution A was combined with 47.1 ml of 1x Stock solution B. The pH was adjusted by using 1x Stock solution B to raise and 1x Stock solution A to lower the pH. This buffer was used as the binding buffer in the native purification protocol.

Native Wash Buffer pH 5.5

20 mM Sodium Phosphate, 500 mM Sodium Chloride, pH 5.5.

For 50 ml: 43.5 ml of 1x Stock solution A was combined with 6.5 ml of 1x Stock solution B. The pH was adjusted by using 1x Stock solution B to raise and 1x Stock solution A to lower the pH. This buffer was used as the high stringency wash buffer in the native purification protocol.

Native Elution Buffer pH 4.0

20 mM Sodium Phosphate, 500 mM Sodium Chloride, pH 4.0.

For 50 ml: 50 ml of 1x Stock solution A was combined with no 1x Stock solution B. The pH was adjusted by using 1x Stock solution B to raise and Phosphoric Acid (H3PO4) to lower the pH. This buffer was used as the low pH wash buffer in the pH elution protocol under native purification conditions.

Appendix VI: JSRV recombinant proteins previously produced

VI.1 Brief description of GST-CA and β-Gal-CA recombinant protein (Palmarini *et al.*, 1995)

JSRV gag gene (bases 953 to 3030 of the nucleotide sequence published by York *et al.*, 1992) was inserted in plasmid pBluescript-Js382. The fragment was excised by *Eco*RI restriction and it was subcloned into plasmids pPS1S (supplied to Moredun by M. Shreiber) and pGeX1λT (Pharmacia) and expressed in *E. coli* host strain NM522 as β-galactosidase (βgal-CA, plasmid pMCA) and glutanione-S- transferase (GST-

CA, plasmid pGCA) fusion proteins respectively. Confirmation that the *gag* gene was in the correct reading frame was obtained by sequencing across the vector-insert junction, as well testing clones for production of β-galactosidase and GST fusion proteins of the appropriate size by western blotting analysis with a goat antiserum to Mason-Pfizer monkey virus major capsid protein (MPMV-CA) (Sharp & Herring, 1983). Transformed bacteria were grown and induced with IPTG for the expression of recombinant proteins. Bacteria were pelleted (5000x g for 10 min) and resuspended in 20 ml of TE (10mM Tris pH7.5, 1mM EDTA). Phenylmethylsulphonylfluoride (2mM) was added before lysing the cell suspension in a French press at 1500 psi (10.35 Mpa). The lysate obtained was sonicated and clarified at 100,000 x g at 4°C for 10 min.

βgal-CA was approximately 9mg/l of bacterial culture. Protein concentration was estimated using BCA Protein Estimation Assay (Pierce), as recommended by the manufacturer.

GST-CA was purified by affinity chromatography using a 4 ml column of glutathione sepharose (Pharmacia) as recommended by the manufacturers.

GST-CA could not be eluted with free glutathione from the sepharose beads as recommended by the manufacturers and therefore animals were immunised with the fusion protein coupled to the beads.

VI. 2 Brief description of GST-SU

JSRV *env* gene (bases 5557 to 6483 of the nucleotide sequence published by York *et al.* 1992) was cloned and inserted in the plasmid pGEM-T Easy (Promega, Southampton,UK). The inserted fragment was excised and sub-cloned into plasmids:

pGEX1λT (Pharmacia, Uppsala, Sweden) The fragment inserted in pGEX1λT was expressed in *E.coli* cells as glutathione S-transferase fusion protein (GST-SU) as described for expression and purification of recombinant JSRV-capsid protein (Palmarini *et al.*, 1995).

Appendix VII: Necropsy procedure for clinical OPA animal or experimental animals

1) collect blood samples in EDTA for PCR or in tubes w/o anticoagulant for collecting serum.
2) Euthanasia: overdose of pentobarbitone
3) Collection of any lung fluid in a sterile jug.
4) Necropsy.

Appendix VIII : Rapid Translation System 500 (RTS-500) (Roche Biochemicals). Procedures.

All the required reagents are supplied with the kit. The different reagent are lyophilized in bottles with caps of different colour and they need to be reconstituted directly prior to use with a Reconstitution Buffer. The plasmids are reconstituted using RNase-Dnase-free water.

1. Reconstitution of lyophilized components:

Solution	Component	Reconstitution procedure
1	*E.coli* lysate ,bottle 1 (red cap)	Reconstitute the lyophilizate with 0,25 ml Reconstitution Buffer, Bottle 5. Mix carefuly without shaking

2	Reaction mix , bottle 2 (green cap)	Reconstitute the lyophilizate with 0.8 ml Reconstitution Buffer, Bottle 5.
3	Feeding mix, bottle 3, blue cap	Reconstitute the lyophilizate with 10 ml Reconstitution Buffer, Bottle 5.
4	Energy mix, Bottle 4, orange cap	Reconstitute the lyophilizate with 10 ml Reconstitution Buffer, Bottle 5.
5	Reconstitution Buffer, Bottle 5, white cap	Ready to use. The solution is stable at 4°C but can also be stored at –20°C.

2. Preparations of working solutions

Solution	Component	Preparation of working solution
7	Feeding solution	Add o.5ml of the reconstituted solution 4 to solution 3 to obtain the Feeding solution
8	Reaction solution	To *E. coli* lysate (solution1) add - 0.75 ml of solution 2, - 50µl of solution 4, - 5-15µg of the DNA template in a maximum volume of 50µl

After preparing the solutions ready the reaction compartment must be filled first with 1 ml of solution 8 and then the feeding compartment can be loaded with 10ml of solution 7. At this point the RTS device can be inserted into the RTS Instrument. The instrument can be set according to the instrument manual.

Appendix IX: BCA method (Pierce)

Bovine serum albumin (BSA) was obtained from Pierce Chemical Company.

1. Make up series of protein standard (bovine serum albumin) concentrations in 2 ml polypropylene tubes (do in duplicate):

Final conc	Volume of albumin (2 mg/mL)	Volume of diluent (water)
200 ug/mL	5 uL	45 uL
400 ug/mL	10 uL	40 uL
600 ug/mL	15 uL	35 uL
800 ug/mL	20 uL	30 uL
1000 ug/mL	25 uL	25 uL
1200 ug/mL	30 uL	20 uL

2. Add 50 uL unknown protein samples to 2 ml polypropylene tubes.
   a. Do in duplicate.
   b. If protein concentration likely to be > 1 mg/mL then dilute sample appropriately in water.
   c. Suggest using serial dilution – ½; ¼; 1/8; 1/16.
3. Make up sufficient solution A and B.
   a. Mix 1 part solution B with 50 parts solution A.
   b. Count up the number of tubes (remember to count standards and duplicates).
   c. You will need at least this many mL of solution A+B.

d. E.g. 6x2 standards + 10 samples x 2 dilutions x 2 duplicates = 52 mLs – suggest making up > 60 mL

4. Add 1 mL solution A/B to each tube and mix
5. Incubate at 37^0C for 20-30 minutes (or can let stand at room temperature for 2 hours)
6. Read absorbance at 562 nM in spectrophotometer using 1 mL plastic cuvettes (only need to use 1 cuvette – wash between samples)
   a. Don't allow time to elapse between readings since reaction continues albeit slowly at room temperature.
7. Use standard curve function of spectrophotometer (linear regression) to calculate protein concentrations (or can use raw data and Excel spreadsheet).

Appendix X: GAPDH PCR

Primers were designed on a consensus sequence between the rat and the human GAPDH. The sense primer (5' TCACCACCATGGAGAAGGCT 3') was based on exon 4 of the human gene and the reverse primer was based on exon 7 (5' TTCATTGTCATACCAGGAAA 3'). The primers were designed to amplify an 818 bp fragment The buffer employed was 10mM-Tris-HCl, 50mM-KCl, 2.5mMMgCl2 (pH8.9) with 200μM each dNTP, 6,25 pmol each primer and 1.25U Taq polymerase (Boehringer). Cycles employed were 94°C for 1 min , 35 cycles at 94°C for 45s, 52°C for 1 min and 72°C for 1 min 30s with a final extension of 72°C for 5 min.

Appendix XI: Methods for DNA extraction from blood

XI.1 Flexigene

Pipet 750 μl Buffer FG1 into a 1.5 ml centrifuge tube. Add 300 μl whole blood and mix by inverting the tube 5 times.

2. Centrifuge for 20 s at 10,000 x *g* in a fixed-angle rotor.

3. Discard the supernatant and leave the tube inverted on a clean piece of absorbent paper for 2 min, taking care that the pellet remains in the tube.

Note: In rare cases the pellet may be loose, so pour slowly. Inverting the tube onto absorbent paper minimizes backflow of supernatant from the rim and sides of the tube onto the pellet.

4. Add 150 μl Buffer FG2/QIAGEN Protease, close the tube, and vortex immediately until the pellet is completely homogenized. Inspect the
tube to check that the content is homogeneous.

Note: When processing multiple samples, vortex each tube immediately after addition of Buffer FG2/QIAGEN Protease. Do not wait until buffer has been added to all samples before vortexing.

Usually 3–4 pulses of high-speed vortexing for 5 s each are sufficient to homogenize the pellet. However, traces of pellet with a jelly-like consistency (often barely visible)
may remain. If these traces are seen, add 30 μl Buffer FG2 and vortex again.

5. Centrifuge the tube briefly (3–5 s), place it in a heating block or water bath, and incubate at 65°C for 5 min.

Note: The sample changes color from red to olive green, indicating protein digestion.

6. Add 150 μl isopropanol (100%) and mix thoroughly by inversion until the DNA

precipitate becomes visible as threads or a clump.

Note: Complete mixing with isopropanol is vital to precipitate the DNA and must be checked by inspection. For samples with very low white blood cell counts, in which the DNA may not be visible, invert the tube at least 20 times.

7. Centrifuge for 3 min at 10,000 x *g*.

Note: If the resulting pellets are loose, centrifugation can be prolonged or a higher *g*-force can be used.

8. Discard the supernatant and briefly invert the tube onto a clean piece of absorbent paper, taking care that the pellet remains in the tube.

Note**:** In rare cases the pellet may be loose, so pour slowly.

If the white blood cell count of the sample was sufficiently high, the DNA should be visible as a small white pellet.

9. Add 150 μl 70% ethanol and vortex for 5 s.

10. Centrifuge for 3 min at 10,000 x *g***.**

Note: If the resulting pellets are loose, centrifugation can be prolonged or a higher *g*-force can be used.

11. Discard the supernatant and leave the tube inverted on a clean piece of absorbent paper for at least 5 min, taking care that the pellet remains in the tube.

Note: In rare cases the pellet may be loose, so pour slowly. Inverting the tube onto absorbent paper minimizes backflow of ethanol from the rim and sides of the tube onto the pellet.

12. Air-dry the DNA pellet until all the liquid has evaporated (at least 5 min).

Note: Avoid over-drying the DNA pellet, since over-dried DNA is very difficult to dissolve.

13. Add 150 µl Buffer FG3, vortex for 5 s at low speed, and dissolve the DNA by incubating for 10 min at 65°C in a heating block or water bath.

XI.2 Puregene

First, 600 µl whole blood are aliquoted into a 2.0 ml microfuge tube containing 900 µl Red blood cells lysis solution, The tube is inverted to mix and incubated for one minute at room temperature. During the incubation the tube is inverted again gently 3 times to mix. For fresh blood collected within 1 hour, the incubation time was increased to 3 minutes to ensure complete red blood cell lysis. The tube is centrifuged at 13,000-16,000 x g for 20 seconds. The supernatant containing the lysed red blood cells is removed. The tube is vortex vigorously to resuspend the pellet; 900 µl of lysis solution are added and the tube is centrifuged at 13,000-16,000 x g for 20 seconds. The supernatant is removed with a pipette, leaving behind the visible white cell pellet and about 10-20 µl of the residual liquid. The tube is vortex vigorously to resuspend the white cells in the residual liquid. 600 µl of Cell Lysis Solution were added to the resuspended cells and pipet up and down to lyse the cells. 200 µl Protein Precipitation Solution were added to the cell lysate. The tube is vortex vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution uniformly with the cell lysate and then centrifuge at 13,000-16,000 x g for 1 minute. The supernatant containing the DNA (leaving behind the precipitated protein pellet) is poured into a clean 2.0 ml microfuge tube containing 600 µl 100% Isopropanol (2-propanol). The sample is then mixed by inverting the tube gently 50 times. After centrifugation (13,000-16,000 x g for 1 minute), the DNA is visible as a white pellet. The supernatant is poured off the supernatant and the tube drained briefly on clean,

absorbent paper. After adding 600 µl 70% Ethanol the tube is centrifuged at 13,000-16,000 x g for 1 minute. Carefully the ethanol is poured off and the tube inverted and drain on clean, absorbent paper for 5 seconds. Finally add 150 µl DNA Hydration solution.

XI.3 Genisol

1. Dilute 2.5 ml of Lysis Buffer 10 time with distilled water to a total volume of 25 ml. Add 1 ml of blood. Mix by inversion and leave for 2 minutes.
2. Centrifuge at about 400 g for 2 minutes.
3. Discard the supernatant taking care not to lose the soft pellet.
4. Add 5 ml of digestion buffer, vortex for 1 minute and incubate until the pellet is not completely dissolved.
5. Add 1.6 ml of precipitation buffer and mix well.
6. centrifuge at 600-1000 g for 2 minutes . Remove the supernatant containg the DNA to a new tube.
7. Add 6.6 ml of 80% (v/v) isopropanol to the supernatant and mix by inversion. The DNA will gradually precipitate in the form of white strands.
8. Pellet the DNA by centrifugation (600-1000g) for 5 minutes and discard the supernatant. At this point the DNA can be transferred to a smaller tube.
9. Wash the DNA with 70% (v/v) ethanol; remove the excess alcohol by discarding the supernatant. Redissolve the DNA in sterile distilled water.

XI.4 DNAzol

1. DNA precipitation

Precipitate DNA from the lysate/homogenate by the addition of 0.5 ml of 100% ethanol per 1 ml of DNAzol used for the isolation. Mix samples by inverting tubes 5-8 times and store at room temperature for 1-3 minutes. Make sure that DNAzol and ethanol mix well to form a homogeneous solution. DNA should quickly become visible as a cloudy precipitate. Remove the DNA precipitate by spooling with a pipette tip. Swirl the DNA onto the tip and attach it to the tube wall near the top of the tube by gently sliding the DNA off the tip. Alternatively, transfer the DNA to a clean tube. Store the tubes upright for about 1 minute and remove from the bottom of the tubes the remaining lysate/homogenate.

2. DNA wash

Wash the DNA precipitate twice with 0.8 - 1.0 ml of 75% ethanol. At each wash, suspend the DNA in ethanol by inverting the tubes 3 - 6 times. Store the tubes vertically for 0.5 - 1 minutes to allow the DNA to settle to the bottom of the tubes and remove ethanol by pipetting or decanting.

4. DNA solubilization

Remove any remaining alcohol from the bottom of a tube using a pipette. Next, dissolve DNA (without drying) in 8 mM NaOH by slowly passing the pellet through a pipette. Alternatively, dissolve DNA in water. However, the alkaline solubilization of DNA occurs faster and assures full solubilization of the DNA precipitate. Add an adequate amount of 8 mM NaOH or water to approach a DNA concentration of 0.2 - 0.3 $\mu g/\mu l$.

XI.5 Dneasy tissue kit method for the whole blood

Before using for the first time, add the appropriate amounts of ethanol (96–100%) to Buffers AW1 and AW2 as indicated on the bottles. Prepare PBS, pH 7.2 (50 mM

potassium phosphate; 150 mM NaCl), for use in step 1.Buffers AW1 and AW2 are supplied as concentrates.

Procedure

1. Add 20 μl proteinase K and 200 μl Buffer AL to100μl of whole blood, mix thoroughly by vortexing, and incubate at 70°C for 10 min. It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing and pipetting to yield a homogeneous solution.

2. Add 200 μl ethanol, and mix thoroughly by vortexing**.** It is important that the sample and the ethanol are mixed thoroughly to yield ahomogeneous solution.

4. Pipet the mixture from step 3 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at 6000 x *g* for 1 min. Discard flow-through and collection tube.

5. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 μl Buffer AW1, and centrifuge for 1 min at 6000 x *g*. Discard flow-through and collection tube.

6. Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 μl Buffer AW2, and centrifuge for 3 min at 20,000 x *g* (14,000 rpm) to dry the DNeasy membrane. Discard flow-through and collection tube.

It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during the following elution.

Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection

tube, then reuse it in another centrifugation for 1 min at 20,000 x *g*.

7. Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube (not provided), and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 6000 x *g* to elute. Elution with 100 µl (instead of 200 µl) increases the final DNA concentration in the eluate, but also decreases the overall DNA yield. A second elution can be performed.

XI.6 Ultraclean

Component	Amount	Description
RNase A	165µl	25 mg/ml RNase A
Solution G1	99 ml	Ammonium Chloride Solution
Solution G2	33 ml	Detergent Solution
Solution G3	11 ml	Ammonium Acetate
100 % Isopropanol	33 ml	100% Isopropanol
70% Ethanol:	30 ml	70% Ethanol in DNA Free Water
Solution G4	11ml	Tris in DNA Free Water.

Protocol

1. Add 300µl of whole blood to 900µl of Solution G1.
2. Invert twice and incubate 5 minutes at room temperature.
3. Invert two times during incubation.
4. Centrifuge for 30 seconds at 13,000xg. Remove supernatant with narrow pipet tip and discard without disturbing white pellet. (About 20µl of supernatant will remain.)

5. Bump vortex to resuspend pellet completely.
6. Check Solution G2. If precipitated, heat to 55° - 65°C for 5 minutes to dissolve. Add 300μl of Solution G2.
7. Pipet up and down to lyse the cells (If cell clumps are still visible incubate at 37°C until no clumps are visible).
8. Add 1.5μl of RNase A, invert 5 times and vortex on low speed for 5 seconds.
9. Add 100μl of Solution G3.
10. Immediately vortex for 15 seconds.
11. Centrifuge 3 minutes at 13,000xg.
12. Remove clear supernatant and transfer to a clean 2.0 ml tube.
13. Add 300μl of 100% Isopropanol.
14. Invert 15 times and incubate at room temperature for 3 min.
15. Centrifuge 1minute at 13,000xg.
16. Pour off supernatant without disturbing pellet, and drain on a paper towel or absorbent material.
17. Add 300μl of 70% Ethanol.
18. Invert tube 5 times to wash.
19. Centrifuge 30 seconds at 13,000xg.
20. Carefully pour off supernatant without disturbing pellet, and drain on a paper towel or absorbent material.
21. Centrifuge for 30 seconds, and remove residual supernatant with narrow pipet tip without disturbing pellet.
22. Add 100μl of Solution G4.

23. Heat in 65°C water bath tapping tube occasionally, until pellet has resuspended (should take no longer than 30-40min).

24. Genomic DNA in tube is now ready to use for any application.

Appendix XII: MALDI-TOF analysis of JSRV RTS SU

The band containing the supposed JSRV RTS SU protein was excised from a SDS-PAGE gel, digested with proteolytic enzymes and then analysed by MALDI-TOF at the Moredun Functional Genomics Unit.

The MALDI-TOF application, followed by database search, confirmed that the 43 kDa band fitted the predicted sequence of JSRV SU protein as shown in the figure XII.1.

Fig.XII.1. MALDI-TOF analysis of JSRV RTS SU.

The band of about 43kDA that was supposed to be JSRVRTS SU was excised from the SDS-PAGE and analysed by MALDI-TOF technique (A). Another band largely present was excised from the SDS-PAGE and analysed by the same technique (B).

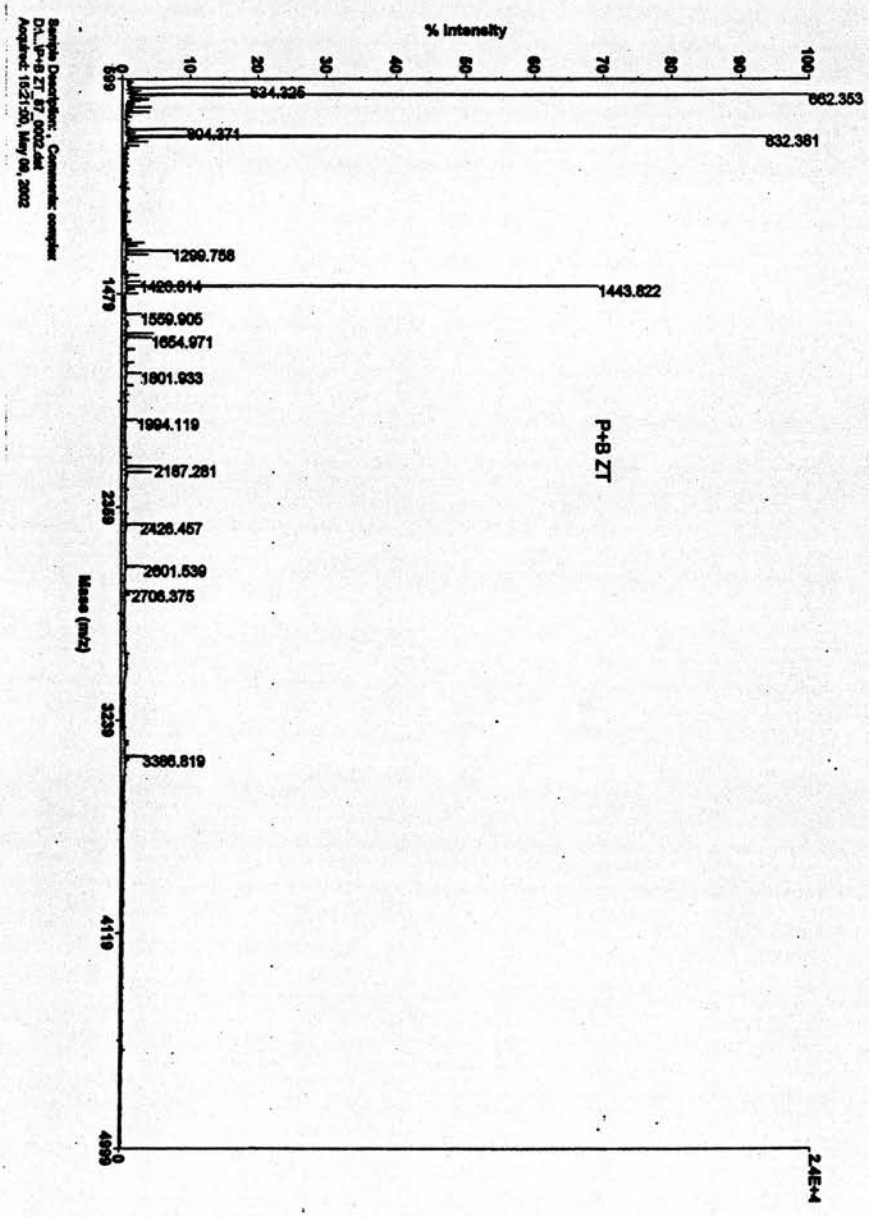

% Intensity
0
10
20
30
40
50
60
70
80
90
100
599
834.326
662.353
804.374
832.361
1299.758
1420.814
1443.822
1559.905
1654.971
1801.933
1994.119
2167.281
P+8 ZT
1479
2359
2426.457
2601.539
2706.375
Mass (m/z)
3239
3386.819
4119
4999
0
2.4E+4
Sample Description: , Comments: complex
D:\...\P+8 ZT_87_0002.dat
Acquired: 15:21:00, May 09, 2002

Fig. XII.1 A)

MS-Fit Search Results

Abort Search

Parameters

Database searched: **Ludwigpr.11.07.2000**
Digest Used: **Trypsin**
Max. # Missed Cleavages: **1**
Composition Include:
Composition Mask Type: **AND**
Peptide N terminus: **Hydrogen**
Peptide C terminus: **Free Acid**
Cysteine Modification: **acrylamide**
Instrument Name: **MALDI-TOF**
Sample ID (comment): **Magic Bullet digest**
Minimum Matches: **4**
Sort Type: **Score Sort**
Considered modifications: **| Peptide N-terminal Gln to pyroGlu | Oxidation of M | Protein N-terminus Acetylated |**
Min Parent Ion Matches: **1**
MOWSE On: **1**
MOWSE P Factor: **0.4**

Pre Search Results

Number of entries in the database: **576474**
Molecular weight search (**1000 - 100000 Da**) selects **548069** entries.
Full pI range: **576474** entries.
Combined molecular weight and pI searches select **548069** entries.
Pre searches select **548069** entries.

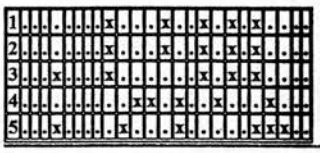
Data Set 1 Results

MS-Fit search selects **1670** entries (results displayed for top **5** matches).

Results Summary

MS-Fit Search Results

	MOWSE Score	#/24(%) Masses Matched	% Cov	% TIC	Mean Err ppm	Data Tol ppm	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein I
1	6151	5 (20)	13.0	20.8	67.8	34.7	515470	70379/8.6	Y18303	SHEEP PULMONARY ADENOMATOSIS VIRUS	(ENV)EN
2	6147	5 (20)	13.0	20.8	67.8	34.7	359884	70419/8.6	Y18302	SHEEP PULMONARY ADENOMATOSIS VIRUS	(ENV)EN
3	2723	5 (20)	12.0	20.8	50.7	104	196418	68730/8.3	AF153615	OVIS ARIES	(env)Env (PDI2..)P
4	2180	5 (20)	8.0	20.8	59.7	60.1	152808	75357/5.3	P20717	RATTUS NORVEGICUS	ARGINI DEIMIN (EC 3.5.3 (PEPTID DEIMIN
5	2087	6 (25)	11.0	25.0	25.4	134	216373	99415/8.2	AJ233397	PSEUDOMONAS PUTIDA	(alkS)Alk regulator.



Detailed Results

1. 5/24 matches (20%).
Acc. #: Y18303 **Species:** SHEEP PULMONARY ADENOMATOSIS VIRUS **Name:** (ENV)ENV PROTEIN.
Index: 515470 **MW:** 70379 **Da pI:** 8.6

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1443.8216	1443.7272	65		288	300	0	(K) YGDVGVTGFLYPR (A)
1704.0007	1703.9220	46		108	123	0	(R) EIVPVYVNDTSLLGGK (S)
1853.1333	1852.9744	86		198	213	0	(K) HLSIGIGIDTPWTLCR (A)
2187.2806	2187.1562	57		267	287	0	(K) LLAAFGHGNSLYLQPNISGSK
2426.4566	2426.2509	85		246	266	0	(R) GQHPPIFSVNTAPIYQTELWK

Click link below to search for cysteine linked fragments.
19 unmatched masses:

Click link below to do a non-specific cleavage search.
19 unmatched masses:

Click link below to search for another component.
19 unmatched masses:

The matched peptides cover 13% (87/622AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 515470

2. 5/24 matches (20%).
Acc. #: Y18302 **Species:** SHEEP PULMONARY ADENOMATOSIS VIRUS **Name:** (ENV)ENV PROTEIN.
Index: 359884 **MW:** 70419 Da **pI:** 8.6

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1443.8216	1443.7272	65		288	300	0	(K) YGDVGVTGFLYPR (A)
1704.0007	1703.9220	46		108	123	0	(R) EIVPVYVNDTSLLGGK (S)
1853.1333	1852.9744	86		198	213	0	(K) HLSIGIGIDTPWTLCR (A)
2187.2806	2187.1562	57		267	287	0	(K) LLAAFGHGNSLYLQPNISGSK
2426.4566	2426.2509	85		246	266	0	(R) GQHPPIFSVNTAPIYQTELWK

Click link below to search for cysteine linked fragments.
19 unmatched masses:

Click link below to do a non-specific cleavage search.
19 unmatched masses:

Click link below to search for another component.
19 unmatched masses:

The matched peptides cover 13% (87/622AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 359884

3. 5/24 matches (20%).
Acc. #: AF153615 **Species:** OVIS ARIES **Name:** (env)Env.
Index: 196418 **MW:** 68730 Da **pI:** 8.3

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
832.3811	832.4140	-39		577	582	0	(K) EFLHMR (V)
1443.8216	1443.7272	65		281	293	0	(K) YGDVGVTGFLYPR (A)
1853.1333	1852.9744	86		191	206	0	(K) HLSIGIGIDTPWTLCR (A)
2187.2806	2187.1562	57		260	280	0	(K) LLAAFGHGNSLYLQPNISGSK
2426.4566	2426.2509	85		239	259	0	(R) GQHPPIFSVNTAPIYQTELWK

Click link below to search for cysteine linked fragments.
19 unmatched masses:

MS-Fit Search Results

Click link below to do a non-specific cleavage search.
19 unmatched masses:

Click link below to search for another component.
19 unmatched masses:

The matched peptides cover **12%** (77/611AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 196418

4. 5/24 matches (20%).
Acc. #: P20717 **Species:** RATTUS NORVEGICUS **Name:** (PDI2..)PROTEIN-ARGININE DEIMINASE TYPE II (EC 3.5.3.15) (PEPTIDYLARGININE DEIMINASE II).
Index: 152808 **MW:** 75357 Da **pI:** 5.3

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1638.9693	1638.8388	80		583	596	0	(R) AFFPNMVNMIVLDK (D)
1654.9711	1654.8337	83	1Met-ox	583	596	0	(R)AFFPNMVNMIVLDK(D)
1758.9177	1758.9067	6.3		241	256	0	(K) YTGGSAELLFFVEGLR (F)
1994.1188	1994.0356	42		580	596	1	(R) QARAFFPNMVNMIVLDK (D)
2601.5387	2601.3469	74		294	315	0	(R) IAPWIMTPNILPPVSVFVCCMK
	2601.3469	74		294	315	0	(R) IAPWIMTPNILPPVSVFVCCMK

Click link below to search for cysteine linked fragments.
19 unmatched masses:

Click link below to do a non-specific cleavage search.
19 unmatched masses:

Click link below to search for another component.
19 unmatched masses:

The matched peptides cover **8%** (55/665AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 152808

5. 6/24 matches (25%).
Acc. #: AJ233397 **Species:** PSEUDOMONAS PUTIDA **Name:** (alkS)Alk gene regulator.
Index: 216373 **MW:** 99415 Da **pI:** 8.2

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
832.3811	832.4317	-61		447	452	1	(K) NKWQEK (G)
1559.9048	1559.7858	76		329	342	0	(R) DFLQGIAWAENPAK (R)
1758.9177	1758.9754	-33		508	523	1	(R) FAELEKVLAQAQAVNK (F)
2426.4566	2426.2695	77		412	431	0	(K) NPIVLICFAWVLYFSQQSAR (A
2601.5387	2601.2812	99		597	618	0	(K) FSEILEFIANHGVTDVFFSVCR
2706.3751	2706.3925	-6.4		375	398	1	(R) WAVGMSEGIILDLSFRQGEID

MS-Fit Search Results

Click link below to search for cysteine linked fragments.
18 unmatched masses:

Click link below to do a non-specific cleavage search.
18 unmatched masses:

Click link below to search for another component.
18 unmatched masses:

The matched peptides cover 11% (102/883AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 216373

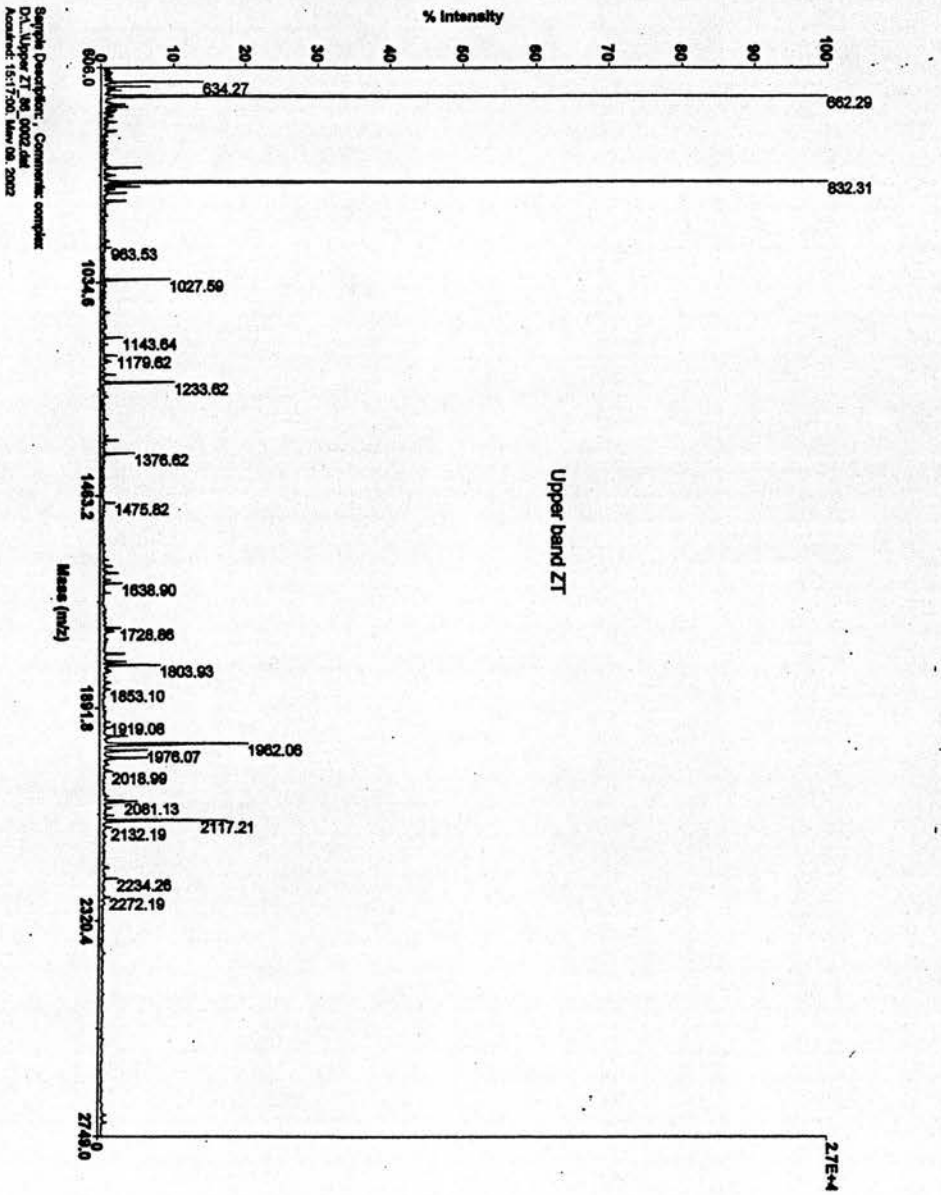

% Intensity
0
10
20
30
40
50
60
70
80
90
100
Sample Description: , Comments: complex
DL_Upper ZT_86_0002.dat
Acquired: 15:17:00, May 09, 2002
Mass (m/z)
606.0
1034.6
1463.2
1891.8
2320.4
2749.0
634.27
662.29
832.31
963.53
1027.59
1143.64
1179.62
1233.62
1376.62
1475.82
1638.90
1728.86
1803.93
1853.10
1919.06
1962.06
1976.07
2018.99
2061.13
2117.21
2132.19
2234.26
2272.19
Upper band ZT
2.7E+4

Fig. XII.1 B)

MS-Fit Search Results

Abort Search

Parameters

Database searched: **Ludwignr.11.07.2000**
Digest Used: **Trypsin**
Max. # Missed Cleavages: **1**
Composition Include:
Composition Mask Type: **AND**
Peptide N terminus: **Hydrogen**
Peptide C terminus: **Free Acid**
Cysteine Modification: **acrylamide**
Instrument Name: **MALDI-TOF**
Sample ID (comment): **Magic Bullet digest**
Minimum Matches: **4**
Sort Type: **Score Sort**
Considered modifications: **| Peptide N-terminal Gln to pyroGlu | Oxidation of M | Protein N-terminus Acetylated |**
Min Parent Ion Matches: **1**
MOWSE On: **1**
MOWSE P Factor: **0.4**

Pre Search Results

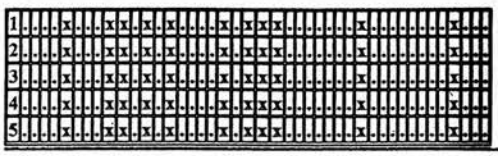
Number of entries in the database: **576474**
Molecular weight search **(1000 - 100000 Da)** selects **548069 entries.**
Full pI range: **576474 entries.**
Combined molecular weight and pI searches select **548069 entries.**
Pre searches select **548069 entries.**

Data Set 1 Results

MS-Fit search selects **11449 entries** (results displayed for top **5** matches).

Results Summary

	MOWSE Score	#/43(%) Masses Matched	% Cov	% TIC	Mean Err ppm	Data Tol ppm	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein I
1	5.415e+006	11 (25)	32.0	25.6	5.01	32.7	114536	43153/5.3	P21694	SALMONELLA TYPHIMURIUM	(TUFA..) ELONG/ FACTOF (EF-TU).
2	5.411e+006	11 (25)	32.0	25.6	5.01	32.7	118450	43183/5.3	P02990	ESCHERICHIA COLI	(TUFA..) ELONG/ FACTOF (EF-TU) 43).
3	5.403e+006	11 (25)	32.0	25.6	5.01	32.7	92868	43252/5.3	S13560	SALMONELLA TYPHIMURIUM	translatio elongatio factor EF - Salmon typhimur
4	5.399e+006	11 (25)	32.0	25.6	5.01	32.7	235212	43284/5.3	J01690	ESCHERICHIA COLI	(tufA) Elongatic factor Tu
5	5.399e+006	11 (25)	32.0	25.6	5.01	32.7	236156	43284/5.3	AF170176	SALMONELLA TYPHIMURIUM LT2	(tufB) Salmonel typhimur translatio elongatio factors T TU) (SW:P21 contains similarity PFam do PF00009 (GTP_EF Score=54 E=4.6e-1 N=1.



Detailed Results

1. 11/43 matches (25%).

Acc. #: P21694 **Species:** SALMONELLA TYPHIMURIUM **Name:** (TUFA..)ELONGATION FACTOR TU (EF-TU).
Index: 114536 **MW:** 43153 Da **pI:** 5.3

m/z Submitted	MH+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1027.5918	1027.5900	1.8		270	279	0	(R) AGENVGVLLR (G)
1218.5315	1218.5643	-27		177	187	0	(K) ALEGDAEWEAK (I)
1233.6157	1233.6169	-0.98		325	333	0	(K) GYRPQFYFR (T)
1376.6180	1376.6334	-11		45	56	0	(R) AFDQIDNAPEEK (A)
1603.8007	1603.7716	18		45	58	1	(R) AFDQIDNAPEEKAR (G)
1728.8569	1728.8445	7.2		304	318	1	(K) FESEVYILSKDEGGR (H)
1780.9271	1780.9454	-10		358	373	0	(K) MVVTLIHPIAMDDGLR (F)
1796.9588	1796.9403	10	1Met-ox	358	373	0	(K)MVVTLIHPIAMDDGLR(F)
1803.9325	1803.8877	25		59	74	0	(R) GITINTSHVEYDTPTR (H)
1962.0613	1962.0224	20		188	204	0	(K) IIELAGFLDSYIPEPER (A)
2117.2119	2117.1647	22		205	223	0	(R) AIDKPFLLPIEDVFSISGR (G)

Click link below to search for cysteine linked fragments.
32 unmatched masses:

Click link below to do a non-specific cleavage search.
32 unmatched masses:

Click link below to search for another component.
32 unmatched masses:

The matched peptides cover **32%** (127/393AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 114536

2. 11/43 matches (25%).
Acc. #: P02990 **Species:** ESCHERICHIA COLI **Name:** (TUFA..)ELONGATION FACTOR TU (EF-TU) (P-43).
Index: 118450 **MW:** 43183 Da **pI:** 5.3

m/z Submitted	MH+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1027.5918	1027.5900	1.8		270	279	0	(R) AGENVGVLLR (G)
1218.5315	1218.5643	-27		177	187	0	(K) ALEGDAEWEAK (I)
1233.6157	1233.6169	-0.98		325	333	0	(K) GYRPQFYFR (T)
1376.6180	1376.6334	-11		45	56	0	(R) AFDQIDNAPEEK (A)
1603.8007	1603.7716	18		45	58	1	(R) AFDQIDNAPEEKAR (G)
1728.8569	1728.8445	7.2		304	318	1	(K) FESEVYILSKDEGGR (H)
1780.9271	1780.9454	-10		358	373	0	(K) MVVTLIHPIAMDDGLR (F)
1796.9588	1796.9403	10	1Met-ox	358	373	0	(K)MVVTLIHPIAMDDGLR(F)

1803.9325	1803.8877	25	59	74	0	(R) GITINTSHVEYDTPTR (H)
1962.0613	1962.0224	20	188	204	0	(K) ILELAGFLDSYIPEPER (A)
2117.2119	2117.1647	22	205	223	0	(R) AIDKPFLLPIEDVFSISGR (G)

Click link below to search for cysteine linked fragments.
32 unmatched masses:

Click link below to do a non-specific cleavage search.
32 unmatched masses:

Click link below to search for another component.
32 unmatched masses:

The matched peptides cover **32%** (127/393AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 118450

3. 11/43 matches (25%).
Acc. #: S13560 **Species:** SALMONELLA TYPHIMURIUM **Name:** translation elongation factor EF-Tu A - Salmonella typhimurium
Index: 92868 **MW:** 43252 Da **pI:** 5.3

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1027.5918	1027.5900	1.8		271	280	0	(R) AGENVGVLLR (G)
1218.5315	1218.5643	-27		178	188	0	(K) ALEGDAEWEAK (I)
1233.6157	1233.6169	-0.98		326	334	0	(K) GYRPQFYFR (T)
1376.6180	1376.6334	-11		46	57	0	(R) AFDQIDNAPEEK (A)
1603.8007	1603.7716	18		46	59	1	(R) AFDQIDNAPEEKAR (G)
1728.8569	1728.8445	7.2		305	319	1	(K) FESEVYILSKDEGGR (H)
1780.9271	1780.9454	-10		359	374	0	(K) MVVTLIHPIAMDDGLR (F)
1796.9588	1796.9403	10	1Met-ox	359	374	0	(K)MVVTLIHPIAMDDGLR(F)
1803.9325	1803.8877	25		60	75	0	(R) GITINTSHVEYDTPTR (H)
1962.0613	1962.0224	20		189	205	0	(K) IIELAGFLDSYIPEPER (A)
2117.2119	2117.1647	22		206	224	0	(R) AIDKPFLLPIEDVFSISGR (G)

Click link below to search for cysteine linked fragments.
32 unmatched masses:

Click link below to do a non-specific cleavage search.
32 unmatched masses:

Click link below to search for another component.
32 unmatched masses:

The matched peptides cover **32%** (127/394AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 92868

4. 11/43 matches (25%).
Acc. #: J01690 **Species:** ESCHERICHIA COLI **Name:** (tufA)Elongation factor Tu.
Index: 235212 **MW:** 43284 **Da pI:** 5.3

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1027.5918	1027.5900	1.8		271	280	0	(R) AGENVGVLLR (G)
1218.5315	1218.5643	-27		178	188	0	(K) ALEGDAEWEAK (I)
1233.6157	1233.6169	-0.98		326	334	0	(K) GYRPQFYFR (T)
1376.6180	1376.6334	-11		46	57	0	(R) AFDQIDNAPEEK (A)
1603.8007	1603.7716	18		46	59	1	(R) AFDQIDNAPEEKAR (G)
1728.8569	1728.8445	7.2		305	319	1	(K) FESEVYILSKDEGGR (H)
1780.9271	1780.9454	-10		359	374	0	(K) MVVTLIHPIAMDDGLR (F)
1796.9588	1796.9403	10	1Met-ox	359	374	0	(K)MVVTLIHPIAMDDGLR(F)
1803.9325	1803.8877	25		60	75	0	(R) GITINTSHVEYDTPTR (H)
1962.0613	1962.0224	20		189	205	0	(K) ILELAGFLDSYIPEPER (A)
2117.2119	2117.1647	22		206	224	0	(R) AIDKPFLLPIEDVFSISGR (G)

Click link below to search for cysteine linked fragments.
32 unmatched masses:

Click link below to do a non-specific cleavage search.
32 unmatched masses:

Click link below to search for another component.
32 unmatched masses:

The matched peptides cover **32%** (127/394AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 235212

5. 11/43 matches (25%).
Acc. #: AF170176 **Species:** SALMONELLA TYPHIMURIUM LT2 **Name:** (tufB)Salmonella typhimurium translation elongation factors TU (EF-TU) (SW:P21694), contains similarity to PFam domain PF00009 (GTP_EFTU, Score=541.8 E=4.6e-159, N=1.
Index: 236156 **MW:** 43284 **Da pI:** 5.3

m/z Submitted	MH^+ Matched	Delta ppm	Modifications	Start	End	Missed Cleavages	Database Sequence
1027.5918	1027.5900	1.8		271	280	0	(R) AGENVGVLLR (G)
1218.5315	1218.5643	-27		178	188	0	(K) ALEGDAEWEAK (I)
1233.6157	1233.6169	-0.98		326	334	0	(K) GYRPQFYFR (T)
1376.6180	1376.6334	-11		46	57	0	(R) AFDQIDNAPEEK (A)
1603.8007	1603.7716	18		46	59	1	(R) AFDQIDNAPEEKAR (G)
1728.8569	1728.8445	7.2		305	319	1	(K) FESEVYILSKDEGGR (H)
1780.9271	1780.9454	-10		359	374	0	(K) MVVTLIHPIAMDDGLR (F)
1796.9588	1796.9403	10	1Met-ox	359	374	0	(K)MVVTLIHPIAMDDGLR(F)

1803.9325	1803.8877	25	60 75	0	(R) GITINTSHVEYDTPTR (H)
1962.0613	1962.0224	20	189 205	0	(K) IIELAGFLDSYIPEPER (A)
2117.2119	2117.1647	22	206 224	0	(R) AIDKPFLLPIEDVFSISGR (G)

Click link below to search for cysteine linked fragments.
32 unmatched masses:

Click link below to do a non-specific cleavage search.
32 unmatched masses:

Click link below to search for another component.
32 unmatched masses:

The matched peptides cover 32% (127/394AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 236156

Appendix XIII: Collection of epidemiology data for statistical analysis

The PCR results regarding JSRV infection in the blood were collected in Excel format in order to facilitate the statistical analysis (Table XIII.1).

Table XIII.1. Collection of epidemiological data for statistical analysis.

The JSRV U3 PCR results for the lamb and ewe populations were tabulated in Excel format. From the left column of the table:

Lamb Num: identification number of the lambs.

LMay 03- Lmay04: in this column the results of the PCR test for each lamb were reported divided according to the date in which the bleed was done. Each animal was scored with " 0 " when the PCR result was negative and with " 1 " when the PCR test was positive. The " * " indicated a missing value.

EweNum: identification number of the ewes.

EDec02-EDec04: in this column the results of the PCR test for each lamb were reported divided according to the date in which the sampling was done. Each animal was scored with " 0 " when the PCR result was negative and with " 1 " when the PCR test was positive. The " * " indicated a missing value.

EweAge: age of the ewes in December 2002.

Ewe/Milk: identification of the cross-breed of the ewe. If Milksheep breed was involved the animal was scored with " 1 " if not with " 0 ".

Ewe/Suff: identification of the cross-breed of the ewe. If Suffolk breed was involved the animal was scored with " 1 " if not " 0 ".

Ewe/Tex: identification of the cross-breed of the ewe. If Texel breed was involved the animal was scored with " 1 " if not with " 0 ".

Table XIII.1. Collection of epidemiology data for statistical analysis.

LambNum	LMay03	LSept03	LJan04	LMay04	EweNum	EDec02	EFeb03	EMay03	ESep03	EJan04	EMay04	EweAge	EweMilk	EweSuff	EweTex
347	0	.	.	.	2	0	0	0	.	1	0	2	1	1	0
232	0	0	0	0	4	1	1	1	1	1	0	3	1	1	0
231	0	1	1	1	4	1	1	1	1	1	0	3	1	1	0
305	1	1	1	1	7	0	0	0	0	0	1	3	1	1	0
296	1	1	0	0	11	0	0	1	0	0	.	2	1	1	0
250	1	1	1	1	12	0	0	0	0	0	1	2	1	1	0
300	1	0	1	1	14	0	0	1	0	0	0	2	1	1	0
359	.	1	1	0	18	0	0	0	0	0	0	2	1	1	0
245	0	1	1	1	20	0	0	0	0	0	0	3	1	1	0
319	1	1	1	1	21	0	0	0	0	1	0	2	1	1	0
246	0	0	0	0	22	0	0	0	0	1	0	3	1	1	0
247	0	.	.	.	22	0	0	0	0	1	0	3	1	1	0
207	0	0	0	0	24	0	0	0	0	1	0	2	1	1	0
344	0	0	0	0	25	0	0	0	0	1	0	3	1	1	0
323	1	1	1	1	26	0	0	0	0	0	0	3	1	1	0
324	0	0	1	.	26	0	0	0	0	0	0	3	1	1	0
325	0	0	0	0	26	0	0	0	0	0	0	3	1	1	0
309	0	0	0	0	29	0	0	0	1	1	0	2	1	1	0
310	0	0	0	1	29	0	0	0	1	1	0	2	1	1	0
238	0	0	1	1	30	0	0	0	1	1	0	3	1	1	0
341	0	1	1	0	31	0	0	0	1	1	0	3	1	1	0
234	1	1	1	.	33	0	0	0	0	1	0	3	1	1	0
339	0	0	0	0	34	0	0	0	0	0	0	3	1	1	0
261	0	0	0	0	35	1	0	0	0	0	0	2	1	1	0
270	0	0	1	0	39	0	0	0	0	0	0	2	1	1	0
291	1	1	1	1	40	0	1	0	1	0	0	2	1	1	0
331	0	.	.	.	42	0	0	0	0	1	0	2	1	1	0
295	1	1	1	1	44	0	1	0	1	0	1	2	1	1	0
333	0	0	1	0	48	0	0	0	0	0	0	2	1	1	0
234a	0	.	.	.	50	0	0	0	0	0	0	2	1	1	0
206	0	0	0	.	51	0	0	0	0	0	0	2	1	1	0
286	0	1	0	0	52	0	0	0	0	0	0	3	1	1	0
287	0	0	0	0	52	0	0	0	0	0	0	3	1	1	0
208	0	0	0	.	54	0	0	0	0	1	0	3	1	1	0
225	0	0	1	1	56	0	0	0	1	1	0	3	1	1	0
336	0	1	1	0	58	0	0	0	1	1	0	2	1	1	0
329	0	1	1	.	59	0	0	0	1	1	0	3	1	1	0
330	0	0	0	0	59	0	0	0	1	1	0	3	1	1	0
235	0	1	1	1	60	0	0	0	0	0	0	3	1	1	0
343	0	.	.	.	62	0	0	0	1	0	0	2	1	1	0
327	0	0	1	.	65	0	0	0	1	1	0	2	1	1	0
328	0	1	0	1	65	0	0	0	1	1	0	2	1	1	0
320	0	1	0	1	66	0	0	0	0	0	0	2	1	1	0
264	0	0	0	.	66	0	0	0	0	0	0	2	1	1	0
357	0	0	0	0	67	0	1	0	0	0	0	2	1	1	0
262	0	0	0	0	72	0	0	0	0	0	0	2	1	1	0
263	0	0	0	.	72	0	0	0	0	0	0	2	1	1	0
301	0	1	1	0	77	0	0	0	0	0	0	2	1	1	0
302	0	1	1	.	77	0	0	0	0	0	0	2	1	1	0
311	0	0	0	1	78	0	0	0	0	0	0	2	1	1	0
236	0	1	1	1	79	0	0	0	0	1	0	2	1	1	0
285	0	.	.	.	81	0	0	0	1	1	0	3	1	1	0
274	0	0	1	1	82	0	1	0	1	1	0	3	1	1	0
255	.	.	1	.	85	0	1	0	0	1	1	3	1	0	1
352	.	0	0	0	88	0	1	.	1	1	1	2	1	1	0
272	0	1	.	.	89	0	1	1	1	1	1	3	1	1	0

240	0	*	*	*	91	0	1	1	1	1	1	3	1	1	0
317	1	*	1	1	93	0	1	*	1	1	0	3	1	1	0
271	0	1	1	0	95	0	1	1	1	0	0	2	1	1	0
252	0	1	1	1	96	0	1	1	1	1	*	3	1	1	0
312	0	*	*	*	98	0	1	*	1	1	1	3	1	1	0
290	0	0	1	0	100	0	0	0	0	0	0	3	1	1	0
266	1	1	1	*	102	1	1	1	1	1	1	3	1	0	1
313	0	1	1	1	103	1	1	1	1	1	1	3	1	0	1
314	1	1	1	0	103	1	1	1	1	1	1	3	1	0	1
268	0	0	1	0	107	0	0	0	0	1	0	3	1	0	1
269	0	*	*	0	107	0	0	0	0	1	0	3	1	0	1
259	0	1	0	*	110	1	1	1	1	1	*	4	1	0	1
260	0	*	*	0	110	1	1	1	1	1	*	4	1	0	1
276	0	0	1	1	111	1	1	1	1	1	1	4	1	0	1
362	0	0	1	0	112	1	1	1	1	1	1	4	1	0	1
204	0	0	1	0	116	1	1	1	1	1	*	4	1	0	1
308	*	0	1	0	117	1	1	1	1	1	1	4	1	0	1
251	0	0	0	0	118	1	1	1	1	1	1	4	1	0	1
221	1	1	1	0	120	1	1	1	1	1	*	4	1	0	1
222	1	1	1	1	120	1	1	1	1	1	*	4	1	0	1
322	0	0	0	0	122	0	0	0	0	*	*	3	1	0	1
281	1	0	1	0	125	0	0	0	1	1	1	4	1	0	1
315	0	0	1	0	127	1	1	1	1	1	1	2	1	0	1
316	1	1	1	0	127	1	1	1	1	1	1	2	1	0	1
346	*	0	0	0	128	1	1	1	1	1	1	4	1	0	1
282	0	1	1	1	131	1	1	1	*	*	*	4	1	0	1
283	0	1	1	1	131	1	1	1	*	*	*	4	1	0	1
284	0	*	*	*	131	1	1	1	*	*	*	4	1	0	1
354	*	0	1	0	132	0	0	0	0	1	0	2	1	0	1
203	0	1	0	0	134	0	0	0	1	1	0	2	1	0	1
350	0	1	0	0	135	1	1	*	1	1	0	3	1	0	1
349	*	0	0	0	135	1	1	1	1	1	0	3	1	0	1
337	1	0	0	0	136	1	1	1	1	1	1	4	1	0	1
338	1	0	0	0	136	1	1	1	1	1	1	4	1	0	1
205	1	1	1	0	137	1	1	1	0	1	1	4	1	0	1
299	1	0	1	0	138	1	1	1	1	1	1	2	1	0	1
321	0	1	0	0	139	1	1	1	1	1	1	4	1	0	1
335	0	0	0	0	143	1	1	1	1	1	*	4	1	0	1
348	0	0	0	0	146	0	0	0	0	1	0	3	1	0	1
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209	0	1	1	*	148	1	1	1	*	*	*	4	1	0	1
210	0	1	1	1	148	1	1	1	*	*	*	4	1	0	1
306	*	0	1	0	150	1	1	1	1	1	1	3	1	0	1
351	0	*	*	*	152	0	0	0	0	1	1	3	1	0	1
248	1	1	1	1	153	0	1	*	1	1	1	2	0	1	1
279	*	0	0	0	154	0	*	0	0	0	1	2	0	1	1
288	0	0	*	*	158	0	1	1	1	1	1	2	0	1	1
244	0	1	1	0	159	0	0	0	1	1	1	2	0	1	1
345	0	0	0	0	160	0	1	*	*	*	*	2	0	1	1
242	0	1	1	1	161	0	0	0	0	0	0	2	0	1	1
217	0	0	1	1	163	1	1	*	*	*	*	2	0	1	1
215	*	0	0	1	168	0	0	*	1	0	0	2	0	1	1
216	*	1	0	1	168	0	0	*	1	0	0	2	0	1	1
218	0	0	*	*	169	0	0	0	0	0	0	2	0	1	1
304	0	1	0	0	170	0	0	*	0	0	0	2	0	1	1
212	0	*	*	*	173	0	0	0	*	*	*	2	0	1	1
211	1	*	*	1	173	0	0	0	*	*	*	2	0	1	1

280	0	1	0	0	176	1	1	*	*	*	*	2	0	1	1
292	*	0	0	0	177	1	1	*	1	0	1	2	0	1	1
258	*	0	1	0	178	0	0	*	1	0	1	2	1	1	0
213	0	1	0	1	184	1	0	*	1	0	0	2	0	1	1
214	1	1	0	1	184	1	0	*	1	0	0	2	0	1	1
342	*	0	1	0	187	0	0	*	0	0	0	2	0	1	1
229	*	0	1	1	188	0	0	*	0	0	1	2	0	1	1
353	0	0	1	*	192	1	1	*	1	0	0	2	0	1	1
253	*	1	0	*	192	1	1	*	1	0	0	2	0	1	1
356	1	1	1	0	194	0	0	0	0	0	1	3	0	1	1
226	0	0	1	1	195	0	0	0	0	0	1	2	0	1	1
219	0	0	0	1	198	1	1	1	1	1	*	2	0	1	1
363	0	*	*	*	200	0	0	*	0	1	*	2	0	1	1

Appendix XIV: Papers published during the course of the thesis

Daniela Salvatori, Marcelo de las Heras, and J. Mike Sharp. (2004). OVINE PULMONARY ADENOCARCINOMA: THE STORY TO DATE. *In Practice*. 26, 387-392.

Daniela Salvatori, Lorenzo González, Patricia Dewar, Christina Cousens, Marcelo de las Heras, Robert G. Dalziel and J. Mike Sharp. (2004). Successful induction of ovine pulmonary adenocarcinoma (OPA) in lambs of different ages and detection of viraemia during the pre-clinical period. *J Gen Virol* 85, 3319-3324.

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Short Communication

Successful induction of ovine pulmonary adenocarcinoma in lambs of different ages and detection of viraemia during the preclinical period

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Ovine pulmonary adenocarcinoma (OPA) can be reproduced consistently in neonatal lambs by intratracheal injection of inocula containing jaagsiekte sheep retrovirus (JSRV). In this study, clinical disease, confirmed pathologically as OPA, was induced in a high proportion of lambs that had been inoculated intratracheally with infectious lung fluid at 1, 3 and 6 months of age. The incubation periods, however, were longer in these three age groups than in 1-week-old lambs that were used as controls. Viraemia was detected in all age groups before onset of clinical signs, but occurred later in older animals. These results suggest an age-dependent susceptibility to OPA that could be determined by the availability of JSRV target cells in the ovine lung. The feasibility of inducing OPA in older lambs and detecting JSRV viraemia in preclinical stages enables improved studies on the pathogenesis, assessment of vaccines, diagnosis and control of the disease.

Ovine pulmonary adenocarcinoma (OPA) is a contagious lung cancer that is caused by an exogenous betaretrovirus, jaagsiekte sheep retrovirus (JSRV), which is distinct from the transcriptionally active endogenous retroviral sequences that are present in the ovine genome (York *et al.*, 1992; Palmarini *et al.*, 2004). The mechanism by which the virus causes oncogenic transformation of lung epithelial alveolar type II (ATII) and Clara cells is still not understood completely. Whilst natural OPA has a long incubation period, with most clinical cases occurring in 2–4-year-old animals (Sharp & DeMartini, 2003), the disease can be induced experimentally very rapidly and efficiently by intratracheal inoculation of newborn lambs with concentrated lung fluid (LF) (Verwoerd *et al.*, 1980; Sharp *et al.*, 1983; De las Heras *et al.*, 2003). However, following a similar intratracheal challenge of 10-week-old lambs with tumour extract, only 25% of them developed scattered neoplastic nodules and none showed clinical disease after 8 months (DeMartini *et al.*, 1987; Rosadio *et al.*, 1988). Similarly, in early transmission studies that were performed in sheep older than 1 year by parenteral inoculation or aerosol exposure, clinical disease was seldom achieved, with incubation periods usually longer than 1 year and pathological changes consisting of confined small tumour nodules (Dungal, 1946; Wandera, 1968).

The neonatal lamb model has been very useful in determining the aetiological role of JSRV in OPA and some aspects of its pathogenesis, but is unsuitable for evaluating the efficacy of potential vaccine preparations and studying pathogenic mechanisms of JSRV infections that occur later in life. These studies also have been hampered by the absence of detectable antibodies to JSRV in affected animals (Sharp & Herring, 1983; Ortín *et al.*, 1998).

The aims of the present study were to investigate whether JSRV infection, OPA lesions and clinical disease could be induced in older lambs and to determine the dynamics of the infection by PCR examination of peripheral blood samples.

Four groups of Scottish Blackface lambs were inoculated with JSRV-containing LF, as confirmed by Western blot and RT-PCR (Palmarini *et al.*, 1995). Ages at inoculation were: 1 week ($n=5$), 1 month ($n=10$), 3 months ($n=10$) and 6 months ($n=10$). All animals were from an OPA-free flock at Moredun Research Institute, UK. Six lambs from the same source were used as unchallenged controls.

LF collected from sheep that were naturally affected with OPA was clarified and stored at −70 °C. In order to remove variation in JSRV titres between batches of LF and to

ensure that each lamb received the same inoculum, several batches of LF from different sheep were thawed, pooled together, aliquotted and stored at −70 °C. Immediately before inoculation of the lambs, the appropriate number of aliquots were thawed and concentrated to give a 12·5-fold concentrate of the original volume (Sharp *et al.*, 1983). Each lamb in each age group received 5 ml of this concentrated LF pool by the intratracheal route, as described previously (Sharp *et al.*, 1983).

Peripheral blood samples were collected into EDTA tubes immediately before challenge and at intervals until necropsy. Peripheral blood leukocytes (PBLs) were obtained by centrifugation after lysis of erythrocytes (García-Goti, 1999) and the cell pellet was stored at −70 °C. DNA extraction was performed by using a DNeasy Tissue kit (Qiagen) following the manufacturer's protocol for animal blood. Blood samples from OPA-free Icelandic sheep were processed simultaneously as negative controls.

A single-round PCR to amplify the U3 region of JSRV was developed, based on the previously reported primers PI and PIII (Palmarini *et al.*, 1996). The new PCR was validated by using DNA from the OPA cell line JS7 (Jassim *et al.*, 1987; DeMartini *et al.*, 2001), diluted in a background of 600 ng DNA from Icelandic sheep PBLs. Optimized PCR conditions were: 5 μl 10 × PCR buffer (Qiagen), 200 μM dNTPs (Roche Diagnostics), 200 nM each primer (MWG) and 1·25 U HotStarTaq polymerase (Qiagen) were added to ultra-pure water (Sigma) to a final volume of 50 μl. To this pre-mix, 600–800 ng test DNA was added and incubated at 94 °C for 16 min, followed by 35 cycles at 94 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s, with a final extension step of 5 min at 72 °C. This JSRV-U3-PCR successfully amplified 10–100 copies of JSRV template that were integrated into JS7 genomic DNA (data not shown). The test samples were analysed in triplicate and considered to be positive if one or more of the replicates gave a band of the correct molecular mass. To confirm the integrity of the DNA, samples that were negative in the JSRV-U3-PCR were tested for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Palmarini *et al.*, 1996).

Animals were monitored daily after experimental infection for clinical signs of respiratory disease that might be suggestive of OPA. As soon as these were identified, lambs were killed humanely. Lambs that did not show any clinical signs were kept for a maximum of 7 months post-infection (p.i.) and then culled. Unchallenged control animals were culled at the end of the experiment, at the same time as the last inoculated lambs. At necropsy, examination of the lungs was performed to identify any gross lesions that might be suggestive of OPA. Neoplastic-like areas or, in their absence, a range of tissue sites from all pulmonary lobes were taken for microscopic examination. Samples were fixed in 10 % neutral-buffered formalin, processed routinely, sectioned at 5 μm and stained with haematoxylin and eosin (HE). Sections from selected lung samples were subjected to immunohistochemical examination for JSRV capsid protein (JSRV-CA) and JSRV surface glycoprotein (JSRV-SU) by using a newly developed antiserum, which was prepared by immunizing rabbits with glutathione S-transferase-SU (GST-SU) and His-tagged SU proteins in Freund's incomplete adjuvant. Recombinant fusion proteins were made by cloning and expressing nt 5557–6483 of JSRV (GenBank accession no. M80216) following standard molecular biology procedures (Sambrook *et al.*, 1989). The fragment inserted in pGEX1λT (Pharmacia) was expressed in *Escherichia coli* cells to generate GST-SU, which was purified by using glutathione/Sepharose beads (Invitrogen). The fragment inserted in pIVEX2.4a (Roche Molecular Biochemicals) was expressed as a His-tagged protein by using the cell-free translation system RTS-500 (Roche Molecular Biochemicals), purified by using ProBond nickel resin (Invitrogen) and eluted following denaturing conditions according to the manufacturer's protocol. Both antibodies were used at 1/1000 dilutions as described previously (Palmarini *et al.*, 1995). Samples of lung and mediastinal lymph node were taken for JSRV-U3-PCR analysis.

None of the unchallenged control lambs developed clinical signs of respiratory disease and their PBLs were negative by JSRV-U3-PCR throughout the experiment. When culled at 14 months of age, neither histopathological lesions of OPA nor proviral DNA was detected in lung or mediastinal lymph-node samples.

In four of five 1-week-old lambs, severe clinical signs that were suggestive of OPA were observed at 70–74 days p.i. (Fig. 1), whereas the other lamb (1W1; Fig. 2) died at 28 days p.i. of an unrelated illness. All five lambs showed

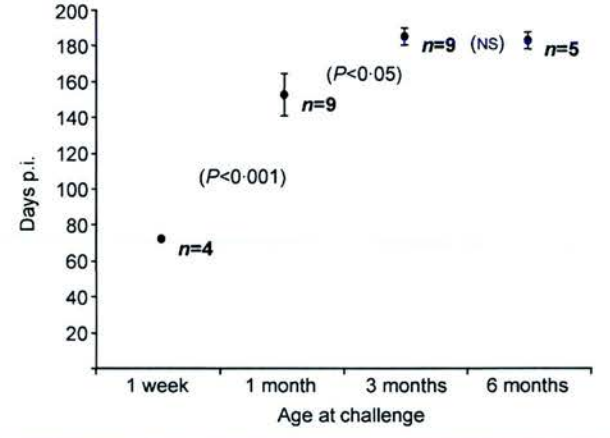

Fig. 1. Incubation period of experimental OPA depending on age at challenge. Incubation period (days from experimental infection to post-mortem) of OPA in four groups of sheep that were challenged at different ages, all of which showed clinical signs of the disease and histopathological confirmation of pulmonary neoplasia. Results are expressed as mean values; bars indicate SEM; statistical differences between groups appear in parentheses. NS, Not significant.

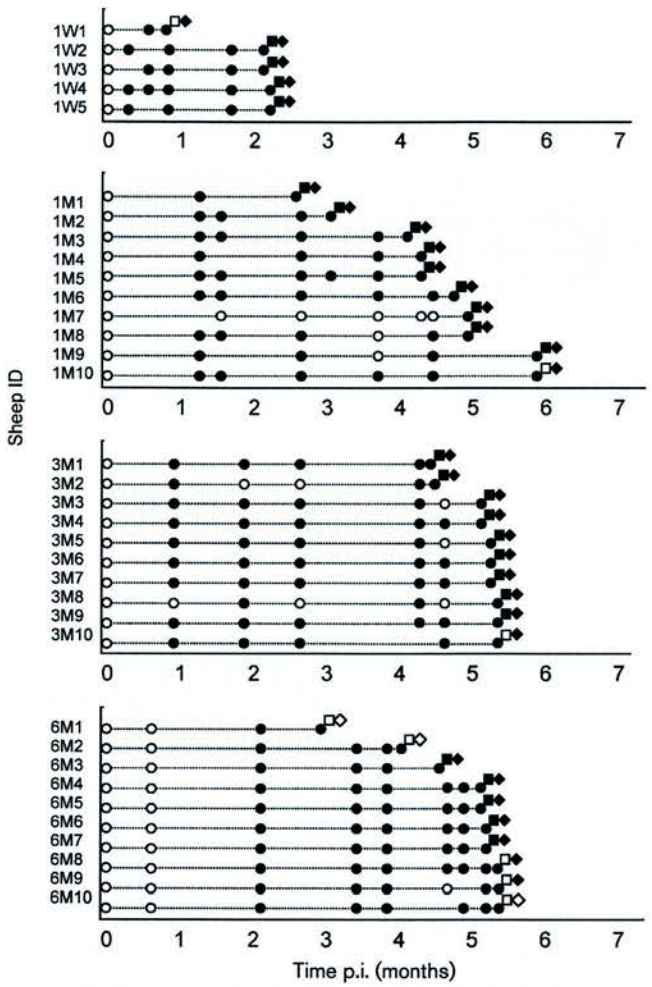

Fig. 2. Evolution of JSRV viraemia as detected by U3 PCR (○, negative; ●, positive) during the course of experimental infection and at culling of sheep showing clinical signs of OPA (■) or not showing such signs (□), and in which gross and/or histopathological lesions of OPA were confirmed (◆) or absent (◇).

gross and microscopic lesions of OPA (Fig. 2) and were consistently positive after infection by JSRV-U3-PCR (Fig. 2).

Nine out of ten 1-month-old lambs showed clinical signs of OPA, with incubation periods ranging from 92 to 209 days (mean, 152 days; Fig. 1). The remaining lamb (1M10; Fig. 2) was asymptomatic when culled at almost 7 months p.i. All ten lambs had macroscopic pulmonary lesions that were confirmed histologically as OPA (Fig. 2) and were JSRV-U3-PCR-positive throughout the incubation period, with the exception of one (1M7; Fig. 2).

Nine out of ten 3-month-old lambs developed signs of OPA, with incubation periods ranging from 159 to 192 days (mean, 185 days; Fig. 1). The other animal (3M10; Fig. 2) was asymptomatic when culled at 192 days p.i. Macroscopic lung lesions were confirmed histologically as OPA in all ten lambs. Whilst JSRV viraemia was detected consistently in six lambs, including the one that remained asymptomatic, the other four (3M2, 3M3, 3M5 and 3M8; Fig. 2) were JSRV-U3-PCR-negative on occasions during the experiment.

Five out of ten 6-month-old lambs developed clinical OPA with incubation periods ranging from 164 to 169 days (mean, 182 days; Fig. 1) and, amongst the other five sheep, three (6M8, 6M9 and 6M10) were asymptomatic when culled at 190–192 days p.i. All five clinically affected sheep and two asymptomatic lambs showed pathological evidence of neoplasia, although in one of them (6M9; Fig. 2), only microscopic lesions were detected. Two lambs (6M1 and 6M2; Fig. 2) died early from OPA-unrelated conditions; in these and in one of the asymptomatic culls (3M10; Fig. 2), neither gross nor microscopic lesions of OPA were found. Within this group of 6-month-old lambs, PBLs from all animals were JSRV-U3-PCR-negative at 3 weeks p.i. but, with the exception of a single sheep (6M9; Fig. 2), they were consistently positive thereafter.

Regardless of age at challenge, all sheep with histologically confirmed OPA showed the same pattern of neoplasia, which consisted predominantly of papillary adenocarcinoma of the alveoli (Fig. 3a, b). Immunohistochemical examinations showed JSRV-CA protein in the cytoplasm of a proportion of ATII neoplastic cells and nodules with the features described in previous studies (Palmarini *et al.*, 1995; Platt *et al.*, 2002). Detection of immunohistochemical reactivity to JSRV-SU was consistent in epithelial cells lining neoplastic alveoli (Fig. 3c) and in polypoid structures in the bronchioles (Fig. 3d). Immunolabelling was strongest at the apical surface of the cells.

JSRV was detected by U3-PCR in all mediastinal lymph-node and lung-tissue samples that were collected from the 35 JSRV-challenged sheep.

The present study demonstrates clearly that JSRV infection can be induced in 100 % of lambs aged 1–6 months at the time of inoculation and that a high proportion of these animals develop clinical signs (62–90 %) and lesions (87–100 %) of OPA. This is in contrast to a previous study on a much smaller scale, in which only a proportion of 10-week-old lambs developed neoplastic lesions (Rosadio *et al.*, 1988). One explanation for this discrepancy and for the success of the current experiment would be a large amount of JSRV in the inoculum, as LF contains more JSRV than tumour extracts (Herring *et al.*, 1983) and JSRV dose and incubation period are correlated inversely (Verwoerd *et al.*, 1980). Nevertheless, some findings in the present study support the notion of an age-related resistance to OPA. Firstly, within those animals that developed clinical OPA, the incubation period was significantly shorter ($P<0{\cdot}001$) for lambs that were inoculated at 1 week of age than for older lambs (Fig. 1) and it was also shorter ($P=0{\cdot}03$) for animals that were challenged at 1 month than for those inoculated at 3 months. Secondly, the development of lesions was delayed in older lambs, as indicated by three

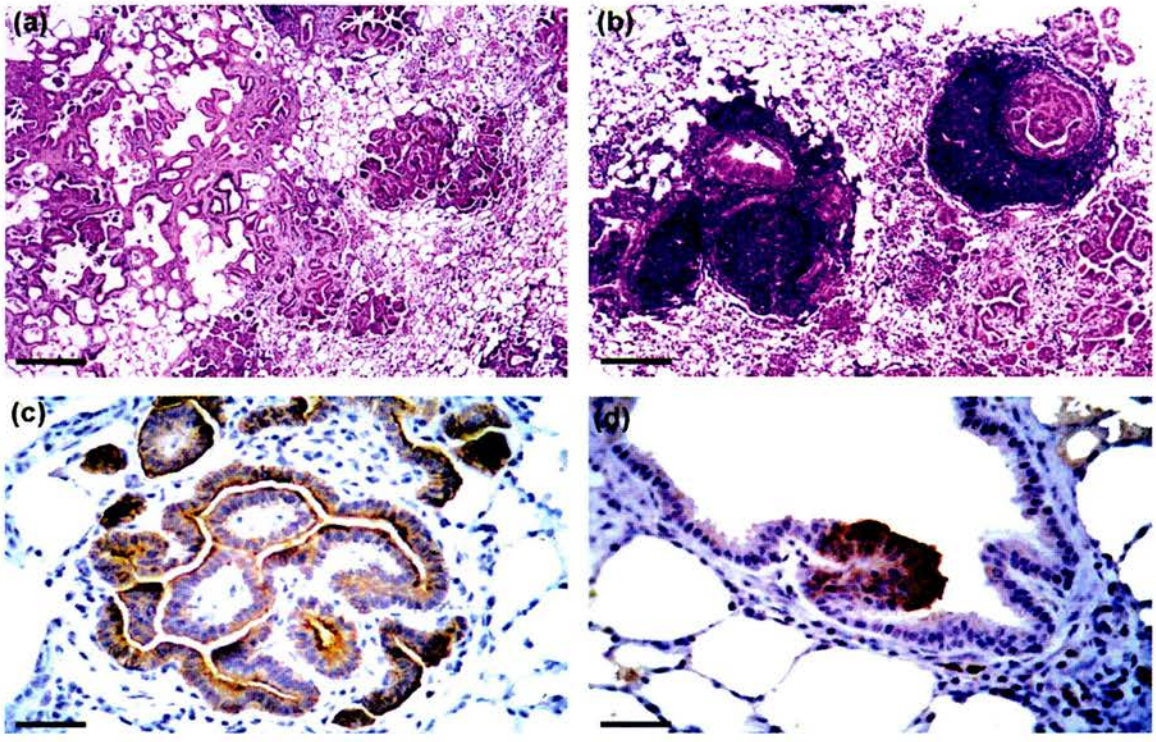

Fig. 3. Histopathology and immunohistochemistry of experimental OPA. (a) Co-existence of papillary and acinary forms of the tumour and presence of desquamated alveolar macrophages in the lung of a lamb inoculated at 1 month of age (HE staining). Bar, 400 μm. (b) Papillary neoplastic proliferations involve alveoli and bronchioles, which also show hyperplasia of associated lymphoid tissue. Para-adenomatous change is conspicuous. Lamb was inoculated at 1 month of age (HE staining). Bar, 200 μm. (c) Papillary alveolar tumour in a lamb challenged at 3 months of age, showing strong immunoreactivity for JSRV-SU protein mainly in the apical border of transformed ATII cells. Haematoxylin counterstaining. Bar, 60 μm. (d) Proliferating cells in a bronchiole show immunoreactivity for JSRV-SU protein (most evident in the apical cytoplasm), whereas non-transformed cells are negative. Note some immunolabelled cells in the interstitium. Lamb was inoculated at 6 months of age. Haematoxylin counterstaining. Bar, 60 μm.

asymptomatic lambs in the oldest age group that had no evidence of OPA lesions and a fourth lamb with only microscopic lesions (Fig. 2). Finally, detectable viraemia occurred later in the group of 6-month-old lambs than in the group of 1-week-old lambs (Fig. 2), and possibly also later than in the 1- and 3-month-old animals, although straight comparisons cannot be made with these two groups, as their first PCR testing on PBLs was slightly delayed.

As the same inoculum was used for all 35 lambs, our results suggest that age at infection has an effect on the development of OPA; this is particularly noticeable between 1 week and 1 month of life.

Other examples of age-related susceptibility to retroviral infections include feline leukemia virus (Hoover *et al.*, 1976; Flynn *et al.*, 2000), certain avian leukosis viruses (Pizer *et al.*, 1992; Stedman *et al.*, 2001), visna/maedi virus (Lairmore *et al.*, 1986; Andresson *et al.*, 1993) and Mason–Pfizer monkey virus (Fine *et al.*, 1975). Whilst, for some of these infections, differences in the ability of neonates and older animals to mount immune responses could account for an age-related susceptibility, this is unlikely for OPA, in view of the lack of a specific humoral immune response to JSRV (Sharp & Herring, 1983; Ortín *et al.*, 1998).

A more plausible explanation for the age effect in OPA would be that the target cells for primary JSRV infection and oncogenesis, ATII pneumocytes and Clara cells of the bronchioles, are present in higher numbers in newborn lambs than in older animals. In rodents and ruminants, the ratio of type I pneumocytes to ATII cells at birth is around 1:2, with ATII pneumocytes accounting for approximately 60 % of alveolar cells (Weibel & Taylor, 1996; Kahwa *et al.*, 1997; Otto, 1997). During the first month of life, the number of ATII cells declines progressively and dramatically, so that they represent only 6 % of alveolar cells at 7 days, 1 % at 3 weeks and 0·2 % at 1 month, with no further significant changes occurring later (Wright & Alison, 1984). Similarly, Clara cells retain fetal characteristics in calves and goat kids that are up to 30 days old, but lose them and become fully differentiated after this time (Castleman & Lay, 1990; Kahwa *et al.*, 2000). We hypothesize that following intratracheal inoculation of

JSRV, primary virus replication would occur in the ATII and Clara cells and that the magnitude of such replication would depend on the availability of these target cells. Thus, higher viral production could be expected in newborn lambs, leading to earlier detection of viraemia and development of neoplastic lesions (and hence a shorter incubation period), and vice versa in older lambs.

In conclusion, we have developed a new experimental model to induce JSRV infection and OPA in aged lambs, which can be monitored by an efficient PCR technique in blood samples. Apart from providing new insights into the pathogenesis of the disease by confirming an age effect in the timescale of OPA-related events, the model will be useful to test the efficacy of potential vaccines. Finally, our PCR results reinforce previous studies on the detection of JSRV infection in subclinically infected sheep (García-Goti, 1999; González *et al.*, 2001), providing an opportunity for future epidemiological research.

Acknowledgements

This work was funded by the European Union Framework Programme 5 (grants QLK2-PL1999-00983 and QLRT-2000-02380) and Scottish Executive Environment and Rural Affairs Department ROAME 66/01. We thank Dr Valgerdur Andrésdóttir, University of Iceland, for negative-control sheep blood samples and the staff of the Clinical Department, Moredun Research Institute, UK, for assistance with animal care.

References

Andresson, Ó. S., Elser, J. E., Tobin, G. J. & 11 other authors (1993). Nucleotide sequence and biological properties of a pathogenic proviral molecular clone of neurovirulent visna virus. *Virology* **193**, 89–105.

Castleman, W. L. & Lay, J. C. (1990). Morphometric and ultrastructural study of postnatal lung growth and development in calves. *Am J Vet Res* **51**, 789–795.

De las Heras, M., González, L. & Sharp, J. M. (2003). Pathology of ovine pulmonary adenocarcinoma. *Curr Top Microbiol Immunol* **275**, 25–54.

DeMartini, J. C., Rosadio, R. H., Sharp, J. M., Russell, H. I. & Lairmore, M. D. (1987). Experimental coinduction of type D retrovirus-associated pulmonary carcinoma and lentivirus-associated lymphoid interstitial pneumonia in lambs. *J Natl Cancer Inst* **79**, 167–177.

DeMartini, J. C., Bishop, J. V., Allen, T. E., Jassim, F. A., Sharp, J. M., de las Heras, M., Voelker, D. R. & Carlson, J. O. (2001). Jaagsiekte sheep retrovirus proviral clone $JSRV_{JS7}$, derived from the JS7 lung tumor cell line, induces ovine pulmonary carcinoma and is integrated into the surfactant protein A gene. *J Virol* **75**, 4239–4246.

Dungal, N. (1946). Experiments with jaagsiekte. *Am J Pathol* **22**, 737–759.

Fine, D. L., Landon, J. C., Pienta, R. J., Kubicek, M. T., Valerio, M. G., Loeb, W. F. & Chopra, H. C. (1975). Responses of infant rhesus monkeys to inoculation with Mason-Pfizer monkey virus materials. *J Natl Cancer Inst* **54**, 651–658.

Flynn, J. N., Hanlon, L. & Jarrett, O. (2000). Feline leukaemia virus: protective immunity is mediated by virus-specific cytotoxic T lymphocytes. *Immunology* **101**, 120–125.

García-Goti, M. (1999). *Estudios sobre la patogenia y diagnóstico de la adenomatosis pulmonary ovina.* PhD thesis, University of Zaragoza, Spain (in Spanish).

González, L., García-Goti, M., Cousens, C., Dewar, P., Cortabarría, N., Extramiana, A. B., Ortín, A., De las Heras, M. & Sharp, J. M. (2001). Jaagsiekte sheep retrovirus can be detected in the peripheral blood during the pre-clinical period of sheep pulmonary adenomatosis. *J Gen Virol* **82**, 1355–1358.

Herring, A. J., Sharp, J. M., Scott, F. M. M. & Angus, K. W. (1983). Further evidence for a retrovirus as the aetiological agent of sheep pulmonary adenomatosis (jaagsiekte). *Vet Microbiol* **8**, 237–249.

Hoover, E. A., Olsen, R. G., Hardy, W. D., Jr, Schaller, J. P. & Mathes, L. E. (1976). Feline leukemia virus infection: age-related variation in response of cats to experimental infection. *J Natl Cancer Inst* **57**, 365–369.

Jassim, F. A., Sharp, J. M. & Marinello, P. D. (1987). Three-step procedure for isolation of epithelial cells from the lungs of sheep with jaagsiekte. *Res Vet Sci* **43**, 407–409.

Kahwa, C. K., Atwal, O. S. & Purton, M. (1997). Transmission electron microscopy of the epithelium of distal airways and pulmonary parenchyma of the goat lung. *Res Vet Sci* **63**, 49–56.

Kahwa, C. K., Balemba, O. B. & Assey, R. J. (2000). The pattern of ciliation and the development of the epithelial lining of the respiratory tract in the neonatal kid: a scanning electron microscopic study. *Small Rumin Res* **37**, 27–34.

Lairmore, M. D., Rosadio, R. H. & DeMartini, J. C. (1986). Ovine lentivirus lymphoid interstitial pneumonia. Rapid induction in neonatal lambs. *Am J Pathol* **125**, 173–181.

Ortín, A., Minguijon, E., Dewar, P., Garcia, M., Ferrer, L. M., Palmarini, M., González, L., Sharp, J. M. & De las Heras, M. (1998). Lack of a specific immune response against a recombinant capsid protein of Jaagsiekte sheep retrovirus in sheep and goats naturally affected by enzootic nasal tumour or sheep pulmonary adenomatosis. *Vet Immunol Immunopathol* **61**, 229–237.

Otto, W. R. (1997). Lung stem cells. *Int J Exp Pathol* **78**, 291–310.

Palmarini, M., Dewar, P., De las Heras, M., Inglis, N. F., Dalziel, R. G. & Sharp, J. M. (1995). Epithelial tumour cells in the lungs of sheep with pulmonary adenomatosis are major sites of replication for Jaagsiekte retrovirus. *J Gen Virol* **76**, 2731–2737.

Palmarini, M., Holland, M. J., Cousens, C., Dalziel, R. G. & Sharp, J. M. (1996). Jaagsiekte retrovirus establishes a disseminated infection of the lymphoid tissues of sheep affected by pulmonary adenomatosis. *J Gen Virol* **77**, 2991–2998.

Palmarini, M., Mura, M. & Spencer, T. E. (2004). Endogenous betaretroviruses of sheep: teaching new lessons in retroviral interference and adaptation. *J Gen Virol* **85**, 1–13.

Pizer, E. S., Baba, T. W. & Humphries, E. H. (1992). Activation of the c-*myb* locus is insufficient for the rapid induction of disseminated avian B-cell lymphoma. *J Virol* **66**, 512–523.

Platt, J. A., Kraipowich, N., Villafane, F. & DeMartini, J. C. (2002). Alveolar type II cells expressing jaagsiekte sheep retrovirus capsid protein and surfactant proteins are the predominant neoplastic cell type in ovine pulmonary adenocarcinoma. *Vet Pathol* **39**, 341–352.

Rosadio, R. H., Lairmore, M. D., Russell, H. I. & DeMartini, J. C. (1988). Retrovirus-associated ovine pulmonary carcinoma (sheep pulmonary adenomatosis) and lymphoid interstitial pneumonia. I. Lesion development and age susceptibility. *Vet Pathol* **25**, 475–483.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sharp, J. M. & Herring, A. J. (1983). Sheep pulmonary adenomatosis: demonstration of a protein which cross-reacts with the major core proteins of Mason–Pfizer monkey virus and mouse mammary tumour virus. *J Gen Virol* **64**, 2323–2327.

Sharp, J. M. & DeMartini, J. C. (2003). Natural history of JSRV in sheep. *Curr Top Microbiol Immunol* **275**, 55–79.

Sharp, J. M., Angus, K. W., Gray, E. W. & Scott, F. M. (1983). Rapid transmission of sheep pulmonary adenomatosis (jaagsiekte) in young lambs. *Arch Virol* **78**, 89–95.

Stedman, N. L., Brown, T. P. & Brown, C. C. (2001). Localization of avian leukosis virus subgroup J in naturally infected chickens by RNA in situ hybridization. *Vet Pathol* **38**, 649–656.

Verwoerd, D. W., Williamson, A.-L. & De Villiers, E.-M. (1980). Aetiology of jaagsiekte: transmission by means of subcellular fractions and evidence for the involvement of a retrovirus. *Onderstepoort J Vet Res* **47**, 275–280.

Wandera, J. G. (1968). Experimental transmission of sheep pulmonary adenomatosis (jaagsiekte). *Vet Rec* **83**, 478–482.

Weibel, E. R. & Taylor, C. R. (1996). Functional anatomy of the human lung. In *Oxford Textbook of Medicine*, 3rd edn, pp. 2593–2609. Edited by D. J. Weatherall, J. G. G. Ledingham & D. A. Warrell. Oxford: Oxford University Press.

Wright, N. A. & Alison, M. A. (1984). Cell proliferation in respiratory epithelia. In *The Biology of Epithelial Cell Populations*, vol. 2, pp. 1068–1078. Oxford: Oxford University Press.

York, D. F., Vigne, R., Verwoerd, D. W. & Querat, G. (1992). Nucleotide sequence of the jaagsiekte retrovirus, an exogenous and endogenous type D and B retrovirus of sheep and goats. *J Virol* **66**, 4930–4939.

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In Practice

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Heartworm disease – potential health risks for the travelling pet

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Peritonitis in dogs and cats: optimising the outcome

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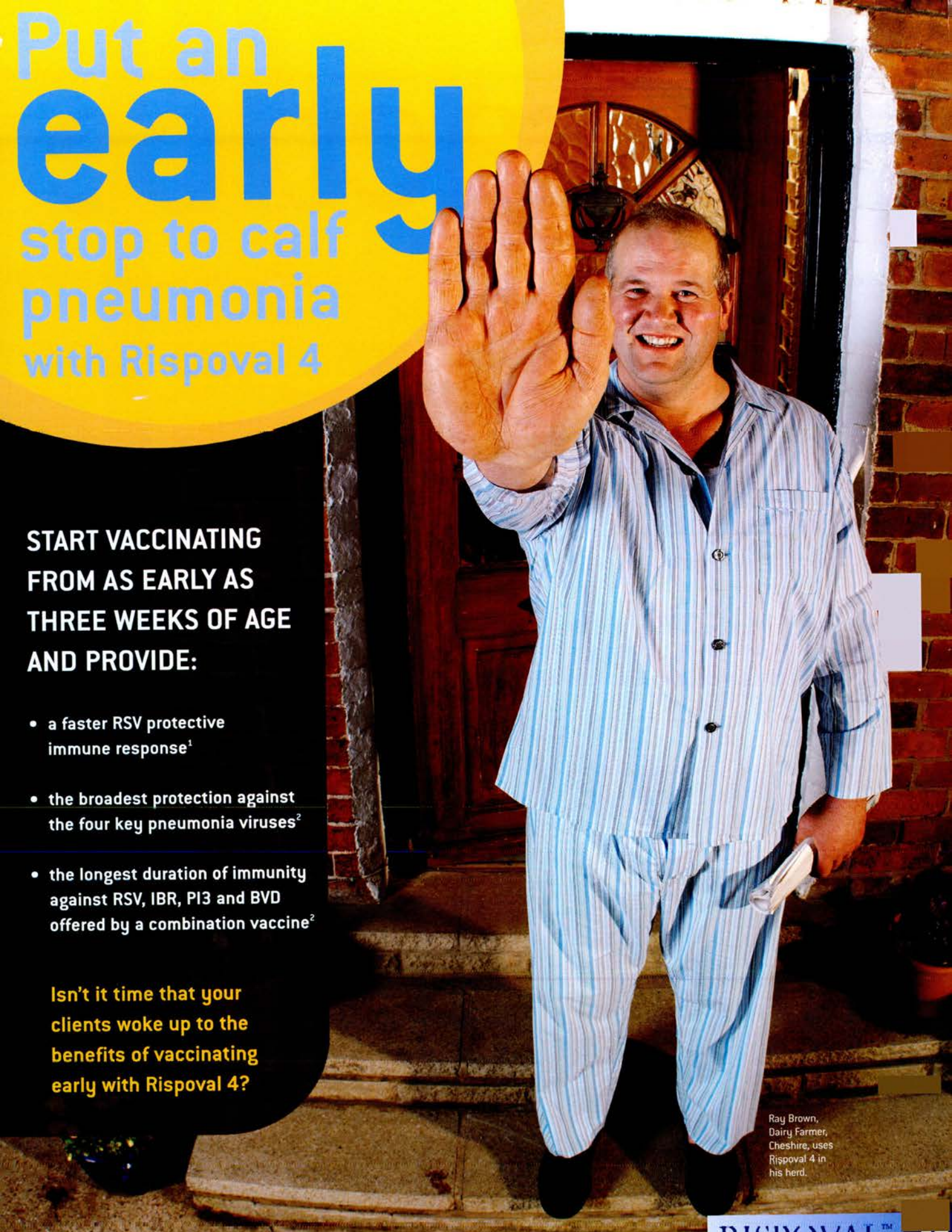

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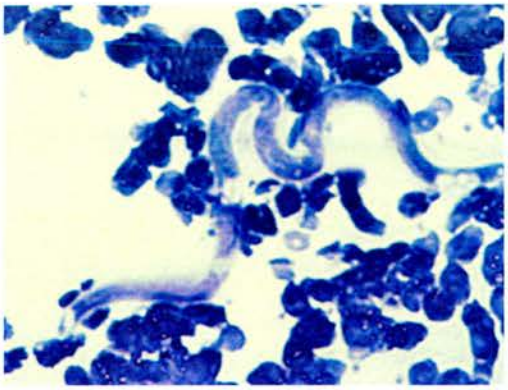


The number and location of *Dirofilaria immitis* within a host will determine the severity and extent of heartworm disease and, in turn, the prognosis.
Picture, Susan Shaw

Disease risks for the travelling pet: Heartworm disease

LUCA FERASIN

Luca Ferasin graduated from the University of Bologna, Italy, in 1992 and undertook a three-year research project in endocrinology at the BBSRC in Cambridge for which he was awarded a PhD in 1996. He was appointed assistant professor at the University of Padova, Italy, first in veterinary physiology, and then in internal medicine. He is currently a lecturer in small animal medicine at the University of Bristol where he teaches on cardiorespiratory diseases of the dog and cat. He holds the RCVS certificate in cardiology.

HEARTWORM disease is a parasitic infection caused by *Dirofilaria immitis*. This is a nematode (roundworm) that lives primarily in the pulmonary arteries and the right heart of dogs and, less commonly, cats and ferrets. The disease may sometimes affect other species, such as wild canids and felids, sealions and, sporadically, humans; however, these species are generally considered dead-end hosts as the parasite does not reach full maturity and cannot complete its biological cycle. Heartworm disease is transmitted by mosquito bites and there are more than 70 species of mosquito that can potentially transmit the infection. This article discusses the epidemiology and pathogenesis of heartworm disease, as well as its diagnosis and management.

EPIDEMIOLOGY

Heartworm disease is present worldwide, but its prevalence varies depending on the canine population, presence of mosquitoes and the climate. Climatic conditions must be sufficiently warm to allow mosquitoes to thrive and *Dirofilaria immitis* larvae to develop in the insect (see box below). For this reason, the prevalence of heartworm disease depends not only on geographical area, but also on the season, and this is a key consideration when screening for the disease or planning a chemoprophylactic schedule.

Although heartworm disease has been diagnosed in the UK in dogs imported from abroad, the disease is unlikely to spread in this country despite the presence of potential vectors and a large canine population. In fact, the low average daily temperature does not allow larval maturation within the mosquito, even in summertime.

Biological cycle of *Dirofilaria immitis*

D immitis adults reach sexual maturity in the pulmonary arteries and right ventricle of the dog. After mating, the female nematodes release immature larvae (L1 or microfilariae) into the circulation. Microfilariae are eventually ingested by mosquitoes during a blood meal. Mosquitoes are not only a vector, but also an obligatory intermediate host, without which heartworm disease could not be transmitted. The ingested microfilariae require a period of maturation in the insect, which varies depending on environmental conditions. This development cannot occur below the threshold temperature of 14°C when the cycle is temporarily suspended until warmer conditions resume. At an average daily temperature of 30°C, maturation can be completed in eight days; however, maturation takes approximately one month at an environmental temperature of 18°C. Transmission of infective larvae is therefore limited to warm months and varies depending on geographical location.

In the mosquito, microfilariae mature to a second larval stage (L2) and, eventually, to the infective stage (L3). When the L3 stage is reached, the larvae migrate from the tubules to the insect's mouth and, during a subsequent blood meal on an appropriate host, enter the bite wound, colonising the local connective tissues. After approximately one week, the larvae mature from L3 to L4 and, following continued migration for two to three months in the subcutaneous tissues, reach the final stage of immature adults (L5). The L5 larvae penetrate a systemic vein and migrate to the right heart and pulmonary arteries within a few days, where they mature and mate after around three to six months, releasing microfilariae into the circulation to complete the biological cycle. The average prepatent period is about six months in dogs and eight months in cats.

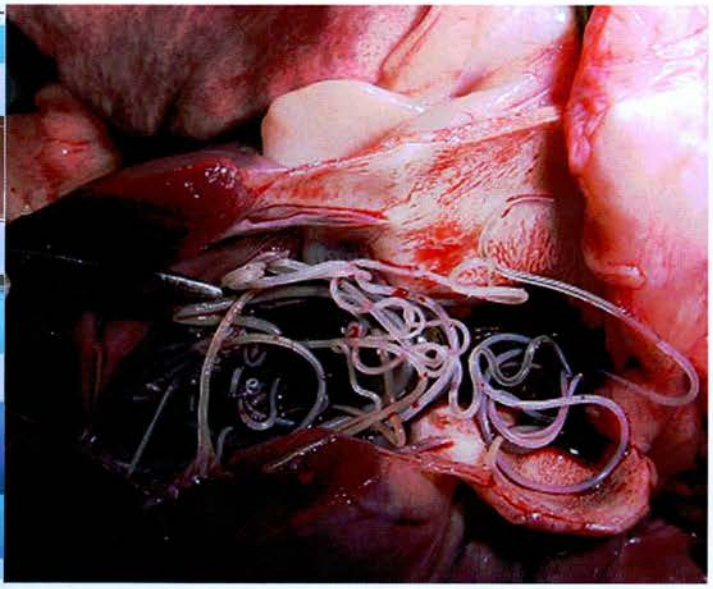
Adult *D immitis* may survive for up to five years in dogs; in cats, the worms develop more slowly and survive for approximately two years. The slower development

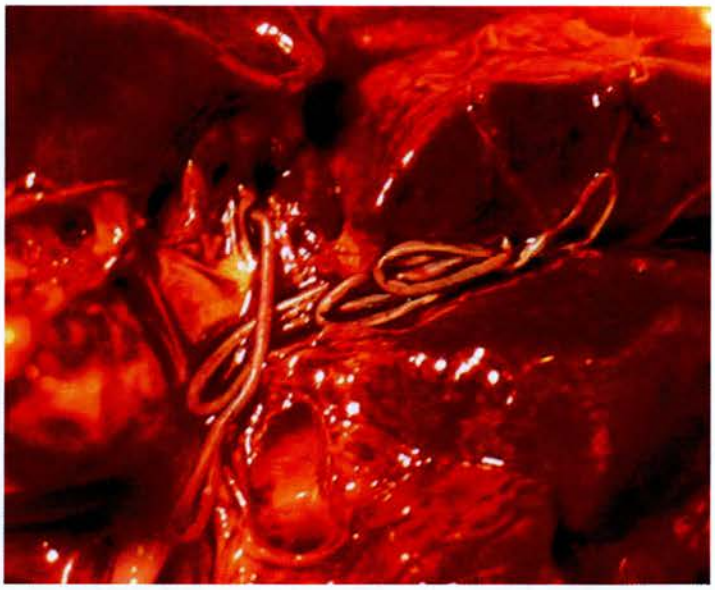
PATHOGENESIS

Heartworm disease is primarily a cardiopulmonary disease. The severity and extent of lesions depends on the number and location of adult worms. The presence of parasites in the pulmonary arteries causes proliferation of the intima, resulting in narrowing and occlusion of the vessels, which leads to pulmonary hypertension. Severe pulmonary arterial disease may cause increased permeability of the lung vessels with periarterial oedema, and interstitial and alveolar cellular infiltrate, which can result in irreversible lung fibrosis. Pulmonary thromboembolism, due to platelet aggregation induced by the parasite or in response to the death (spontaneous or induced by adulticidal treatment) of adult worms, is another possible sequela of heartworm disease.

In some severe cases, worms can migrate to the right ventricle, right atrium and caudal vena cava. This retrograde migration induces incompetence of the tricuspid valve which, in association with concurrent pulmonary hypertension, is responsible for the clinical signs of

Adult heartworms (black arrows) in the pulmonary arteries with thromboembolic lesions (white arrow). Picture, Dr Luigi Venco

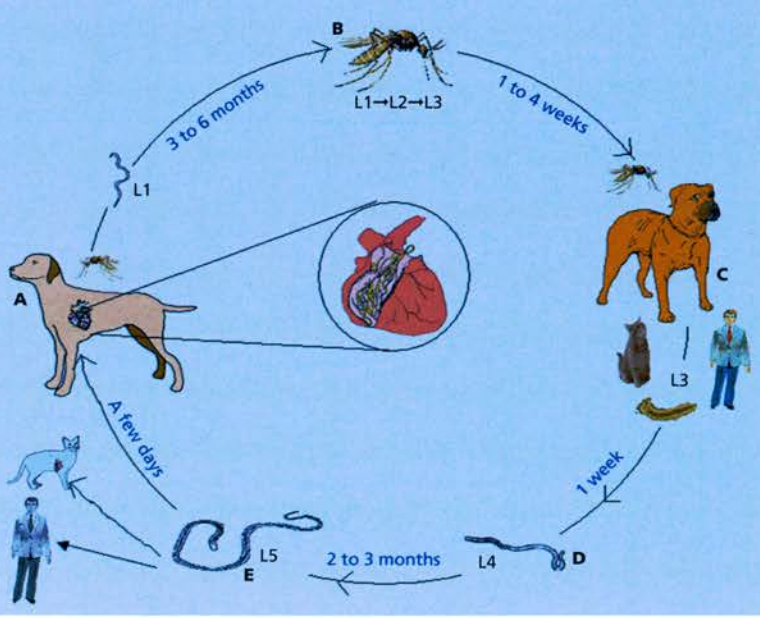




Adult heartworms in (left) the right ventricle of an infected dog and (right) the lung of an infected cat. Pictures, Dr Luigi Venco

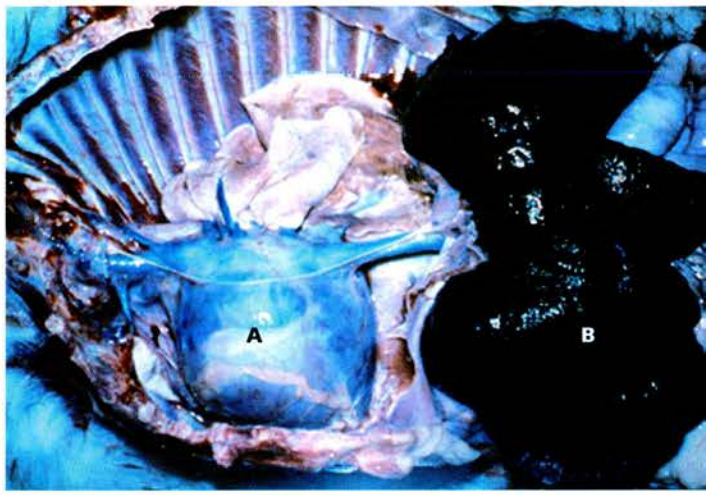
and shorter survival of the parasite in cats contributes to lower worm burdens in this species. Furthermore, microfilaraemia is uncommon in cats (occurring in less than 20 per cent of cases) and, when present, is only transitory. Therefore, cats are poor reservoirs of infection, as *D immitis* larvae are less likely to mature to adults in this species and, when present, are short-lived.

Dirofilaria infection without microfilaraemia is known as occult heartworm disease.



Life cycle of *Dirofilaria immitis*. (A) *D immitis* adult worms live in the pulmonary arteries and right ventricle of the definitive host. When sexually mature, they mate and release immature larvae (L1 or microfilariae) into the circulation. Microfilaraemia is rare in cats and humans. (B) Microfilariae can be ingested by a mosquito during a blood meal and mature in the insect to L2 and eventually the infective stage L3. (C) L3 larvae can penetrate the local connective tissues of the host during a subsequent blood meal. (D) The larvae mature from L3 to L4. (E) L4 larvae mature in the subcutaneous tissues until they reach the pre-adult stage (L5). The L5 larvae migrate to the right heart and pulmonary arteries where they mature and mate, releasing microfilariae into the circulation to complete the biological cycle. Modified from Ferasin and Knight (2004)

Dog with heartworm infection and ascites secondary to the development of right-sided heart failure. Picture, Dr Luigi Venco



Postmortem findings in a dog with caval syndrome. The right side of the heart (A) is dramatically distended and the liver (B) appears enlarged (hepatomegaly) and engorged as a consequence of blood stasis occurring secondarily to caval hypertension. Picture, Susan Shaw

right-sided heart failure (eg, jugular distension, liver congestion, ascites). In addition, red blood cell membranes may rupture as the cells flow through the mass of parasites, causing haemolysis and haemoglobinaemia. The concomitant presence of acute right-sided heart failure and intravascular haemolysis is referred to as caval syndrome. Severe cases of caval syndrome can also be characterised by the presence of adult worms in the caudal vena cava, thromboembolic events and disseminated intravascular coagulation. Caval syndrome is less common in cats due to the lighter worm burden.

Immune-mediated glomerular disease is also commonly reported in dogs with heartworm disease. It is characterised by protein-losing nephropathy, which results in proteinuria, hypoalbuminaemia and, eventually, a reduced antithrombin III plasma concentration, which may predispose the patient to the development of pulmonary thromboembolism. The antigen that causes this immune-complex disease is unknown, but could be a substance released by circulating microfilariae.

In cats, heartworm disease generally induces a diffuse pulmonary infiltrate and signs of eosinophilic pneumonia. The death of adult worms may cause acute pulmonary embolism with severe haemorrhage and oedema of the affected lobe. Occasionally, immature nematodes can migrate to sites other than the heart and pulmonary arteries, causing ectopic infection. Localisation of *D immitis* has been reported in the eye, central nervous system, systemic arteries and subcutaneous tissues. Ectopic infections are more commonly seen in cats than in dogs, suggesting that the parasite is not well adapted to feline hosts.

CLINICAL SIGNS

Heartworm disease tends to develop slowly and gradually and clinical signs are usually only present in cases with a high worm burden and/or when the host has a significant allergic response to the parasite. Frequently, clinical signs of *D immitis* infection only manifest during intense exercise and, therefore, sedentary patients may never show overt signs of heartworm disease. In dogs, coughing is the most common clinical sign, followed by tachypnoea and dyspnoea, exercise intolerance, chronic weight loss and syncope. In severe cases, haemoptysis can be present as a possible consequence of pulmonary vessel rupture. Jugular distension, hepatomegaly, ascites and marked exercise intolerance are typical signs of concurrent right-sided heart failure. In such patients, a systolic heart murmur or split-second heart sound can be heard on thoracic auscultation.

Although the majority of infected cats are asymptomatic, cases of sudden death without any premonitory clinical signs have been reported. Sometimes, the sudden death is preceded by an acute respiratory crisis, probably due to a parasitic embolism and obstruction of a major pulmonary artery. When present, clinical signs of heartworm disease in cats are generally vague and non-specific. These may include anorexia, lethargy, coughing, vomiting, dyspnoea, syncope and collapse. In some cases, the respiratory signs are very similar to those generally observed in feline asthma.

Caval syndrome represents a severe form of heartworm disease both in dogs and cats. This is characterised by respiratory distress, intravascular haemolysis and haemoglobinuria, signs of right-sided heart failure and, frequently, disseminated intravascular coagulation.

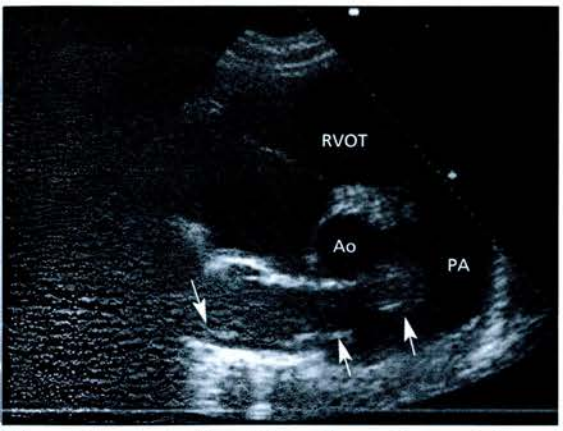
DIAGNOSIS

Diagnostic investigations are justified only if there is a previous history of exposure to mosquitoes in an area where *D immitis* is likely to be present.

CLINICAL DIAGNOSIS

Cases of heartworm disease may be accompanied by eosinophilia and basophilia, especially in the early stages of infection. Microfilariae can also occasionally be seen on examination of a blood smear. Serum biochemistry may show changes related to secondary organ involvement (eg, increased urea, creatinine, liver enzymes). However, a standard laboratory work-up is usually insufficient to make an aetiological diagnosis. A high eosinophilic population may be found on bronchoalveolar lavage, especially in cats, although differential diagnosis from feline asthma is often difficult.

Survey radiographs of the thorax may show a bulge at the level of the main pulmonary artery, enlarged and tor-



Echocardiogram of a dog with heartworm disease; right parasternal short axis view at the level of the aorta (Ao). Some adult worms are visible as hyperechoic structures (arrows) in the main pulmonary artery (PA). RVOT Right ventricular outflow tract. Picture, Dr Luca Benvenuti

Thoracic radiograph of a dog with heartworm disease. The presence of adult heartworms in the pulmonary arterial circulation causes enlarged and tortuous pulmonary arteries (arrows) and a mixed interstitial/alveolar pattern (arrowheads) affecting, in particular, the caudal lung fields. Picture, Susan Shaw

tuous pulmonary arteries and an interstitial and/or alveolar pattern. The right side of the heart may be enlarged and the caudal vena cava, hepatomegaly and ascites can be observed in cases of severe infection with caval syndrome. The vascular changes are less dramatic in cats, and radiographic abnormalities (eg, overinflation of the lung, bronchial or alveolar pattern) resemble those observed in cases of feline asthma or *Aelurostrongylus abstrusus* infection. Occasionally, adult heartworms can be visualised on echocardiography at the level of the main pulmonary artery, right ventricle and right atrium. The echogenicity of the body wall of the parasite gives rise to a double-lined hyperechoic appearance. In severe cases, echocardiography may also reveal signs of pulmonary hypertension (eg, right ventricular hypertrophy, right atrial dilation and tricuspid insufficiency).

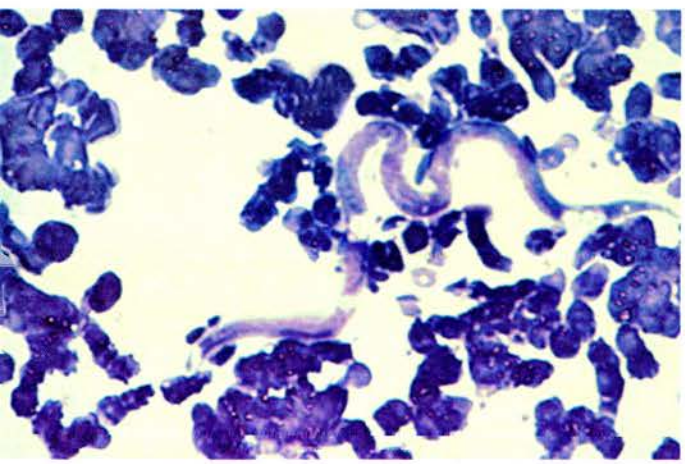
SPECIFIC TESTS

Direct microscopic examination

If present, microfilariae can be easily identified on direct microscopic examination of a drop of fresh blood because they can vigorously move the surrounding red blood cells. However, although this method offers an easy and inexpensive diagnosis, it is not sufficiently sensitive, especially when there is a low concentration of microfilariae in the bloodstream. Filtration methods (eg, Difil-test; EVSCO Pharmaceuticals, Buena, New Jersey, USA) and the modified Knott's test (haemolysis, centrifugation and staining with methylene blue) are more sensitive and allow morphological examination of the microfilariae. In cats, given the absence or short-term presence of circulating microfilariae, these tests have little practical value.

Antigen tests

ELISA antigen tests detect specific circulating proteins released by the reproductive tract of mature female worms. These are available as either in-house or laboratory tests and their sensitivity and specificity approach 100 per cent (see top table on page 354). The antigen levels become undetectable eight to 12 weeks after adulticidal therapy and this should be taken into account when re-screening for heartworm disease or evaluating the response to treatment. Small worm burdens, the presence of immature females or male-only infections are common causes of low antigen titres and false negative results, especially in cats, where these circumstances occur more frequently. In dogs, specific aggluti-



An L1 larva (microfilaria) identified using the modified Knott's test. The larva is surrounded by red blood cells and its dark colour is due to the methylene blue staining (x 100 magnification). Picture, Susan Shaw

Some in-house antigen diagnostic kits available for canine *Dirofilaria* infection. (A) SNAP Canine HW; IDEXX Laboratories; (B) SoloStep CH; Heska; (C) Witness HW; Synbiotics

SEROLOGICAL TESTS FOR THE DETECTION OF HEARTWORM ANTIGENS IN DOGS AND CATS

Test kit	*Manufacturer*	*Type*	*Mean sensitivity (%) (range)*	*Mean specificity (%) (range)*	*Species*	*Type of sample*
PetChek	IDEXX Laboratories	ELISA (microwell)	76 (66-98)	97 (90-99)	Dog	Serum, plasma
SNAP Canine HW	IDEXX Laboratories	ELISA (membrane)	67 (53-98)	98 (92-100)	Dog	Serum, plasma, whole blood
SNAP Feline HW	IDEXX Laboratories	ELISA (membrane)	73 (49-91)	99 (98-100)	Cat	Serum, plasma, whole blood
SoloStep CH	Heska	Lateral flow immunochromatography	60 (44-97)	98 (92-100)	Dog	Serum, plasma
AbboScreen	Abbott Laboratories	Lateral flow immunochromatography	52 (35-94)	96 (89-99)	Dog	Serum, plasma
DiroChek	Synbiotics	ELISA (microwell)	71 (59-98)	94 (86-97)	Dog	Serum, plasma
DiroChek	Synbiotics	ELISA (microwell)	79 (54-94)	98 (96-99)	Cat	Serum, plasma
Witness HW	Synbiotics	Lateral flow immunochromatography	71 (69-92)	94	Dog	Serum, plasma, whole blood
VetRed*	Synbiotics	Haemoagglutination	65-94	?	Dog	Whole blood

*No longer available, ? Published data not available
From Ferasin and Knight (2004)

nation and immunochromatography techniques are also available. An antigen test performed too soon after infection (first six to seven months) may yield false negative results because of the low antigen levels in the circulating blood.

Antibody tests

Antibody tests are currently available for routine screening of feline heartworm infection, either as in-house or laboratory tests (see table below). The presence of antibodies provides information about previous exposure, but not necessarily current infection. Consequently, antibody tests are more useful to help rule out infection rather than confirm it. These tests are no longer used in dogs given their low specificity and the availability of highly reliable antigen tests.

PCR-based tests

Polymerase chain reaction (PCR)-based tests offer a very sensitive and specific diagnostic tool for routine identification of mature and immature adult worms, especially in unconventional hosts. However, at present, these tests are not widely available.

TREATMENT

ADULTICIDAL TREATMENT

The decision as to whether or not to treat a dog with heartworm disease and the relative prognosis depend on the severity of the infection. In an attempt to assess this, animals may be classified as:

- CLASS 1. Patients with subclinical or no clinical signs;
- CLASS 2. Patients with mild to moderate clinical signs and mild to moderate radiographic changes;
- CLASS 3. Patients with severe clinical signs (eg, persistent cough, haemoptysis, dyspnoea, right-sided heart failure) and severe radiographic changes.

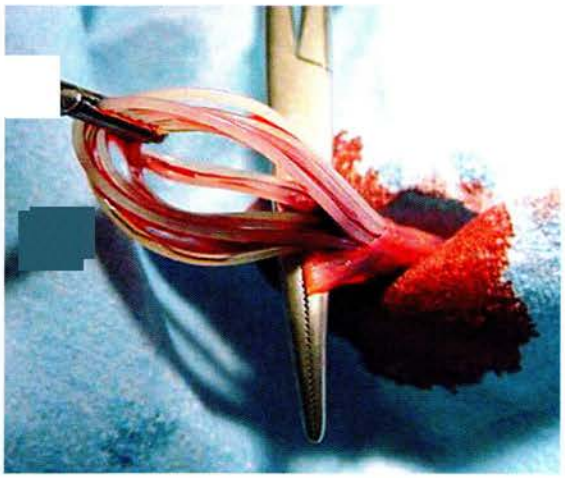
The prognosis for dogs in class 1 following adulticidal treatment is generally very good. Dogs in class 2 may have a positive outcome. Animals in class 3 have a guarded prognosis due to the high risk of pulmonary thromboembolism; in these cases, the potential complications of adulticidal treatment should be thoroughly discussed with the owners.

In cats, adulticidal treatment may be dangerous even in patients with low grade infection, and pulmonary thromboembolism due to premature death of parasites

SEROLOGICAL TESTS FOR THE DETECTION OF HEARTWORM ANTIBODIES IN CATS

Test kit	*Manufacturer*	*Type*	*Mean sensitivity (%) (range)*	*Mean specificity (%) (range)*	*Type of sample*
SoloStep FH (5 minutes incubation)	Heska	ELISA (membrane)	32 (13-57)	99 (97-100)	Serum, plasma, whole blood
SoloStep FH (10 minutes incubation)	Heska	ELISA (membrane)	47 (24-71)	94 (91-97)	Serum, plasma whole blood
SoloStep FH (20 minutes incubation)	Heska	ELISA (membrane)	84 (60-97)	85 (81-89)	Serum, plasma, whole blood
Assure FH	Synbiotics	ELISA (wand)	68 (43-87)	93 (89-95)	Serum, plasma
Witness HW	Synbiotics	Lateral flow immunochromatography	?	?	Serum, plasma, whole blood

? Published data not available
From Ferasin and Knight (2004)



Adult heartworms can be removed from the right side of the heart and pulmonary artery using flexible crocodile retrieval forceps inserted through a jugular vein. Picture, Dr Luigi Venco

is a common complication. Considering that some cats may undergo spontaneous clinical remission if parasites die naturally, periodic monitoring of the patient is more appropriate than adulticidal treatment. Cats with respiratory signs can be treated symptomatically in the same way as patients with feline asthma. Cage rest, oxygen supplementation, fluid therapy, bronchodilators and injectable steroids (eg, dexamethasone) can be used to stabilise acutely ill cats. Adulticidal treatment should only be considered for cats in a stable condition that fail to respond to supportive treatments.

Adulticidal treatment involves the administration of a new generation arsenical compound, melarsomine dihydrochloride (Immiticide, Merial; this product is not licensed for use in the UK). Melarsomine is less nephrotoxic and hepatotoxic than its predecessor thiacetarsamide, and has a higher efficacy. Melarsomine is injected intramuscularly into the lumbar muscles at a recommended dose of 2·5 mg/kg, repeated after 24 hours. However, only a single dose should be administered to class 3 dogs, to kill just a proportion of worms and hence minimise the risk of pulmonary thromboembolism. If the patient remains stable, the standard adulticidal protocol can be administered one month later. In the week following the administration of melarsomine, the likelihood of pulmonary thromboembolism can be minimised with cage rest and corticosteroids at anti-inflammatory doses.

If adulticidal treatment is declined by the owner, monthly administration of prophylactic doses of ivermectin may represent a reasonable option because it will prevent further infection and may kill some adult nematodes.

Patients with severe caval syndrome may benefit from physical removal of worms from the right side of the heart and the main pulmonary artery using flexible crocodile or basket-type retrieval forceps. This procedure is complex, requires general anaesthesia and fluoroscopic imaging, but may reduce the risk of thromboembolism following subsequent adulticidal treatment.

MICROFILARICIDAL TREATMENT

Circulating microfilariae should be eliminated four to six weeks after successful adulticidal treatment. There are no approved drugs for microfilaricidal treatment. However, a single administration of ivermectin (50 µg/kg) or milbemycin oxime (500 µg/kg) has been shown to be highly effective in eliminating microfilariae from the circulation within a few hours. Moxidectin and selamectin are also known to be potent microfilaricides but, at present, there is little experience of their use in the clinical setting. Oral prednisolone (1 to 2 mg/kg) administered with microfilaricidal drugs may control anaphylactic reactions which may occur secondarily to the sudden death of a large number of microfilariae.

PREVENTION

Prophylaxis of heartworm disease should be considered for all dogs and cats living in endemic areas during the transmission period. For example, in northern Italy, where a high prevalence of heartworm disease has been reported, mosquitoes are only present during the summer and, therefore, prophylaxis is recommended from May to October.

The currently approved prophylactic drugs are listed in the table below. However, only milbemycin oxime (Program Plus; Novartis) and selamectin (Stronghold;

CHEMOPROPHYLACTIC DRUGS AVAILABLE FOR THE PREVENTION OF HEARTWORM DISEASE

Tradename	*Manufacturer*	*Active ingredient*	*Species*	*Recommended administration*	*Route of administration*
Heartgard (Cardotek)	Merial	Ivermectin	Dog, cat	Monthly	Oral (chewable tablets)
Heartgard Plus (Cardotek Plus)	Merial	Ivermectin (+pyrantel)	Dog	Monthly	Oral (chewable tablets)
Advantage Duo	Bayer	Ivermectin (+imidacloprid)	Dog	Monthly	Topical ('spot-on')
Interceptor	Novartis	Milbemycin oxime	Dog	Monthly	Oral (flavoured tablets)
Program Plus* (Sentinel)	Novartis	Milbemycin oxime (+iufenuron)	Dog	Monthly	Oral (flavoured tablets)
Proheart (Guardian)	Fort Dodge	Moxidectin	Dog	Monthly	Oral
Proheart 6 (Guardian 6)	Fort Dodge	Moxidectin microspheres	Dog	Six-monthly	Injectable
Stronghold* (Revolution)	Pfizer	Selamectin	Dog, cat	Monthly	Topical ('spot on')
Filaribits Plus	Pfizer	Diethylcarbamazine citrate (+oxibendazole)	Dog	Daily	Oral

*Licensed in the UK for the prevention of heartworm
From Ferasin and Knight (2004)

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Pets travelling from the UK

When pets travel from the UK to areas where heartworm disease has been reported, prophylactic intervention should be considered. If a dog or cat has been exposed to mosquitoes in an endemic area for less than one month, a single administration of a prophylactic agent immediately after its return to the UK should be sufficient to prevent the disease. Conversely, for longer stays in endemic areas, owners should administer the drug on a monthly basis, starting within a month of their departure.

Subcutaneous dirofilariasis

Subcutaneous dirofilariasis is primarily caused by *Dirofilaria (Nochtiella) repens*, which parasitises domestic dogs in Europe (especially Italy, Spain, Greece, the former Yugoslavia and France), Africa and Asia, and bears in the USA and Canada. *D repens* is also transmitted by a variety of mosquito species and its biological cycle is very similar to that of *D immitis* except that adult worms live in subcutaneous tissues. The clinical significance of adult worms in dogs and cats has not been clearly defined, but nodular skin lesions, abscesses and immunological reactions have been described. Treatment is achieved by surgical removal of adult worms from the skin lesions. Although there are no drugs currently licensed for the prevention of *D repens* infection, prophylaxis can be achieved using the same chemoprophylactic regimen used for *D immitis*.

Human dirofilariasis

Although humans are considered incidental hosts, *D immitis* and *D repens* infections have been reported in people and represent an important zoonotic risk, especially in endemic areas. In recent studies conducted in southern Europe, a high prevalence (26 to 37 per cent) of clinically healthy people were found to be positive for antibodies against *D immitis*. *D immitis* may cause pulmonary injuries in humans when an immature parasite dies after having entered the right ventricle, causing embolisation and small lung infarctions, which may appear as solitary nodules on thoracic radiography. *D repens* is occasionally found in people in various body locations including the conjunctiva, eyelids, scrotum, inguinal area, breasts, arms and legs. The diagnosis of human dirofilariasis depends mainly on microscopic evaluation of the morphological characteristics of the nematode in histopathological specimens.

Pfizer) are licensed in the UK for the prevention of heartworm disease. Preventive drugs kill migrating larvae of *D immitis* up to the sixth week of infection. Therefore, they all provide a high degree of protection when administered on a monthly basis. If a patient misses one or more doses in the prophylactic schedule, it should be tested for heartworm disease after six months. The risk of incomplete protection due to owner non-compliance can be eliminated with a recently introduced sustained-release injectable formulation of moxidectin (Proheart 6; Fort Dodge).

High doses of ivermectin and milbemycin oxime are potentially toxic in approximately one-third of collies. However, side effects are not observed in these breeds when prophylactic drugs are administered at the recommended doses.

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Further reading

COURTNEY, C. H. & ZENG, Q. Y. (2001) Comparison of heartworm antigen test kit performance in dogs having low heartworm burdens. *Veterinary Parasitology* **96**, 317-322

EXECUTIVE BOARD OF THE AMERICAN HEARTWORM SOCIETY (2004) Guidelines for the diagnosis, prevention and management of heartworm (*Dirofilaria immitis*) infection in dogs and cats. http://heartwormsociety.org

FERASIN, L. (2002) Dirofilariasis. In Proceedings of the 45th British Small Animal Veterinary Association Annual Congress. Birmingham, April 4 to 7. pp 190-192

FERASIN, L. & KNIGHT, D. (2004) Filarial infections. In Arthropod borne Infectious Diseases of the Dog and Cat. Eds S. E. Shaw and M. J. Day. London, Manson Publishing (In press)

KNAUER, K. W. (1998) Human dirofilariasis. *Clinical Techniques in Small Animal Practice* **13**, 96-98

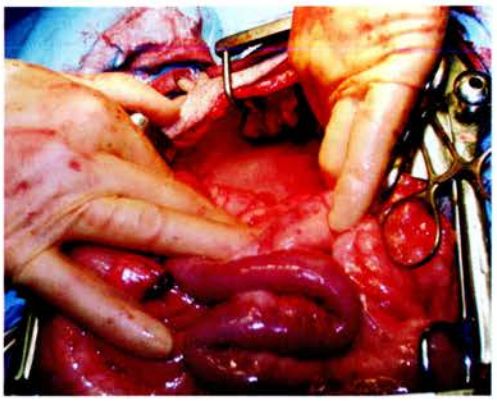
KNIGHT, D. H. & LOK, J. B. (1998) Seasonality of heartworm infection and implications for chemoprophylaxis. *Clinical Techniques in Small Animal Practice* **13**, 77-82

MILLER, M. W. (1998) Feline dirofilariasis. *Clinical Techniques in Small Animal Practice* **13**, 99-109

SIMON, F., PRIETO, G., MORCHÓN, R., BAZZOCCHI, C., BANDI, C. & GENCHI, C. (2003) Immunoglobulin G antibodies against the endosymbionts of filarial nematodes (*Wolbachia*) in patients with pulmonary dirofilariasis. *Clinical and Diagnostic Laboratory Immunology* **10**, 180-181

SNYDER, P. S., LEVY, J. K., SALUTE, M. E., GORMAN, S. P., KUBILIS, P. S., SMAIL, P. W. & GEORGE, L. L. (2000) Performance of serologic tests used to detect heartworm infection in cats. *Journal of the American Veterinary Medical Association* **216**, 693-700

STRICKLAND, K. N. (1998) Canine and feline caval syndrome. *Clinical Techniques in Small Animal Practice* **13**, 88-95



Collection of purulent fluid within the abdominal cavity due to ruptured pyometra. Turbid fluid is suggestive of bacterial peritonitis

Management of peritonitis in dogs and cats

YVONNE McGROTTY AND ROSS DOUST

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PERITONITIS is localised or diffuse inflammation of the peritoneal cavity and is most commonly associated with bacterial contamination from the gastrointestinal tract. Mortality associated with this condition is high, and rapid diagnosis and early surgical and/or medical intervention is essential to optimise the outcome. This article describes the diagnostic tests, and various medical and surgical options available for the treatment of peritonitis in small animals, with emphasis on the management of bacterial (septic) peritonitis and uroperitoneum (urine peritonitis). In addition, it discusses the factors that influence the prognosis and outcome of affected cases.

CLASSIFICATION OF PERITONITIS

Peritonitis is the severe and often fatal inflammation of the peritoneal lining and abdominal viscera and can be classified as either primary or secondary. Primary generalised peritonitis occurs as a result of haematogenous spread of an infectious agent to the peritoneum. Secondary generalised peritonitis results from direct bacterial or chemical contamination of the abdominal cavity and is a more common presentation in dogs (see box below). Frequent causes of secondary peritonitis include penetrating abdominal wounds, rupture of a hollow viscus or surgical contamination. Secondary peritonitis can be further classified as either septic or aseptic. Septic causes are often associated with rupture or dehiscence of surgical wounds of the gastrointestinal tract and, as a result, Gram-negative bacteria are commonly isolated from the peritoneal cavity in such cases. Aseptic causes include pancreatitis, neoplasia and sterile foreign bodies (eg, surgical swabs).

Chemical peritonitis results from leakage of bile, urine or pancreatic secretions into the abdominal cavity. Abdominal trauma or necrotising cholecystitis can lead to rupture of the biliary system and subsequent leakage of toxic bile salts into the peritoneal cavity. Uroperitoneum is usually mild, unless bacterial contamination is present; however, electrolyte abnormalities (hyperkalaemia) associated with uroperitoneum can cause severe clinical signs. Release of pancreatic enzymes causes severe peritonitis and initiates a systemic inflammatory response.

Causes of secondary peritonitis

Aseptic

- Iatrogenic (eg, surgical swabs, talc)
- Blood
- Urine
- Bile
- Neoplasia
- Pancreatitis
- Sclerosing encapsulating peritonitis

Septic

- Leakage of gastrointestinal tract contents (rupture, dehiscence)
- Urinary tract rupture (with bacterial infection)
- Reproductive tract rupture (pyometra)
- Biliary tract rupture (with bacterial infection)
- Penetrating abdominal wounds
- Iatrogenic (bacteria introduced during surgery)

DIAGNOSIS

Clinical signs associated with peritonitis can be vague and non-specific. Haematology results can be variable. However, in some cases, serum biochemistry may help to identify the type of peritonitis present. Bile peritonitis results in increased concentrations of total bilirubin and liver enzymes, while uroperitoneum is associated with increased urea, creatinine and potassium concentrations. Serum protein concentrations are often decreased in cases of bacterial peritonitis and also in cases of aseptic peritonitis as a result of protein exuding into abdominal fluid.

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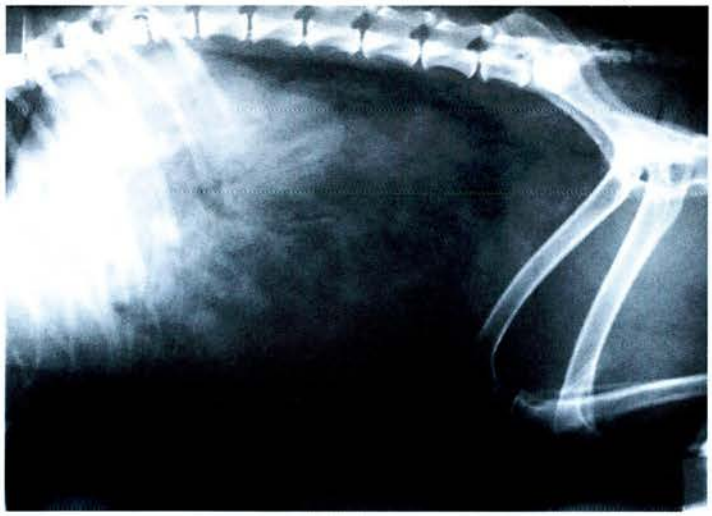


Lateral abdominal radiograph demonstrating loss of contrast due to free abdominal fluid

Lateral radiograph showing increased abdominal contrast due to free abdominal air

Abdominal radiographs may demonstrate a loss of contrast due to the presence of an effusion. Free abdominal gas is suggestive of gastrointestinal tract rupture or penetrating abdominal injury and is always an abnormal finding unless the animal has had recent abdominal surgery or abdominocentesis using an open needle. Free abdominal air can be identified on radiographs for 14 days or occasionally longer following abdominal surgery.

Abdominocentesis is a very useful tool for the diagnosis of peritonitis (see box on the right), but can produce negative results in early cases or in those with localised peritonitis. Approximately 5 to 6 ml/kg of abdominal fluid is required to produce a positive abdominal tap. The peritoneal cavity of healthy animals contains <1 ml/kg of clear yellow transudate which reduces friction between the abdominal organs. Abdominal ultrasound is a more sensitive means of detecting smaller volumes of peritoneal fluid and is used to identify an appropriate site for abdominocentesis in cases with localised peritonitis. Alternatively, diagnostic peritoneal lavage can be performed if abdominocentesis is negative, but there is a high index of suspicion for peritonitis (see box on the right).

Turbid fluid is suggestive of septic peritonitis. Green fluid suggests bile peritonitis and a significant volume of clear yellow fluid may indicate urine leakage. The fluid can be classified as transudate, modified transudate or exudate using the criteria outlined in the table on the right. Total solids can be measured using a refractometer and give an estimate of the protein content of the fluid. All fluid samples require further cytological and bacteriological analysis.

Abdominocentesis

- Clip and surgically prepare the skin around the umbilicus over the ventral midline of the abdomen
- Insert an 18 to 20 gauge needle or intravenous catheter just caudal and lateral to the umbilicus at a 30° to 40° angle
- A four-quadrant tap is recommended (cranial and caudal sites on both the left and right sides of the midline). Care should be taken to avoid the spleen and bladder
- Allow fluid to drain freely from the needle into a sterile container (EDTA tubes for cytology and cell counts, fluoride oxalate tubes for glucose, and plain tubes for biochemistry and bacteriology)

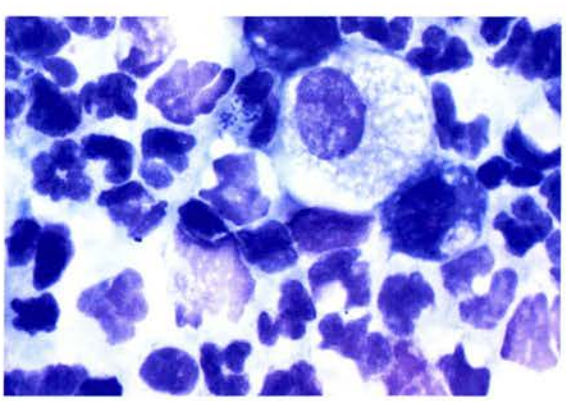
Diagnostic peritoneal lavage

If no fluid is obtained using the above technique, diagnostic peritoneal lavage is carried out as follows:

- Infuse 22 ml/kg of lactated Ringer's solution (warmed to body temperature) into the abdomen via the needle/catheter
- Gently rock the animal to encourage distribution of the fluid within the abdominal cavity
- Collect 10 to 20 ml of fluid from the needle/catheter by gravity flow

CLASSIFICATION OF FLUIDS

	Total protein (g/litre)	*Cell count ($x10^9$/litre)*	*Cell types*	*Possible cause(s)*
True transudate	<25	<1	Mononuclear	Hypoproteinaemia
Modified transudate	25-50	0·5-10	Lymphocytes, monocytes, red blood cells, neutrophils	Congestive heart failure Portal hypertension Neoplasia
Exudate	>30	>5	Neutrophils, mononuclear, red blood cells	Peritonitis



Septic abdominal exudate demonstrating toxic neutrophils with intracellular bacteria

Bacterial peritonitis may be diagnosed by demonstrating the presence of degenerate or toxic neutrophils with intracellular bacteria in the peritoneal fluid and this finding warrants surgical exploration of the abdomen. An abdominal fluid glucose concentration of <2·80 mmol/litre is consistent with a septic effusion. However, a difference of >1·12 mmol/litre between the concentration of glucose in the blood and abdominal fluid is considered a more reliable indicator of a septic effusion than abdominal fluid glucose alone (Boncynski and others 2003).

Indications for exploratory abdominal surgery

- Intracellular bacteria identified in peritoneal fluid
- Penetrating abdominal injury
- Vegetable fibres in peritoneal fluid
- Peritoneal fluid creatinine concentration >serum creatinine concentration
- Peritoneal fluid potassium concentration >serum potassium concentration
- Peritoneal fluid bilirubin concentration >serum bilirubin concentration
- Free air in abdominal cavity (normal for up to 14 days following abdominal surgery)
- Haemoabdomen unresponsive to medical management (ie, continued bleeding despite medical intervention and exclusion of coagulopathies)

Uroperitoneum can be confirmed by demonstrating higher concentrations of creatinine and potassium in the effusion than serum concentrations obtained at the same time. If urinary tract rupture is suspected, contrast studies should be performed to identify the site of the rupture prior to surgical correction. Measures to reduce serum potassium levels should be implemented before anaesthesia and surgery are considered (see later), as hyperkalaemia has dramatic cardiovascular effects.

Bile peritonitis is confirmed by demonstrating higher concentrations of bilirubin in the abdominal fluid than in the serum. Increased amylase and lipase concentrations in abdominal fluid compared to serum are suggestive of pancreatitis, but pancreatitis often occurs in conjunction with other types of peritonitis.

Surgical exploration of the abdominal cavity is warranted in many cases of peritonitis in order to identify and correct the underlying cause (see box above).

MANAGEMENT OF UROPERITONEUM

Uroperitoneum occurs following rupture of the urinary tract (eg, trauma, bladder obstruction) and often results in severe electrolyte abnormalities. Hyperkalaemia associated with uroperitoneum can have profound cardiovascular consequences and must be resolved prior to anaesthesia and surgical intervention. Treatment of severe, life-threatening hyperkalaemia is outlined in the box below.

Rupture occurs more frequently in the lower urinary tract (bladder and urethra). A positive contrast retrograde urethrogram (vaginourethrogram) is obtained to identify a rupture in this area (see page 365). A non-ionic, water soluble contrast agent should be used to minimise exacerbation of the chemical peritonitis. In cases where the retrograde study is inconclusive, an intravenous urogram can be performed to identify upper urinary tract (kidney or ureter) rupture. If no site of rupture can be identified with these studies and a diagnosis of uroabdomen has been confirmed, it may be that a small rupture has occurred, and has already healed. In these cases, exploratory surgery may not be necessary and treatment with urinary diversion and intravenous fluid therapy for five to seven days may be more appropriate.

Management of hyperkalaemia

- Sodium chloride 0·9 per cent (up to 40 to 60 ml/kg, rapid intravenous infusion)
- Calcium gluconate 10 per cent (0·5 to 1·0 ml/kg, slow intravenous infusion)
- Glucose 50 per cent (1 to 2 ml/kg intravenously)
- Regular insulin (1 unit/kg intravenously) and glucose (2 g/unit of insulin intravenously)
- Sodium bicarbonate 8·4 per cent (0·5 to 1 ml/kg, slow intravenous infusion)

Note that not all of the above measures will be required. Most cases respond to a combination of intravenous fluids and glucose. Calcium gluconate reduces the effects of hyperkalaemia on the myocardium while other methods aimed at reducing potassium take effect.

Surgical technique

For lower urinary tract rupture, an indwelling urinary catheter is placed to remove urine and bypass the area of leakage. Urethral leaks, for example, are first treated with urinary diversion for seven to 10 days. If the urine is leaking from the upper urinary tract (ie, the kidneys or ureters), it should be removed from the abdomen by abdominocentesis three to four times a day, depending on the rate of accumulation. With effective urine diversion, hyperkalaemia should resolve within 12 to 24 hours with aggressive fluid therapy alone.

Once the site of leakage has been confirmed, a midline coeliotomy is performed to repair the bladder, urethra or ureter. In cases where a ruptured ureter cannot be repaired, the options are either to re-implant the ureter in the bladder wall or, as a last resort, to perform a ureteronephrectomy. If healing of a urethral leak does not occur following urinary diversion, repair of the urethra may be attempted or, if the damage is too severe, a urethrostomy may be performed (perineal or prepubic, depending on the length of viable urethra proximally).

Bladder wall ruptures are repaired by routine debridement of any tissue of questionable viability and closure of the resultant cystotomy incision in a single or double layer with simple interrupted or continuous appositional suture patterns. Care must be taken around the dorsal-caudal aspect and at the site of insertion of the ureters, but otherwise debridement should be aggressive. The abdomen should be thoroughly lavaged prior to closure.

MANAGEMENT OF BACTERIAL PERITONITIS

INITIAL TREATMENT

Almost all patients presenting with bacterial peritonitis will be hypovolaemic as a result of fluid and protein exudation into the abdomen. As a result, most patients are poor candidates for anaesthesia and surgery and require aggressive fluid therapy to restore cardiovascular stability prior to definitive surgical treatment.

Fluid therapy

Shock rates of crystalloids (up to 90 ml/kg/hour in the dog and 60 ml/kg/hour in the cat) are often required initially; care should be taken in cases with haemoabdomen as the increase in blood pressure may result in further haemorrhage (hypotensive resuscitation to a mean arterial pressure of around 60 mmHg is less likely to cause bleeding). Bolus crystalloid therapy (10 to 20 ml/kg over 20 minutes) may also be used and may be safer as it is less likely to result in volume overload. Additional boluses can be administered depending on the clinical response. The use of natural or synthetic colloids (eg, hydroxyethyl starches) may be beneficial. Most patients with a septic effusion will be hypoproteinaemic as a

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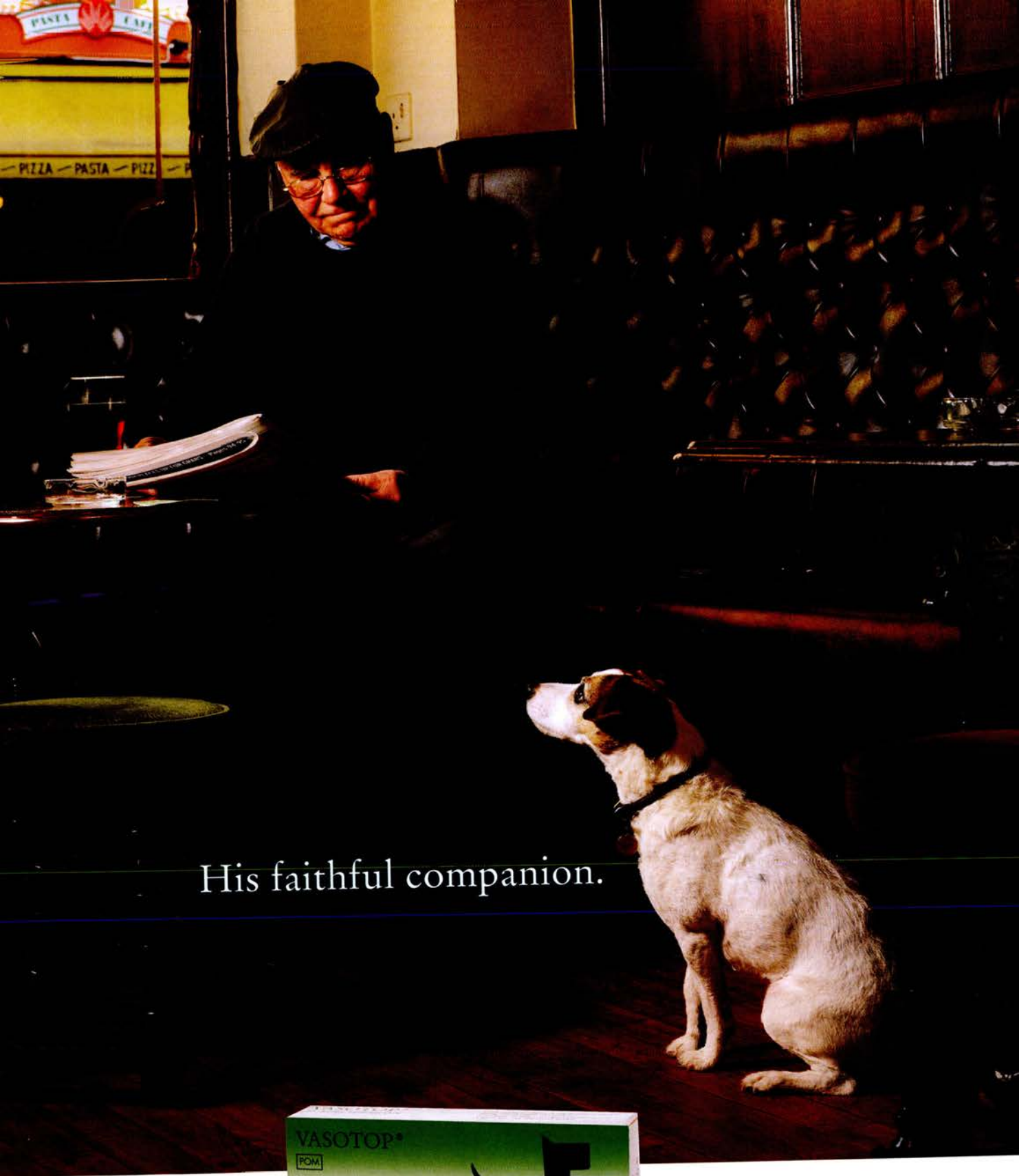

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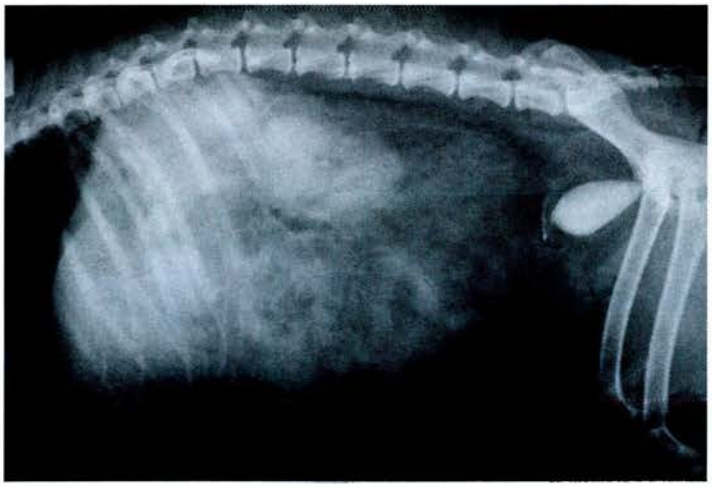

Positive contrast cystogram demonstrating leakage of contrast

Close-up view of the image on the left showing leakage of contrast

result of protein loss into the abdominal fluid. High fluid rates and volumes in hypoproteinaemic patients can lead to oedema as a result of decreased oncotic pressure and so synthetic or natural colloids are often indicated. Excessive volumes of colloids can lead to volume overload, coagulopathies and haemodilution.

Antimicrobials

An empirical choice of broad-spectrum antimicrobials should be administered systemically once a diagnosis of bacterial peritonitis is confirmed. A combination of antimicrobials with activity against Gram-negative, Gram-positive and anaerobic bacteria should be administered intravenously while awaiting the results of culture and sensitivity testing of the peritoneal fluid (see box, above right). The most common isolates from cases of bacterial peritonitis are *Escherichia coli*, and *Clostridium* species which reflect the high incidence of gastrointestinal rupture. *E coli* is the most common isolate in cases with bile peritonitis.

Heparin therapy

The use of heparin therapy to reduce fibrin formation is controversial. Heparin was shown to improve survival in dogs with experimental peritonitis (Gupta and Jain 1985).

SURGICAL TREATMENT

Aims

The main aims of surgical treatment of bacterial peritonitis are to:

- Remove the underlying source of infection;
- Reduce the bacterial population within the peritoneal cavity;
- Remove foreign material, and the cells and mediators of inflammation;
- Prevent recurrence.

Surgical exploration of the abdomen is required to identify and remove the source of infection in all cases of bacterial peritonitis.

Surgical technique

Delayed wound healing and an increased incidence of suture line disruption may occur in cases of septic peritonitis and, hence, surgical techniques and material selection may need to be modified. Suture materials that provide prolonged support are recommended, and non-absorbable materials may be considered. Monofilament suture materials are preferred as bacteria may colonise the interstices of braided materials and form colonies that are protected from the immune system. Catgut is definitely contraindicated in cases of septic inflammation as it degrades more rapidly than normal. Simple interrupted appositional suture patterns should be used. Serosal patching may be considered to prevent potential leakage from a hollow viscus in areas where an omental patch would be appropriate in the non-inflamed abdomen.

A midline coeliotomy from the xiphoid to the pubis is mandatory in all cases to allow for a complete examination of the abdomen. Effusion is removed by suction and the abdomen is explored thoroughly to identify the site(s) of contamination. Exploration may be performed by dividing the abdomen into specific regions (quadrants) or, more commonly, by examination of the individual organs, including the diaphragm and body wall. The examination is completed even if a lesion is identified and then the underlying cause is corrected.

Necrotic and fibrinous areas are debrided and the abdomen thoroughly lavaged with copious volumes of warm, sterile isotonic fluid prior to closure. Initially, a volume of 200 to 300 ml/kg is recommended, but lavage should continue until the return fluid is clear. As much fluid as possible should be removed before closure. Debridement and lavage decreases the levels of intra-abdominal bacteria, foreign material contaminants, inflammatory mediators and peritoneal fibrin. Lavage fluid left in the abdomen impairs immune function by reducing chemotaxis of inflammatory cells and also prevents antibiotics reaching effective concentrations.

A number of approaches have been described for the management of bacterial peritonitis in small animals following surgical correction of the underlying cause (see box on the right).

Antimicrobial combinations

- Amoxycillin/clavulanic acid (20 mg/kg intravenously every eight hours) + metronidazole (10 mg/kg intravenously every 12 hours)
- Cefuroxime (20 mg/kg intravenously every eight hours) + metronidazole (10 mg/kg intravenously every 12 hours)
- Ampicillin (22 mg/kg intravenously every eight hours) + fluoroquinolone (5 mg/kg intravenously every 24 hours). This combination is not licensed for intravenous use

Methods used to manage peritonitis

- Open peritoneal drainage
- Sump-Penrose drainage
- Closed suction drainage
- Peritoneal lavage

OPEN PERITONEAL DRAINAGE

Open peritoneal drainage treats the abdomen as an abscess and helps to remove bacteria, foreign material, and inflammatory cells and mediators. The procedure is indicated where there is severe systemic illness, ineffective drainage with conventional techniques or extensive suppuration or necrosis of the deep layers of the abdominal incision. Although in many cases, open peritoneal drainage is considered to be a superior form of treatment for severe peritonitis, it should only be attempted where adequate 24-hour monitoring and intensive care facilities are available. In addition, due to the high incidence of complications associated with the technique (see box below) there should be provision for synthetic and natural colloid administration.

Surgical technique

The abdomen is surgically explored and the cause of the peritonitis surgically corrected. Copious amounts of warmed saline should be used to lavage the abdominal cavity and then removed by suction. The midline abdominal incision is divided into cranial and caudal segments. The falciform fat is removed to allow for more effective drainage. Closure of the caudal segment of the wound is routine, with apposition of the external abdominal muscular fascia, subcutaneous fat and skin. The cranial half to two-thirds of the wound is loosely sutured with a simple continuous or simple interrupted suture pattern, leaving a gap between the wound edges. The gap between the sutures should be sufficient to allow a finger to be inserted into the abdomen.

The open wound is covered with sterile gauze followed by a sterile disposable nappy, which is secured in place with gauze bandages. Male dogs are catheterised to prevent urinary contamination of the open wound. Sterile dressings are changed at least every four to six hours initially or as soon as there is fluid strikethrough. Further dressing changes are performed once daily as a minimum, or more frequently if the dressing becomes saturated. Soiled dressings are weighed to monitor fluid loss from

Complications associated with open peritoneal drainage

- Hypoproteinaemia
- Hypokalaemia
- Fluid losses via the wound
- Herniation of abdominal contents
- Nosocomial infection
- Adhesion formation

Criteria for secondary closure

- Decreased volume of fluid draining from the abdominal wound
- Absence of intracellular bacteria in the abdominal fluid
- Reduced cellularity of the abdominal fluid

Drainage may continue for one to 14 days (average four days). If intracellular bacteria are still present after five days, re-exploration of the abdomen is indicated.

Gaps are left between sutures to allow a finger to be inserted into the abdomen to aid adhesion break down

The wound for open peritoneal drainage is covered with sterile gauze (above) and then by a sterile nappy (right)

the open wound. The animal is encouraged to stand during dressing changes and a sterile drape is placed underneath in case herniation of abdominal contents should occur. Herniated guts are gently pushed back into the abdomen. Repeated extensive herniations may indicate that a repeat operation is required and that the size of the opening should be reduced. Adhesions are broken down once daily by inserting a sterile gloved hand between the sutures. Opioid analgesics (eg, pethidine, morphine) are administered before adhesion break down is attempted.

An alternative technique is to place suture loops in the cranial muscular fascia so that a single length of umbilical tape can be threaded through the loops for loose closure, in a bootlace pattern with the incision edges separated for drainage. The animal is anaesthetised once or twice daily for dressing changes; the tape is removed, the abdomen thoroughly lavaged (as above) and a fresh tape replaced.

POSTOPERATIVE MONITORING

All animals undergoing open peritoneal drainage require 24-hour monitoring and intensive nursing. Monitoring of patients with bacterial peritonitis managed using open peritoneal drainage should include paying close attention to the following parameters:

- Vital signs (temperature, pulse, respiration, mucous membrane colour, capillary refill time);
- Urine output;
- Packed cell volume/total solids;
- Electrolytes;
- Albumin;
- Arterial blood pressure;
- Bodyweight and nutritional status;
- +/- Central venous pressure.

In addition, adequate analgesia should be provided using systemic opioid analgesics or epidural anaesthesia.

ABDOMINAL DRAINAGE

A variety of different drain materials and designs have been used in veterinary and human medicine. Drains are used in humans to maintain drainage of intra-abdominal abscesses, to control a fistula that cannot be exteriorised or for continuous postoperative lavage. Most drains do not effectively drain the entire cavity and are susceptible to blockage of the lumen or exit site by omentum; furthermore, nosocomial infection is a significant risk in affected animals.

The two drains used most successfully in small animals are the triple lumen sump drain and the Parker peritoneal dialysis catheter, both of which are available commerically. Alternatively, triple lumen sump drains may be constructed from a Penrose drain and a Foley catheter. The tip of the Foley catheter, including the balloon, is cut off and the tube fenestrated. Fenestrations are made along the length of the Penrose drain, which is placed over the Foley catheter and secured with an encircling suture at the external end.

Drains are placed along the ventral abdominal wall, and sutured to the abdominal wall using a Chinese finger trap pattern. Triple sump drains are attached to a closed suction system that allows continuous suction of the cavity. Air is allowed into the abdominal cavity, via the former balloon lumen, to facilitate drainage. Indwelling drains allow the infusion of lavage fluid on a regular basis, although in an abdominal cavity with fibrinous adhesions the lavage fluid may not be distributed throughout the entire cavity. Omentectomy may reduce problems with drain obstruction, but may potentiate infection. An alternative is to surgically secure the omentum onto areas of potential leakage away from the drain.

There is no evidence that the postoperative complications associated with open peritoneal drainage (with the exception of herniation) are reduced by the use of drains.

NUTRITIONAL SUPPORT

Nutritional support is essential in all cases of peritonitis as these animals are usually catabolic as a result of sepsis, hypoproteinaemia and a reduced nutritional intake. The use of naso-oesophageal, oesophagostomy, gastrostomy or jejunostomy tubes is encouraged. Enteral feeding stimulates the gut and can help prevent paralytic ileus associated with abdominal surgery and infection. Parenteral nutrition can be used in cases where enteral nutrition is not possible, although this approach is associated with higher complication rates.

SUMMARY

Peritonitis, regardless of the underlying cause, is associated with a high mortality rate. Irrespective of whether patients are managed medically or surgically, a high standard of perioperative care is vital to optimise the outcome. In most cases, 24-hour monitoring is essential along with access to natural and synthetic colloids for cases of septic peritonitis. If open peritoneal drainage is considered an appropriate therapeutic option, the clinician is encouraged to refer the patient to a hospital that has adequate facilities for perioperative monitoring and intensive care.

Gauze bandages are placed to secure the sterile gauze and nappy used to cover the wound in animals undergoing open peritoneal drainage

References

BONCYNSKI, J. J., LUDWIG, L. L., BARTON, L. J., LOAR, A. & PETERSON, M. E. (2003) Comparison of peritoneal fluid and peripheral blood pH, bicarbonate, glucose and lactate concentration as a diagnostic tool for septic peritonitis in dogs and cats. *Veterinary Surgery* **32**, 161-166

GUPTA, S. & JAIN, P. K. (1985) Low-dose heparin therapy in experimental peritonitis. *European Surgical Research* **17**, 167-172

Further reading

BOSSCHA, K., VAN VROONHOVEN, Th. J. M. V. & VAN DER WERKEN, Ch. (1999) Surgical management of severe secondary peritonitis. *British Journal of Surgery* **86**, 1371-1377

DONNER, G. S. & ELLISON, G. W. (1986) The use and misuse of abdominal drains in small animal surgery. *Compendium on Continuing Education for the Practicing Veterinarian* **8**, 705-714

GREENFIELD, C. L. (1998) Open peritoneal drainage for peritonitis. In Current Techniques in Small Animal Surgery, 4th edn. Ed M. J. Bojrab. Baltimore, Williams & Wilkins. pp 330-335

HOSGOOD, G. (1998) Closed peritoneal drainage. In Current Techniques in Small Animal Surgery, 4th edn. Ed M. J. Bojrab. Baltimore, Williams & Wilkins. pp 335-341

HOSGOOD, G. & SALISBURY, S. K. (1988) Generalized peritonitis in dogs: 50 cases (1975-1986). *Journal of the American Veterinary Medical Association* **193**, 1448-1450

KING, L. G. (1994) Postoperative complications and prognostic indicators in dogs and cats with septic peritonitis: 23 cases (1989-1992). *Journal of the American Veterinary Medical Association* **204**, 407-414

LANZ, O. I., ELLISON, G. W., WEICHMAN, G. & VAN GILDER, J. (2001) Surgical treatment of septic peritonitis without abdominal drainage in 28 dogs. *Journal of the American Animal Hospital Association* **37**, 87-92

McGROTTY, Y. L., KNOTTENBELT, C. M., DOUST, R. T., RAMSEY, I. K. & SULLIVAN, M. (2003) Complications of open peritoneal drainage in nine dogs with gastrointestinal leakage. *Irish Veterinary Journal* **56**, 354-360

SWANN, H. & HUGHES, D. (2000) Diagnosis and management of peritonitis. *Veterinary Clinics of North America: Small Animal Practice* **30**, 603-615

Animal health, productivity and welfare are inextricably linked. Loose housing in straw yards provides a high level of cow comfort, but is often a risk factor for *Streptococcus uberis* mastitis

Healthcare in the modern dairy herd

DAI GROVE-WHITE

Dai Grove-White graduated from Liverpool in 1975. After a period in large animal practice in Wales, he spent some time in the Middle East and Africa where he worked mainly on large scale dairying projects. He returned to Wales in 1985 to work in large animal practice before moving to Liverpool University as a lecturer in farm animal studies. He holds the diploma in bovine reproduction from Liverpool and was awarded an FRCVS for a thesis on calf diarrhoea. He is an RCVS specialist in cattle health and production.

AS the UK dairy industry continues to change, the role of the veterinary surgeon on the modern dairy farm has evolved and is increasingly that of an adviser, encompassing all aspects of health, productivity and welfare. Together with other specialists, such as nutritionists, housing consultants and breeding companies, the practitioner's involvement spans all areas of farm management. Undoubtedly, veterinary intervention in key areas such as farm assurance, fertility, nutrition, and disease monitoring and prevention can have a significant impact on the productivity of a unit. This article reviews these areas, providing examples of where veterinary effort may best be directed. Future articles in *In Practice* will elaborate on specific elements of veterinary input into the dairy enterprise.

IMPLICATIONS OF THE DRIVE FOR PROFITABLE MILK PRODUCTION

The UK dairy industry is going through a period of major restructuring, with many producers giving up farming altogether. Since 1996, 25 per cent of dairy producers have left the industry, 7 per cent of which gave up during 2002 mostly because of low milk prices and the 2001 outbreak of foot-and-mouth disease (FMD). The remaining producers have tended to expand herd size in order to reduce overheads and remain profitable. One survey of costs conducted last year (HSBC/ADAS 2003) showed the gap between the top and bottom 25 per cent of farms to be widening. Total costs per litre of milk produced were 16·9 pence per litre (ppl) for the top 25 per cent compared with 21·9 ppl for the bottom 25 per cent. The top 25 per cent remained in profit (+3·1 ppl) while the middle 50 per cent broke even (+0·1 ppl) and the bottom 25 per cent lost money (–3·2 ppl).

It may therefore be concluded that, for dairy farming to remain viable in the UK, production costs must be kept under control, especially as milk prices are predicted to fall to 15 ppl by 2007 (Colman and Harvey 2004). The veterinary surgeon looking after the modern dairy herd plays a key role in this as both clinical and subclinical disease will have a major impact on production costs. The costs of the major diseases in dairy animals have been well quantified and targets are available (Esslemont and Kossaibati 2002) which, if achieved, should ensure profitable milk production.

Many cows are housed in buildings best described as 'slums'

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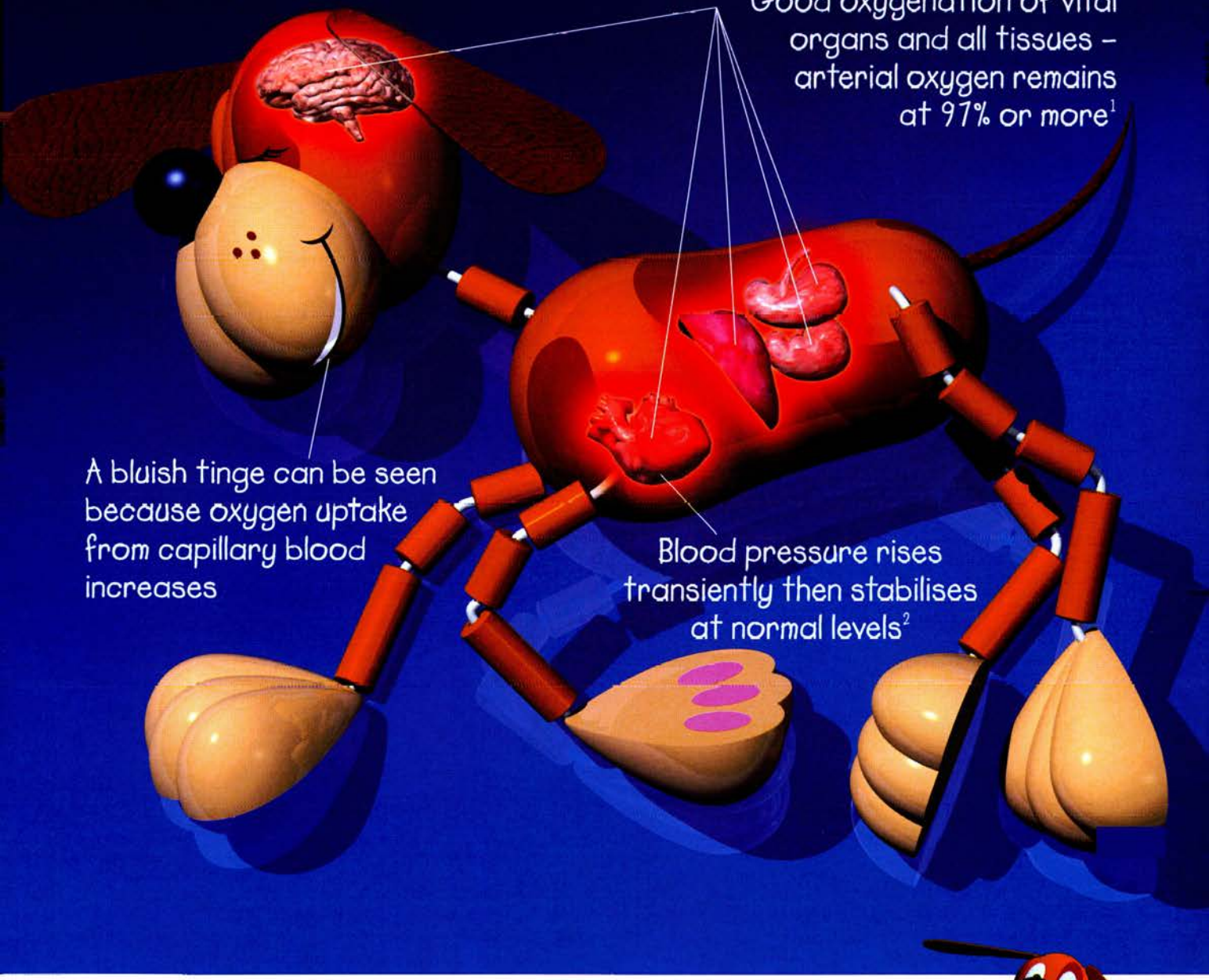

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A full clinical examination should be carried out on all sick cows

Animal welfare is central to profitable milk production. Neglect of welfare in terms of housing and feeding will inevitably lead to falling profitability which invariably results in a further decline in welfare standards until, finally, the business becomes non-viable and shuts down, to the detriment of both animals and producers. The health of the modern dairy cow is largely a reflection of her environment and management in relation to the physiological demands placed on her with regard to lactation and reproduction. Consequently, effective healthcare should revolve around providing an environment and management system that satisfies the basic requirements of the cow. A herd that truly enjoys the 'five freedoms' (Webster 1994) is unlikely to have major health problems and, furthermore, is likely to be profitable.

However, many cows are housed in cubicle buildings erected 25 to 30 years ago when cattle were considerably smaller. The result is poor cubicle acceptance and significantly reduced lying times. This is associated, all too often, with unacceptably high levels of lameness which have adverse effects on production and profitability. Current farm profitability is such that it is usually impossible to invest in major rebuilding projects, thereby condemning those cows to life in buildings that if they were used for people would be referred to as 'slums' (J. Hughes, personal communication).

THE SICK COW: THE TIP OF THE ICEBERG

The nature of veterinary work within the cattle industry has changed immensely over the past 40 years, with increased emphasis being placed on the whole herd and less on the individual sick cow, which is also worth less in monetary terms. This regrettable trend means that the individual sick cow invariably represents just the 'tip of the iceberg', with subclinical or inapparent losses dwarfing those due to actual clinical disease. Studies in the USA have suggested that, in many herds, the economic losses associated with Johne's disease are mainly due to poor production and premature culling of unproductive animals, rather than culling of clinical cases (Chiodini and others 1984). A similar situation applies to many other diseases, including mastitis, bovine virus diarrhoea (BVD) and so on.

While, in general, the veterinary knowledge of farm staff has increased with expanding herd size, this may not always be the case. Farmers and stockpersons have an ethical and moral obligation to ensure that any animal showing ill health is examined properly by a veterinary surgeon. Indeed, a full veterinary examination should be performed on all clinical cases seen and samples taken, as required. It is interesting to note, for example, that while traumatic reticulitis is now a relatively uncommon diagnosis in dairy cattle practice, it has been reported as a frequent postmortem finding (Grohn and Bruss 1990). This may be due to the reluctance of farmers to present cases early or of some veterinary surgeons to carry out a full clinical examination, including an assessment of ruminal and reticular motility (Williams 1955).

Lameness is a major problem on almost all dairy units although its prevalence is often underestimated by farmers. Treatment is usually provided by the farmer or a lay foot trimmer. In such cases, the attending veterinary surgeon may have little information about the lameness situation, as treatment records only give an indication of the prevalence of severely lame animals. Ideally, lameness scoring of the entire herd should be carried out by the veterinary surgeon in conjunction with the herdsman at least twice a year to assess true prevalence (Manson and Leaver 1988). This offers a valuable training opportunity, allowing herdsmen to learn how to recognise lameness.

Digital dermatitis was first reported in the UK in 1985 and is now a major cause of lameness in dairy herds

Automatic scrapers are a well recognised risk factor for digital dermatitis

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WORKING TO ENSURE THE CREDIBILITY OF FARM ASSURANCE AND HERD HEALTH PLANS

A major development in all sectors of UK agriculture over the past decade has been the concept of farm assurance. This has been driven by buyers and fuelled by consumer concerns following the BSE epidemic, with the major buyers, food processors and supermarkets having taken leading roles. Initially, there was a multiplicity of dairy schemes with varying standards, but these have now been unified under the banner of the National Dairy Farm Assurance Scheme (NDFAS). Since January 2003, all producers supplying the major dairies and supermarkets have had to belong to a recognised scheme. Such schemes have a broad remit to ensure the safety of produce, traceability of the retail product back to the farm of origin, and high standards of animal health and welfare. However, there is evidence to suggest that membership of a farm assurance scheme per se has no significant effect on lameness prevalence compared with farms that are not assured, suggesting that membership in itself may have little effect on pre-existing welfare problems (Main and others 2002). This issue needs to be addressed urgently if farm assurance is to retain credibility in the eyes of both the producer and the consumer.

A central plank of livestock farm assurance is the construction of a herd health plan for all classes of stock on the farm. The British Cattle Veterinary Association (BCVA) has played a major role in developing the concept of herd health plans and has put together protocols and training courses for veterinary surgeons wishing to develop herd health plans for clients. The BCVA herd health plan is increasingly recognised as the industry standard. Essentially, a herd health plan states the agreed actions that a farmer will take with regard to identification, recording, treatment and prevention of disease on a particular farm. To be credible, a herd health plan must be tailor-made for each farm, taking into account the specific circumstances pertaining to a unit. All farmers are required by law to record all veterinary drug usage, but there is no legal obligation to record the reasons why a drug is administered. The construction of a herd health plan provides the clinician with a useful opportunity to review husbandry and disease on a farm and is usually educational for both the farmer and the veterinary surgeon.

Training of farm staff is likely to become a key role for the modern veterinary surgeon

A herd health plan in itself is, however, of little value unless it is implemented correctly. It should therefore be reviewed periodically and altered, as required. It must be stressed that recording of disease events is essential to an effective herd health plan and offers the veterinary surgeon a real opportunity to identify disease trends and act accordingly. The BCVA herd plan is evolving rapidly (Orpin and Sibley 2002) and now includes farm risk assessment for the major infectious diseases and a 'disease cost calculator' which allows both the cost of disease and the financial benefits of veterinary intervention to be estimated.

ROUTINE FERTILITY WORK: A GOOD STARTING POINT

Dairy cow fertility is an area where regular veterinary intervention can pay dividends. In an attempt to maintain income, milk yields and herd size have both increased while labour has been shed on many farms. These trends have conspired to produce a marked decline in fertility over the past 15 years, which has been estimated to be in the region of 1 per cent per annum (Royal and others 2000, Washburn and others 2002, McGuirk 2004). The financial losses associated with fertility are well quantified (Esslemont and Kossaibati 2002). In most herds, optimal financial benefits are associated with a calving index of about 370 days and an overall culling rate of 18 per cent, with a failure to conceive culling rate (FTC-CR) of around 7 per cent. Based on data from the National Milk Records, the average UK calving index is 406 days and the overall culling rate is about 30 per cent, with an FTC-CR of about 17 per cent.

The genetic base of the UK dairy herd has changed markedly over the past 20 years with the importation of Holstein genetics from North America and this has driven milk yield up while lactation curves have tended to flatten. There is evidence that, in high yielding herds with flat lactations, it may be advantageous to extend the calving index (Arbel and others 2001). This extension has certainly occurred in many UK herds. However, the question of whether it is the farmer or the cows that dictate fertility policy and the length of the calving index

Farmer education

As herd size increases and the value of the individual animal decreases, there is more pressure on farmers to perform many veterinary tasks themselves (eg, diagnosis and treatment of toxic mastitis with intravenous infusions, and treatment of lame cows). It is therefore essential that, in such cases, the attending veterinary surgeon ensures that animal welfare does not suffer. This involves training and educating farmers; clinicians can either undertake this themselves, or suggest that farmers attend a recognised training course on, for example, foot care or ultrasound examination. In the USA, veterinary surgeons are closely involved with training farm staff in specified tasks and it is likely that this trend will cross the Atlantic. This aspect of the practitioner's workload is expected to increase due to the difficulty in recruiting skilled dairy staff.

should always be asked; all too often, it is the latter. While the direct losses due to culling may easily be calculated, involuntary high culling rates due to infertility or disease mean that there is less scope for genetic improvement in the herd through voluntary culling on the basis of production or type indices.

Fertility work involving the recording of calving and other fertility-associated events, and subsequent analysis using specialist computer software (eg, Daisy, Interherd), has increasingly become the backbone of dairy practice. However, far too often, fertility is taken in isolation (the 'PD and go' approach) whereas in reality it is usually a sensitive indicator of underlying deficits in the management, nutrition or housing of the herd; for example, lameness has a profound effect on fertility indices (Collick and others 1989) while the value of condition scoring as a monitor of nutritional status and its effects on fertility are well recognised (Biggadike and Drew 2001).

The routine fertility visit is an established aspect of veterinary work in a dairy herd and can serve as the starting point from which elements relating to other areas of the enterprise can be built up. Such a visit should extend beyond merely examining cows per rectum to include regular inspection of the herd in order to assess condition scores and husbandry practices (Borsberry 2002). Recording of condition scores and rumen fill scores of animals presented at routine fertility visits is desirable. Simple observation of cows in their environment (for example, noting whether animals are cudding or lying down, and also the consistency of their faeces) can also provide a valuable insight into nutrition and housing.

STRATEGIC AND COST-EFFECTIVE MONITORING OF DISEASE

Monitoring of disease on a farm is essential if effective control is to be achieved. This is done primarily by accurate record keeping of all disease events and their regular review by a veterinary surgeon, which, as mentioned earlier, is an integral part of a successful herd health plan. Such an approach should alert the farmer and his professional advisers that a problem may be developing and trigger a focused investigation of the problem. This constitutes passive surveillance. Active surveillance, meanwhile, involves regular collection and laboratory testing of samples, thus allowing trends in subclinical disease to be identified.

Bulk milk serological analysis

Bulk milk serology is increasingly being used for the diagnosis and monitoring of the infectious disease status of the dairy herd (Pritchard 2001). At present, bulk milk ELISA testing for BVD, leptospirosis and infectious bovine rhinotracheitis (IBR) is available, and it is likely that, in the future, testing will include other diseases such as Johne's disease and neosporosis. Such testing offers cost-effective monitoring of disease status if performed at regular intervals (eg, every three months). Results are usually interpreted quantitatively to estimate the proportion of seropositive cows. However, caution must be exercised when interpreting results as the bulk milk ELISA reading is a reflection of the immune status of the animals currently being milked and this is continually changing as animals enter or leave (ie, at drying off) the lactating herd. It should be borne in mind that high seropositivity does not necessarily indicate active infection as antibodies may persist for life in animals with BVD and IBR.

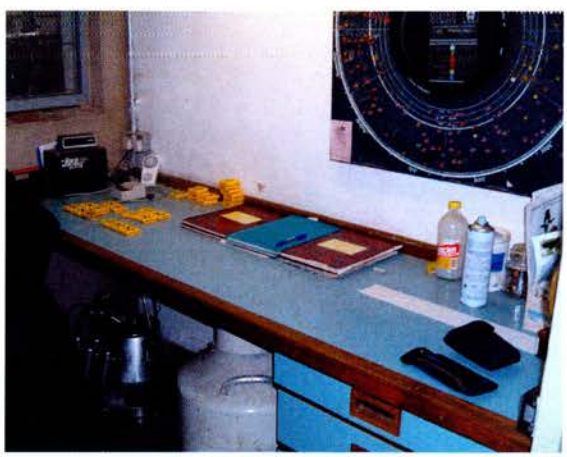
Cohort sampling (eg, of first lactation heifers) may be useful in the interpretation of whole herd bulk milk results as it allows conclusions to be drawn about whether antibodies are historic or whether the infection is active in the herd. Similarly, testing a cohort of unvaccinated animals in a herd previously vaccinated can help to establish whether the infection is still present and whether vaccination should be resumed. Bulk milk testing allows a farmer to ascertain the disease status of potential herd replacements cheaply before purchasing animals by requesting bulk milk serology results from the vendor.

Milk quality analysis

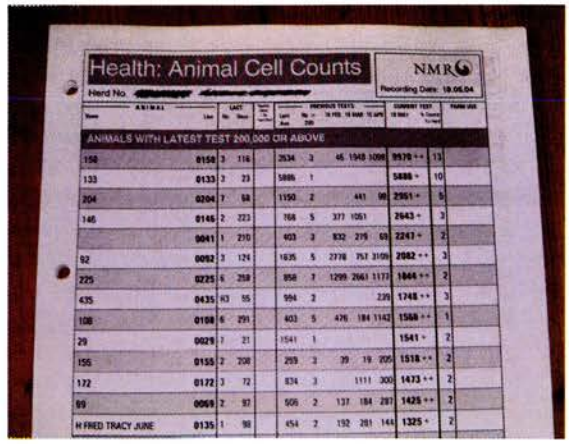
Milk quality analysis is carried out regularly by milk purchasing companies and offers an insight into the nutritional status of the herd. Milk protein is considered to be a useful indicator of long-term dietary energy supply while butterfat is an indicator of dietary fibre. Care is required when interpreting results as a number of factors (eg, stage of lactation, herd yield) may influence both milk protein and butterfat content. Some dairies routinely use bulk milk urea measurements as an aid to assessing dietary protein and energy content. These must be interpreted with extreme caution because, as with bulk milk ELISAs, they primarily reflect the make-up of the herd at the time of sampling, rather than the adequacy of the diet.

Bulk milk somatic cell counts (BMSCC) and Bactoscan readings provide valuable information regarding mastitis and dairy hygiene. In fact, a rising cell count will often alert the farmer to a developing problem of subclin-

Routine fertility visits should not be based on the 'PD and go' approach



Good record keeping is essential on the modern dairy farm


Health: Animal Cell Counts

NMR

Herd No.

ANIMALS WITH LATEST TEST 200,000 OR ABOVE

Monitoring of monthly individual cell counts is valuable in the control of mastitis

ical mastitis without a marked change in the incidence of clinical cases being observed. However, BMSCC are not a reliable indicator of infection by Gram-negative coliforms and there is some debate about whether low cell counts may in fact be a significant risk factor for *Escherichia coli* mastitis (Green 1998). Somatic cell counts reflect the incidence of mastitis in a herd, whether it be clinical or subclinical, and also provide a direct measure of milk quality. It is for this reason that dairy companies pay a bonus for low cell counts (rather than for any welfare considerations as is usually assumed). It is ironic that mastitis control was only taken seriously by many farmers when dairies started to first pay them a premium on the basis of cell counts and then introduced penalties if these counts were not low enough.

The majority of dairy farmers receive monthly individual cow somatic cell counts (ICSCC) and these, if used correctly, offer an invaluable tool for monitoring the acquisition of new intramammary infections and the effect of therapeutic intervention, as well as for identifying problem cows for intensive therapy or culling. An ICSCC of over 200,000 cells/ml indicates an intramammary infection. If this threshold is exceeded on three consecutive recordings before cows are dried off, the count has a sensitivity of 80 per cent and a specificity of 62 per cent for detecting Gram-positive intramammary infections (Bradley 2002). Routine bacteriology of clinical cases is useful for monitoring and investigating mastitis and may provide helpful information about the selection of intramammary antibiotics. As a matter of course, many farmers now collect pre-treatment milk samples from clinical cases and store them deep frozen. It is recommended that 25 per cent of such samples are cultured to enable trends in infection to be detected. In the event of a mastitis problem requiring an in-depth investigation, frozen samples provide a useful source of historical data.

Blood sampling

Blood sampling in cattle is conducted on either an individual animal or group basis for the diagnosis or monitoring of infectious disease, or as an aid to assessing nutritional adequacy.

While bulk milk serology is relatively inexpensive, the interpretation of results is often difficult because, as discussed, titres may reflect past events and have little bearing on the current health status of a herd. Selected cohort blood sampling can often clarify whether milk titres for, say, BVD or leptospirosis reflect historic events only or whether infection remains active in the herd (Houe 1995).

Blood sampling may also reveal the existence of subclinical disease within a herd, but caution must be exercised with regard to sample size selection. The minimum sample size required to establish the presence or absence of a disease in a population depends on the sensitivity and specificity of the test being performed as well as the likely within-herd prevalence of the disease in question. In many cases, whole herd testing is required (eg, for Johne's disease where the ELISA has a low sensitivity, ranging from 10 to 50 per cent depending on the stage of infection in the individual animal tested).

Samples may be taken to assist in the diagnosis of clinical disease (eg, abortion, milk drop syndrome and pneumonia) where affected animals may be tested for antibodies to selected disease agents. Paired serology is useful in assessing the contribution of a particular pathogen to the current disease situation; for instance, in the case of a pneumonia outbreak, a rising titre to a viral pathogen suggests the involvement of that particular pathogen, while a static or falling titre suggests previous exposure which is possibly unrelated to the incident under investigation.

Failure of passive transfer of colostral antibodies is well recognised as being a key factor in incidents of disease in young calves. Investigation of any disease outbreak in neonatal calves should always include blood sampling of a number of healthy and diseased calves for immunoglobulin estimation. The most widely used test is the zinc sulphate turbidity test, but others include the sodium sulphite turbidity test and estimation of total protein concentration using a refractometer.

Faeces

Examination of faecal samples is not usually carried out routinely in dairy cattle. However, regular faecal egg counting is being increasingly advocated in sheep as an aid to effective parasite control and this might be of value for the control of parasitic gastroenteritis in calves, especially on organic enterprises where routine anthelmintic administration is prohibited.

Faecal sampling of diarrhoeic calves is frequently undertaken to identify the likely pathogens involved. However, single samples from diarrhoeic animals are of little diagnostic value, and samples should be taken from both diarrhoeic and healthy calves in the affected group. An organism is only implicated in a disease outbreak if it is isolated at a higher frequency from diarrhoeic calves compared with healthy ones (Reynolds and others 1986).

Faecal culture remains the gold standard for the identification of animals infected with *Mycobacterium avium* subspecies *paratuberculosis* (Johne's disease), but the cost is prohibitive. Workers in Holland have suggested that culture of pooled faecal samples, taken from 10 animals, may offer a cost-effective alternative to using the ELISA in Johne's disease control programmes (Kalis and others 1999).

NUTRITION: ADDRESSING COMMON PROBLEMS AND MISCONCEPTIONS

The lactating dairy cow is under considerable nutritional stress, especially in early lactation when dry matter intake is not yet maximal. Negative energy balance, a common finding in cows at this stage of the production cycle, is a major factor in the poor reproductive perfor-

'Sorting' of a total mixed ration diet by cows may result in severe nutritional problems

mance seen in many herds. Nutritional status may be easily monitored by regular inspection and condition scoring of animals. The aim is to limit the loss of condition during early lactation so that a condition score of only 0·5 is lost between calving and peak lactation. Any greater loss may indicate a nutritional problem.

Assessment of faecal score, rumen fill and cleanliness score offers a valuable insight into rumen function and housing (Hughes 2001). Metabolic profiles are useful for monitoring nutritional status and in the investigation of nutritional problems (Ward and others 1995).

Subacute ruminal acidosis is a widespread and often undiagnosed nutritional disorder which appears to be increasing with the large-scale adoption of feeding maize silage and total mixed rations (TMRs). This syndrome is characterised by poor production, reduced butterfat, poor fertility and increased lameness (Grove-White 2004).

The importance of long fibre in the diet of cattle is well recognised. Particle size may be assessed using a Penn State Particle Forage Separator (Lammers and others 1996), although diets containing large amounts of grass silage may need to be air-dried before testing. It may be useful to carry out an estimation of long fibre content at intervals over a 24-hour period to assess whether cows are 'sorting' the ration (ie, picking out and eating small high energy particles and avoiding the long fibre). Sorting is particularly important in situations where there is competition at the feed barrier.

Attention must be paid not only to diet composition, but also the manner of feeding with regard to feed barriers, and so on. TMRs are designed to be available 24 hours a day. At least 5 per cent of the ration should remain at the barrier before more food is provided. This 'stale food' should be removed and discarded; it should not be fed to young replacement stock as this represents a breach of biosecurity, particularly with regard to Johne's disease. Common problems are that not enough food is placed in front of the cows or that it is not pushed up to the barrier so that cows can actually eat it. Such simple errors may be missed unless the veterinary surgeon visits the farm before feeding commences (or even in the middle of the night!).

Trace element deficiencies are encountered relatively frequently, especially in growing animals where they may be associated with poor growth rates. There are likely to be associations between trace element deficiencies and reproductive performance although there are few reports in the peer-reviewed literature demonstrating this. Conversely, it is likely that trace element deficiencies are often wrongly blamed for poor reproductive performance when the actual problem relates to gross nutritional deficiency (ie, negative energy balance) or poor heat detection.

BIOSECURITY: COMING OUT OF THE DARK AGES

The 2001 FMD epidemic in the UK highlighted the importance of biosecurity in modern livestock agriculture. Compared with the pig and poultry industries, which have long recognised the importance of biosecurity, the dairy sector remains in the 'dark ages' although awareness is increasing to some extent with the advent of herd health plans. While a full review of biosecurity measures is beyond the remit of this article, there are some basic principles which need to be considered:

- Biosecurity may operate at a national, regional, local or farm level. Diseases of interest may be infectious or non-infectious (eg, foodborne toxicosis);
- Biocontainment refers to the control of disease agents already on the farm (eg, prevention of calfhood infection with *M avium* subspecies *paratuberculosis* via adult faeces, colostrum or 'waste milk');
- Risk analysis (hazard analysis and critical control points – HACCP) should be performed before designing a biosecurity plan. This should include an assessment of:
  - Which diseases are of interest/relevance;
  - The likelihood of the disease entering the unit;
  - The consequences of disease entry. This may depend on the existing disease status of the unit;
  - Which biosecurity measures can be instituted;
  - The likely efficacy of the biosecurity measures put in place;
  - Cost;
- The disease status of the herd should be established prior to setting up the biosecurity plan. There may be little point in disease prevention if infection is already present in the herd;
- Disease monitoring should be carried out at regular intervals in order to detect disease early on.

Biosecurity plans should encompass some or all of the following components:

- Purchase policies of livestock, foods, and so on;
- Physical or chemical barriers:
  - Siting of farm buildings and routing of farm traffic;
  - Disinfection and hygiene measures;
  - Quarantine;
  - Fomites (eg, farm staff, visitors, lorries);
  - Role of vectors (eg, birds, insects);

Wild animals and birds may represent risks to biosecurity

■ Testing of animals prior to entry onto the premises or into the herd. It is essential that the limitations of any test performed are recognised and understood. For example, there is little point in performing an ELISA for Johne's disease on a young bull before purchase as the sensitivity of the test is likely to be between 10 and 20 per cent. In such cases, assurance regarding the Johne's disease status of the bull can only be obtained by testing the herd of origin or purchasing from a recognised Johne's disease-free herd;

■ Vaccination prior to entry into the herd (eg, against BVD, IBR) where the home herd is endemically infected;

■ Treatment prior to entry. For example, the administration of suitable anthelmintics to minimise the introduction of resistant nematodes and footbathing cattle for digital dermatitis.

VETERINARY FEES: A WAY FORWARD?

The current fee structure of veterinary practice is usually based on either charging a set fee for work carried out or an hourly rate for time spent on the farm. Neither of these fee structures encourage the involvement of the practitioner in advisory work – the farmer often feels reluctant to pay for advice received and the clinician is unlikely to carry out work for which there will be little remuneration. One possible solution is to negotiate an annual contract for all work (exclusive of drugs) based on a set charge per litre of milk produced, taking milk quality into account (eg, 0·5 ppl). This provides an incentive for the practitioner as his/her income rises as production improves alongside improved health status. Conversely, income declines if production declines. The veterinary surgeon therefore becomes a stakeholder in the business, to the advantage of all concerned.

Responsible use of medicines

Traditionally, the majority of farmers have obtained medicines from the attending veterinary practice although the bulk of anthelmintics are now generally sold via farmers' merchants. However, since the recent report by the Competition Commission, the situation is likely to change, with farmers obtaining prescriptions from the attending veterinary surgeon and the medicines being dispensed elsewhere. Irrespective of the source of medicines, herd health plans should indicate what drugs may be purchased and used, and all such products must be stored and used correctly and safely. The attending veterinary surgeon should advise on such matters.

It is likely that the price of medicines will fall quite considerably as a result of the changes in prescribing. In the light of public concern about drug residues and antibiotic resistance originating from food animal usage, it is hoped that this reduction in price will not result in increased use of antibiotics in food animals. The attending veterinary surgeon must be vigilant in encouraging good husbandry as a means of disease control rather than drug usage. The use of antibiotics in undifferentiated neonatal calf diarrhoea is one area where prescribing practices may be described as irresponsible regarding the transfer of antibiotic resistance, as the aetiological agents involved are mainly viral or protozoal (Reynolds and others 1986, Snodgrass and others 1986) and also because there is no strong evidence base supporting their use.

References

ARBEL, R., BIGUN, Y., EZRA, E., STURMAN, H. & HOJMAN, D. (2001) The effect of extended calving intervals in high-yielding cows on milk production and profitability. *Journal of Dairy Science* **84**, 600-608

BIGGADIKE, H. & DREW, B. (2001) Body condition score and fertility. *UK Vet* **6**, 46-52

BORSBERRY, S. (2002) Approach to the routine herd health visit. *In Practice* **24**, 274-281

BRADLEY, A. J. (2002) Making better use of milk samples: monitoring and investigating herd mastitis. *Cattle Practice* **10**, 105-112

CHIODINI, R. J., VAN KRUININGEN, H. J. & MERKAL, R. S. (1984) Ruminant paratuberculosis (Johne's disease): the current status and future prospects. *Cornell Veterinarian* **79**, 218-262

COLLICK, D. W., WARD, W. R. & DOBSON, H. (1989) Association between different types of lameness and fertility. *Veterinary Record* **25**, 103-106

COLMAN, D. & HARVEY, D. (2004) Future of UK Dairy Farming. www.defra.gov.uk/foodrin/milk/colman-harveyreport.pdf

ESSLEMONT, R. J. & KOSSAIBATI, M. A. (2002) The costs of poor fertility and disease in UK dairy herds. In Trends in DAISY Herds Over Ten Seasons. Milton Keynes, Intervet UK. pp 99-116

GREEN, M. (1998) Coliform mastitis: an evolving problem. *Cattle Practice* **6**, 91-93

GROHN, Y. T. & BRUSS, M. I. (1990) Effect of diseases, production and season on traumatic reticulitis and ruminal acidosis in cattle. *Journal of Dairy Science* **73**, 2355-2363

GROVE-WHITE, D. H. (2004) Rumen healthcare in the dairy cow. *In Practice* **26**, 88-95

HOUE, H. (1995) Epidemiology of bovine viral diarrhoea virus. *Veterinary Clinics of North America: Food Animal Practice* **11**, 521-548

HUGHES, J. (2001) A system for assessing cow cleanliness. *In Practice* **23**, 517-524

HSBC/ADAS (2003) Third annual HSBC Bank and ADAS spotlight survey. Cited by Farmers Weekly Interactive. www.fwi.co.uk

KALIS, C. H. J., BARKEMA, H. W. & HESSELINK, J. W. (1999) Certification of dairy herds as free of paratuberculosis using culture of strategically pooled faecal samples. In Proceedings of the Sixth International Colloquium on Paratuberculosis. February 14 to 18, Melbourne. pp 55-59

LAMMERS, B. P., BUCKMASTER, D. R. & HEINRICHS, A. J. (1996) A simple method for the analysis of particle size of forage and total mixed rations. *Journal of Dairy Science* **79**, 922-928

McGUIRK, B. (2004) Breeding for improved cow fertility. *In Practice* **26**, 272-275

MAIN, D. C. J., WHAY, H. R., GREEN, L. E. & WEBSTER, A. J. F. (2002) Farm assurance and on-farm welfare. *Cattle Practice* **10**, 181-182

MANSON, F. J. & LEAVER, J. D. (1988) The influence of dietary protein and of hoof trimming on lameness in dairy cattle. *Animal Production* **47**, 191-199

ORPIN, P. G. & SIBLEY, R. J. (2002) Herd health planning: where do we go from here? *Cattle Practice* **10**, 73-78

PRITCHARD, G. C. (2001) Milk antibody testing in cattle. *In Practice* **23**, 542-549

REYNOLDS, D., MORGAN, J., CHANTER, N., JONES, P., BRIDGER, J., DEBNEY, T. & BUNCH, K. (1986) Microbiology of calf diarrhoea in southern Britain. *Veterinary Record* **119**, 31-34

ROYAL, M., MANN, G. E. & FLINT, A. P. F. (2000) Strategy for reversing the trend towards subfertility in dairy cows. *Veterinary Journal* **160**, 53-60

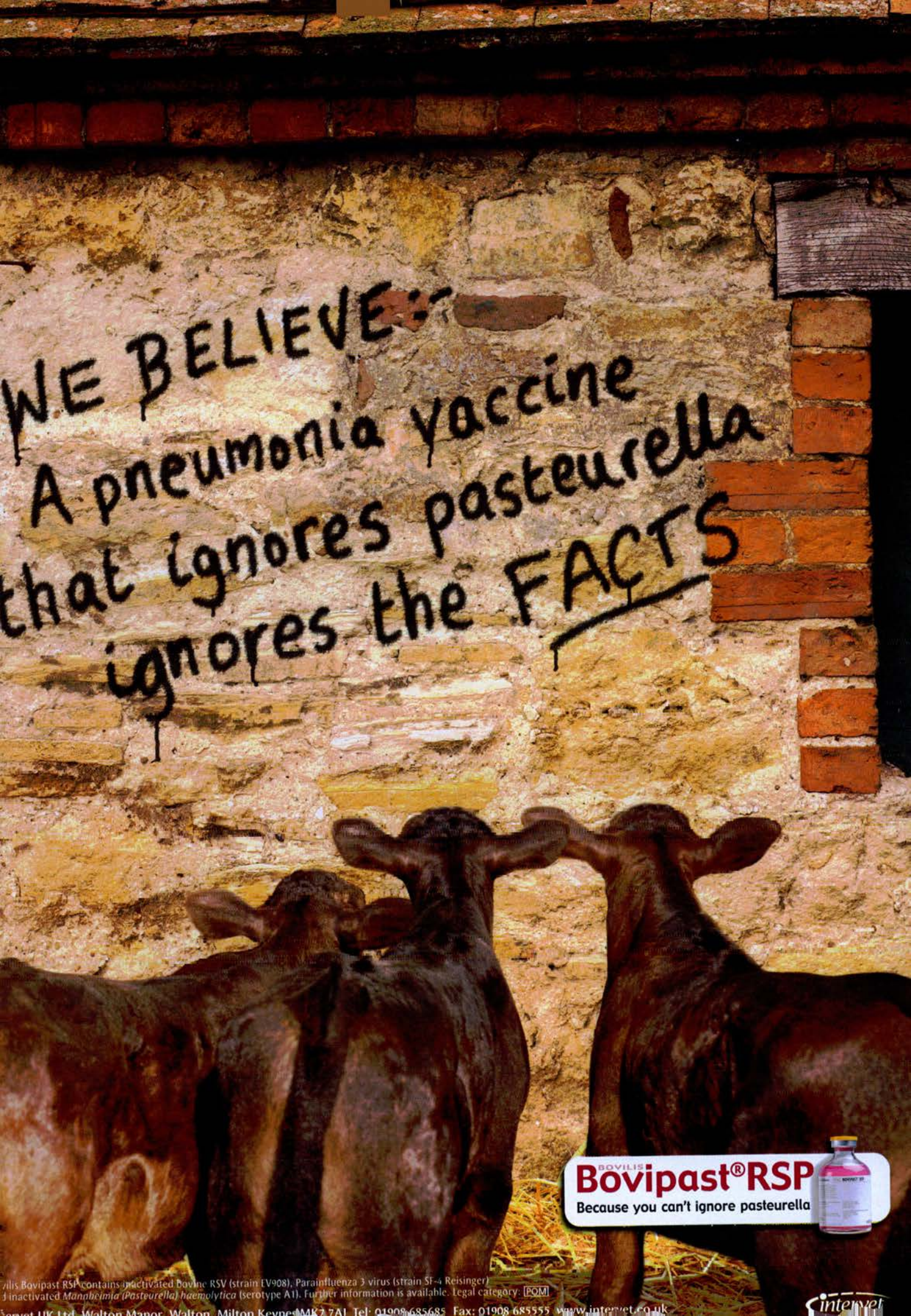
SNODGRASS, D. R., TERZOLO, H., SHERWOOD, D., CAMPBELL, I., MENZIES, J. & SYNGE, B. (1986) Aetiology of diarrhoea in young calves. *Veterinary Record* **119**, 31-34

WARD, W. R., MURRAY, R. D., WHITE, A. R. & REES, E. M. (1995) The use of blood biochemistry for determining the nutritional status of dairy cows. In Recent Advances in Animal Nutrition. Eds P. C. Garnsworthy and D. J. A. Cole. Nottingham, Nottingham University Press. pp 29-51

WASHBURN, S. P., SYLVIA, W. J., BROWN, C. H., McDANIAL, B. T. & McALLISTER, A. J. (2002) Trends in reproductive performance in Southeastern Holstein and Jersey DHI herds. *Journal of Dairy Science* **85**, 244-251

WEBSTER, A. J. F. (1994) Animal welfare. In A Cool Eye Towards Eden. Oxford, Blackwell Science. pp 10-16

WILLIAMS, E. I. (1955) A study of reticulo-ruminal motility in adult cattle in relation to bloat and traumatic reticulitis with an account of the latter condition as seen in general practice. Part 1. A study of reticulo-ruminal motility in adult cattle with reference to the eructation of ruminal gases and bloat. *Veterinary Record* **67**, 907-911



ilis Bovipast RSP contains inactivated bovine RSV (strain EV908), Parainfluenza 3 virus (strain SF-4 Reisinger) inactivated *Mannheimia (Pasteurella) haemolytica* (serotype A1). Further information is available. Legal category: POM

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A number of factors influence whether a horse with colic is a medical or potential surgical case

Decision making in the management of the colicky horse

DEBRA ARCHER

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COLIC is one of the most commonly encountered emergency conditions of the horse seen in practice. Many owners are aware of the potentially life-threatening consequences of colic and such cases can be stressful to deal with, particularly for inexperienced practitioners or those who deal infrequently with horses. Decisions about which diagnostic tests to perform and the most suitable treatment options are important in ensuring that appropriate action is taken. Although many cases of colic seen in first opinion practice are mild and recover spontaneously or following medical treatment, it is vital to identify those animals that may require surgery, if this is an option for the owner. For such patients, early referral is essential as this will maximise the chance of a successful outcome. This article discusses the factors which should be considered in this decision-making process.

MANIFESTATION OF ABDOMINAL PAIN

Colic is the behavioural manifestation of pain that is localised in the abdomen and is most often related to the gastrointestinal tract. In most cases, pain results from a disturbance in normal gut motility. Horses suffering from colic may exhibit varying degrees of pain, ranging from mild to severe, or depression. Pain may arise from one or a combination of intestinal spasm, mucosal irritation, distension of the bowel wall by gas or ingesta, tension on the mesentery or ischaemia of the intestinal wall. Such pain is termed visceral. Obstruction of the gastrointestinal tract is broadly divided into simple and strangulating obstructions. Many textbooks describe the pathogenesis of these types of obstruction and the course of time over which they manifest. It is important to note that strangulating obstructions develop rapidly and can result in death within 24 to 36 hours.

Telltale signs of colic that has been present for several hours include disturbed bedding and abrasions, particularly around the head and over the tuber coxae

Is it colic?

A variety of conditions may mimic the signs of gastrointestinal-associated pain (so-called 'false colic', see box on the right). It is important to differentiate between colic and false colic in order to ensure that appropriate treatment is administered. In some cases, this can be a challenge but valuable information can be obtained from the history and by observing the horse before starting a clinical examination.

Causes of 'false' colic

- Laminitis
- Rhabdomyolysis
- Urinary tract obstruction/rupture
- Dystocia/uterine torsion
- Pleuritis/pleuropneumonia
- Liver disease
- Aortoiliac thrombosis
- Splenic lesions
- Central nervous system disease (eg, tetanus)
- Ovulation/granulosa cell tumour

HISTORY TAKING

A good history provides valuable information and helps to determine the possible cause of colic. Certain risk factors (eg, age, recent box rest) are associated with particular types of colic. The duration and severity of colic may be difficult to establish when an affected horse has been found in the morning. Where a strangulating lesion has been present for several hours, acute signs of pain may become less obvious and the horse may appear quiet and dull due to the progressively worsening bowel wall necrosis and endotoxaemia.

Where bouts of colic are recurrent in nature, it is important to determine whether the episodes are changing in frequency, duration or severity. Initially, the priority is to assess whether the current episode is potentially life-threatening. If medical therapy is successful, further investigations should then be undertaken.

Specific information that should be obtained

- Signalment
- When the colic was first observed
- Signs of colic observed
- When the horse was last seen to be normal
- Any recent episodes of colic
- Recent changes in management or exercise
- Worming history (including prophylaxis against tapeworms)
- Recent dental examination
- Current medications (eg, non-steroidal anti-inflammatory drugs [NSAIDs])
- Whether the mare is pregnant and the stage of gestation (or recent foaling)

CLINICAL EXAMINATION

The severity of the clinical signs and possible cause of colic should be determined before making a decision on how to manage an affected case. The initial clinical examination should be quick but thorough and systematic, and should include an assessment of heart and respiratory rates, mucous membrane colour, hydration status, rectal temperature and abdominal distension, and auscultation for abdominal borborygmi (intestinal sounds). For safety reasons, if the horse is in extreme pain and likely to make violent movements, sedation may be justified before starting an examination. It is useful to assess the horse's heart rate before the administration of any sedatives or butylscopolamine (Buscopan Compositum; Boehringer Ingelheim) due to their transient effects on the resting heart rate, which make further comparisons more difficult.

Such information provides a picture of the systemic status of the horse and also a baseline against which the results of a repeat examination can be compared. In many cases, a specific diagnosis cannot be made. Regardless of the diagnosis, continued deterioration of the horse's parameters despite medical therapy indicates the need for potential surgical intervention.

RECTAL EXAMINATION

Rectal examination should always be performed in any horse exhibiting moderate to severe, or persistent, pain despite analgesia. In mild cases of colic seen for the first time, rectal examination may be omitted, particularly if the horse is fractious or strains relentlessly. Rectal examination will not be possible in foals or very small ponies. Fractious horses should be sedated; xylazine is useful for its short-term sedative and analgesic effects. Spasmo-

Colic in the pregnant mare

It is important to establish whether colic in the pregnant mare is related to the gastrointestinal tract or directly to the uterus and fetus (eg, impending abortion or parturition). Most analgesic drugs are not specifically licensed for use in the pregnant mare and the lack of such data relating to the mare can make it difficult to decide on the most appropriate drugs to use. Most data have been extrapolated from other species and the effects of many drugs on the fetus are inconclusive. In general terms, sufficient analgesia should be administered to control the signs of pain. In a mare with a surgical lesion, the priority is to provide analgesia and anti-endotoxic therapy and to get the patient to a referral centre as soon as possible.

Colic in the foal

Diagnosis of colic in the foal can be challenging and it can be difficult to determine the need for surgical intervention. Common conditions in the neonatal foal include meconium impaction and gastric ulceration. In addition, other conditions, such as ruptured bladder, can be confused with signs of true colic in the neonatal animal. Imaging modalities, such as ultrasonography and radiography, can be useful in the evaluation of these cases. Neonatal foals can deteriorate quickly so they should be monitored carefully if medical therapy is undertaken; they should be referred immediately if there is no response to treatment or if the foal's condition continues to deteriorate. No NSAIDs are licensed for use in foals less than six weeks of age, but they can be used at lower dose rates. Care should be taken to ensure the foal is adequately hydrated before NSAIDs are administered.

Suitable NSAIDs include flunixin and carprofen. Stress in conjunction with the use of NSAIDs can result in gastroduodenal ulceration; therefore, anti-ulcer therapy should be administered concurrently using a proton pump inhibitor (omeprazole) or one of the H_2-antagonists (eg, cimetidine or ranitidine).

Nasogastric intubation is important diagnostically and for gastric decompression

lytics or instillation of a local anaesthetic into the rectal lumen can aid rectal examination in a horse that persistently strains, thereby reducing the risks of causing a rectal tear.

Rectal examination may be conclusive (eg, pelvic flexure impaction, small intestinal distension, tight taenial bands or gas-distended large colon) but, in many cases of mild colic, no abnormalities will be found. In horses showing more severe signs of colic, failure to identify an abnormality does not rule out a surgical lesion (for example, the lesion may be out of reach). In any case of colic that is non-responsive to analgesia, repeat rectal examination can be helpful to detect any changes and the results of this examination should be considered together with the results of other clinical tests.

NASOGASTRIC INTUBATION

Nasogastric intubation should always be carried out in a horse with moderate to severe, or persistent, colic despite analgesia. This may not be warranted on the first visit to a case exhibiting mild signs of colic. In most horses, only a small amount of fluid can normally be retrieved (usually <500 ml). A net reflux of 2 litres or more is often significant and indicates the potential need for surgery. Reflux is usually caused by physical (eg, pedunculated lipoma) or functional (eg, dysautonomia or ileus) obstruction of the small intestine or, sometimes, by large colon displacement resulting in duodenal compression. Horses cannot vomit, so nasogastric intubation is important in the diagnosis of gastric distension as it allows the stomach to be decompressed. Decompression also provides pain relief and prevents rupture of the stomach, which is invariably fatal.

ABDOMINOCENTESIS

Abdominocentesis can be performed relatively easily with minimal equipment (see box, below left) and can provide vital information about acute and recurrent cases of colic. Rectal examination should be performed initially to identify gross distension of a viscus. Abdominocentesis in such cases (eg, a heavily pregnant mare or gross small intestinal or large colon distension) can cause laceration of a viscus if a needle is used; the use of a teat cannula can reduce this risk. Where rectal findings are indicative of severe intestinal disease, the results of abdominocentesis may not alter the decision to send a horse to a referral facility. In such cases, referral should not be delayed until obvious changes in peritoneal fluid occur (by which time the disease process is usually advanced). In foals, the thin intestinal walls may be easily lacerated with a needle; therefore, if a sample of peritoneal fluid is required, ultrasonography of the abdomen is preferable in the first instance before a teat cannula is employed.

The use of peritoneal taps for distinguishing between cases of medical and surgical colic will be discussed in a forthcoming article in *In Practice*.

HAEMATOLOGY AND BIOCHEMISTRY

In practical terms, haematological and biochemical evaluation is unnecessary in cases that obviously require surgery and the priority is to get the horse to the referral facility as soon as possible. However, packed cell volume, systemic total protein and peripheral white blood cell count are basic parameters that should be measured in less critical cases and serial measurements can help in the assessment of a response to therapy. More extensive biochemical and haematological profiles are justified in the investigation of recurrent colic.

Abdominocentesis

Site

- Midline or to the right of midline
- Most dependent portion of the abdomen

Preparation

- Clip a small area and clean with a surgical scrub solution

Equipment

- Sterile gloves
- EDTA and plain blood tubes
- 18 gauge, 3·75 cm needle or teat cannula (local anaesthetic and No 15 scalpel blade is required if using a teat cannula)

Normal results

- COLOUR. Straw coloured/colourless
- VISCOSITY. Clear
- TOTAL PROTEIN. ≤20 to 25 g/litre
- WHITE BLOOD CELL COUNT. ≤5 x 10^9/litre

(above) Equipment needed for abdominocentesis and (right) collection of peritoneal fluid

REPEAT EXAMINATION

Repeat examination is essential in cases of colic that do not respond to analgesia, particularly if moderate to potent analgesics have been given and the cause of colic cannot be determined. A full re-evaluation of the patient should be performed. Changes in a horse's haematological and biochemical parameters, or findings on rectal examination, nasogastric intubation and abdominocentesis can help to determine whether continued medical therapy is justified or if potential surgical intervention is required. Some owners do not appreciate the significance of recurrent, albeit sometimes less severe, signs of pain once the effects of analgesia wear off. It is therefore useful to arrange a repeat examination one to two hours later, or sooner, if signs of pain recur; at the very least, the veterinary surgeon should telephone the owner to get an update on the horse's progress to decide if a re-evaluation is necessary.

ADVICE TO THE OWNER

The cause of one-off episodes of colic may be clear from the history and appropriate advice should be given regarding the prevention of further episodes. In horses that are not regularly wormed, it is worth submitting samples for faecal worm egg and larval counts and an analysis of tapeworm burden (serum ELISA) to rule out these potential causes of colic. Teeth should be checked,

particularly where regular dental care is not undertaken. Further investigations are not usually necessary in most one-off episodes unless the signs of colic recur.

ANALGESIA

The choice of analgesic is one of the most important decisions that need to be made regarding the management of a colicky horse. Analgesia should be sufficient to relieve the signs of visceral pain, but should not alter the ability to detect persistent or increasing pain. Drugs such as flunixin and detomidine provide potent relief of visceral pain, but can mask signs of continued pain if the animal is not monitored carefully. Failure to respond to these more potent analgesics can indicate the need for surgery or euthanasia. Where potent analgesia has been given, the results of a rectal examination, nasogastric intubation and abdominocentesis will usually indicate the need for surgery, hence highlighting the importance of a thorough repeat examination in such cases.

NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

Flunixin meglumine

Flunixin meglumine provides good to excellent analgesia. Its anti-endotoxic effects will reduce the degree of pain and lessen alterations in mucous membrane colour and heart rate in strangulating lesions. Hence, this drug should be used with caution where signs of mild pain are evident or if no definitive diagnosis has been reached on the initial examination.

Ketoprofen

Ketoprofen provides good visceral analgesia and has anti-endotoxic properties. It is not as potent an analgesic as flunixin, but may still mask the signs of mild to moderate visceral pain.

A variety of drugs are licensed for use in horses with colic, but they vary in potency and the duration of analgesia they provide

Phenylbutazone

Phenylbutazone provides mild visceral analgesia, similar to the effects of metamizole. It is therefore useful for colic cases exhibiting mild signs of pain.

Other NSAIDs

Other NSAIDs, such as carprofen, vedaprofen and eltenac, are licensed for use in horses with musculoskeletal pain and soft tissue inflammation. However, their use in cases of visceral pain has not been fully evaluated (ie, their potency is unknown).

ALPHA$_2$-AGONISTS

Xylazine provides good to excellent visceral analgesia, but is relatively short acting. Therefore, it is useful for horses with painful colic because it allows sufficient time to perform a full clinical examination safely, without masking the effects of pain for prolonged periods of time. Detomidine has more potent sedative and analgesic effects and should be used with caution; a failure to respond to detomidine, or a recurrence of signs within an hour, indicates the potential need for surgery. Romifidine provides good, but less potent visceral analgesia.

ANALGESIC DRUGS LICENSED FOR USE IN HORSES WITH COLIC

Active ingredient and tradenames	*Dose and administration*	*Approximate duration of analgesia*	*Comments*
Flunixin meglumine Finadyne (Schering-Plough) Meflosyl (Fort Dodge) Binixin (Bayer) Cronyxin (Bimeda)	0·25-1·1 mg/kg every 12 hours iv or po	One to 24 hours, depending on the cause and severity of the pain	Anti-endotoxic dose 0·25 mg/kg every six to eight hours
Ketoprofen Ketofen (Merial)	2·2 mg/kg every 24 hours iv	Similar to flunixin	
Phenylbutazone Equipalazone (Arnolds)	Up to 4·4 mg/kg iv or po	Up to 14 hours	Many potential side effects, but rarely seen in practice; do not exceed stated dose (low therapeutic index)
Butylscopolamine/metamizole Buscopan Compositum (Boehringer Ingelheim)	5 ml/100 kg iv		Combined NSAID and spasmolytic; transient increase in heart rate following administration (parasympatholytic effect)
Xylazine Virbaxyl (Virbac) Rompun (Bayer) Chanazine (Chanelle) Xylacare (Animalcare) Xylapan (Vétoquinol)	0·2-1·0 mg/kg iv	10 to 40 minutes depending on the dose used	Contraindicated in the first and last month of pregnancy. Available as 2 per cent and 10 per cent solutions
Detomidine Domosedan (Pfizer)	0·01-0·02 mg/kg iv or im	One to three hours	Do not use in the last month of pregnancy
Romifidine Sedivet (Boehringer Ingelheim)	0·04-0·08 mg/kg iv	0·5 to three hours	Do not use in the last month of pregnancy
Butorphanol Torbugesic (Fort Dodge)	0·1 mg/kg iv	0·5 to three hours	Often combined with alpha$_2$-agonists at lower dose rates
Pethidine Pethidine Injection (Arnolds)	2·0 mg/kg im	20 to 60 minutes	Only licensed for use in spasmodic colic

po Orally, iv Intravenously, im Intramuscularly

All of these drugs may be combined at lower dosages with butorphanol, which provides useful sedation in the field and allows a rectal examination to be performed safely. However, not all of these combinations are licensed for use in horses with colic.

OPIATES

Butorphanol provides good visceral analgesia and will not mask the signs of severe pain for long periods of time. Excitatory effects can be seen, but tend to be less evident in horses exhibiting signs of pain. Pethidine is licensed for use in cases of spasmodic colic and provides short-term analgesia.

SPASMOLYTICS

Butylscopolamine is a spasmolytic and mild analgesic that acts mainly on the smooth muscle of the gastrointestinal and urinary tracts. It is combined with the NSAID metamizole, a weak visceral analgesic. It is useful in horses with mild, spasmodic colic and the degree of analgesia will not mask signs of more severe pain.

OTHER MEDICAL THERAPIES

LAXATIVES

Laxatives are most commonly indicated in the management of large colon impactions. They help to increase the softness of ingesta and may be combined with fluid therapy in more severe cases. Laxatives include mineral oil (liquid paraffin), osmotic laxatives (magnesium sulphate and sodium chloride), dioctyl sodium sulphosuccinate and psyllium hydrophilic mucilloid.

FLUID THERAPY

Oral fluids

Administration of oral fluids via a nasogastric tube can be useful in any horse with colic, although mild cases that respond to analgesia will rapidly correct any fluid deficits without intervention. Oral fluids should never be administered in any case in which reflux occurs.

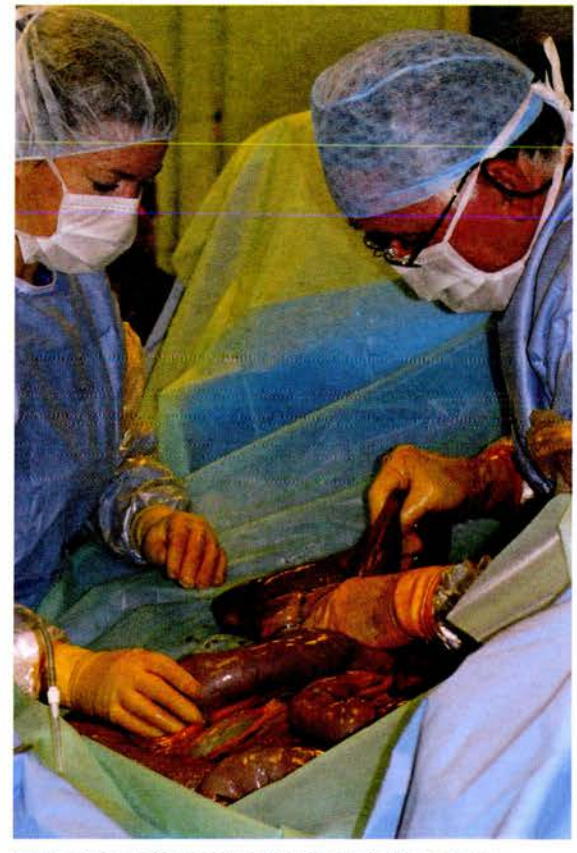
Intravenous fluids

Intravenous fluid therapy can be useful in the medical management of some cases of colic, particularly large colon impactions that do not respond to laxatives or oral fluid therapy. Ideally, intravenous therapy should be undertaken in facilities where the catheter site and fluid rate can be monitored closely by veterinary staff. Intravenous fluid therapy is critical in the management of surgical colic cases, but generally it is far better to get the horse to a referral facility quickly rather than waste time placing an intravenous catheter or collecting the necessary equipment to do so. Large volumes of fluid need to be administered to have a significant effect, which is impractical in many cases. If there is a delay in obtaining transport, particularly if the referral facility involves a journey of several hours or more, fluid therapy may be administered during this time.

KEY DECISIONS

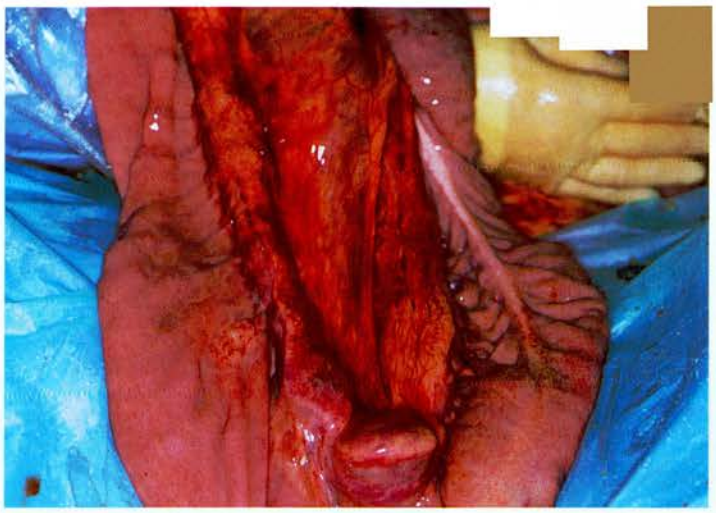
IS SURGICAL INTERVENTION REQUIRED?

The question of whether surgical intervention is required is one of the key decisions which need to be made in the management of the colicky horse. Although most horses seen in first opinion practice will have mild colic of unknown origin that responds to medical therapy alone, approximately 7 per cent of cases will have a surgical lesion. A number of factors will help to determine whether the horse should be treated medically or referred for potential surgery. In many cases, the lack of response to medical therapies will help to decide this. Some cases will fall into the 'in-between' category. Where referral is an option, it is better to refer cases early rather than delay further by which time the decision regarding the need for surgery may be easy, but valuable hours may then be lost during transport.



Improvements in anaesthetic and surgical techniques have contributed to improved success rates for the surgical treatment of colic over the past 20 years

Large colon torsion showing severe infarction of the colon wall and tears in the serosa. In this case, surgical correction was not possible due to the degree of strangulation and the location of the torsion



Large colon torsion of shorter duration than that pictured on the left, exhibiting less severe compromise of the bowel wall. Resection was unnecessary in this case and the horse made a full recovery following surgery

Indications for continued medical therapy

- Mild to moderate pain
- Good response to mild analgesics
- Heart rate <50 beats per minute
- Normal packed cell volume and total protein
- Negative findings on rectal examination
- Continuous or improving intestinal motility
- No nasogastric reflux
- Resolving or no abdominal distension
- Grossly normal peritoneal fluid with normal total protein and white blood cell count

DECISION TO CONTINUE MEDICAL THERAPY

Large colon impactions are often encountered in practice and most will respond to treatment with analgesics, oral fluids and laxatives. Those that show no sign of improvement over 24 to 48 hours may require more aggressive medical therapy (eg, intravenous fluid therapy). Practitioners should be aware that this condition may occasionally occur secondarily to another lesion, such as concurrent large colon displacement or impaction of the right dorsal colon. These cases can often only be resolved by surgical intervention.

Indications for referral

- Severe, unrelenting pain despite the use of analgesia
- Recurrence of pain following the administration of moderate to potent analgesia
- Heart rate persistently >60 beats per minute
- Net gastric reflux of >2 litres
- Positive findings on rectal examination
- Alterations in peritoneal fluid
- Progressive deterioration in mucous membrane colour
- Progressive reduction in intestinal motility
- Progressive abdominal distension

Strangulation of a short portion of small intestine by a pedunculated lipoma. This type of lesion is most commonly seen in older ponies

REFERRAL

REFERRAL FACILITIES

A large number of equine clinics in universities and private practices provide facilities for surgical and intensive medical treatment of colic. The decision to refer can sometimes be difficult to make, but advice can always be sought from these clinics. In some horses, the need for surgery is obvious, but this should not deter the referral of animals that show equivocal signs. The facilities in the stable yard or in the field may preclude a full clinical examination and referral clinics may have other more sophisticated diagnostic or imaging equipment that can be of great help in deciding whether or not to operate. Even if initial medical treatment is instituted, the horse can be monitored intensively and surgery can be undertaken quickly if the patient's condition deteriorates suddenly. Although the horse's clinical signs may have improved dramatically on arrival at a referral facility (the 'therapeutic box ride'), most owners are usually delighted that their horse does not need (expensive) surgery and this situation is preferable to the surgical case in which referral is delayed, which may make the prognosis hopeless, despite surgery.

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A case for euthanasia?

Surgery may be ruled out by the owner for financial or personal reasons. The priority in such cases is to provide sufficient analgesia if a definitive diagnosis cannot be made. Failure to respond to potent analgesia and a continued deterioration in the horse's condition indicates progressive endotoxic shock and the need for euthanasia. Rupture of a viscus may result in a dramatic decrease in the degree of abdominal pain. Such cases rapidly succumb to severe endotoxic shock, showing marked tachycardia, cyanosis of the mucous membranes and profuse sweating. The patient will often exhibit 'boarding' of the abdomen due to peritonitis and peritoneal fluid contaminated with green ingesta will be obtained. These animals should be euthanased immediately.

CONTACT WITH THE REFERRAL FACILITY

The facility to which a horse is to be referred should always be contacted before a case is sent and referral costs and medical therapy discussed. Cases of colic with pyrexia and/or diarrhoea may indicate impending colitis. Salmonellosis is always a potential consideration in these horses and, consequently, some clinics may prefer such animals to be treated on their own premises or will admit them into isolation.

OWNER INFORMATION

In cases of severe, unrelenting colic, referral should be discussed at an early stage and transport arrangements made (to avoid any unnecessary and frustrating delays in getting a horse to a suitable facility). Costs should always be discussed with the owner first and the insurance company should be notified at the earliest opportunity where applicable. The owner or transporter should be given contact telephone numbers and clear directions to the facility. Updates are usually appreciated by the referral facility if any unforeseen delays occur.

PROGNOSIS FOLLOWING SURGERY

The management of the surgical colic patient has improved significantly over the past 20 years. Early referral, improved management of the patient under anaesthesia and postoperatively, as well as advances in surgical techniques have all contributed to improved surgical success rates. The practitioner is essential in identifying the need for surgery and referring the horse at an early stage before the effects of endotoxaemia worsen. The horse's chances of survival postoperatively are also dictated by a number of other, often interrelated, factors including the duration of the colic, the duration of surgery and the need for intestinal resection. Certain types of surgical colic (eg, large colon torsion and epiploic foramen entrapment) may have an inherently worse prognosis. A precise diagnosis can be difficult to make preoperatively; therefore, it can be difficult to offer the owner a prognosis until exploratory laparotomy has been undertaken. While most studies have concentrated on horse survival until discharge from the hospital (ie, short-term survival), several studies are currently looking at the long-term survival of these cases and the problems encountered following discharge. It is hoped that information from such studies will provide owners and practitioners with a better understanding of what to expect in the long term in horses that have been treated surgically for colic.

Acknowledgements
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Further reading

MAIR, T., DIVERS, T. & DUCHARME, N. (2002) Manual of Equine Gastroenterology. Philadelphia, W. B. Saunders

MALONE, E. & GRAHAM, L. (2002) Management of gastrointestinal pain. *Veterinary Clinics of North America: Equine Practice* **18**, 133-158

PROUDMAN, C. J., SMITH, J. E., EDWARDS, G. B. & FRENCH, N. P. (2002) Long-term survival of equine surgical colic cases. Part 1. Patterns of mortality and morbidity. *Equine Veterinary Journal* **34**, 432-437

SINGER, E. R. & SMITH, M. A. (2002) Examination of the horse with colic: is it medical or surgical? *Equine Veterinary Education* **14**, 87-96

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Typical gross pathology of ovine pulmonary adenocarcinoma (OPA). Currently, the only means of controlling OPA is by regular inspection of affected flocks, with culling of suspected cases. However, work is underway to develop improved diagnostic procedures and it is hoped that this will change the overall approach to disease control

Ovine pulmonary adenocarcinoma: the story to date

DANIELA SALVATORI, MARCELO DE LAS HERAS AND MIKE SHARP

TUMOURS are generally uncommon in domestic livestock, but small ruminants are unusual because two neoplastic diseases – ovine pulmonary adenocarcinoma (OPA) and enzootic nasal adenocarcinoma of sheep and goats – can affect many animals in countries and flocks where they are endemic. These diseases can be transmitted experimentally, but do not seem to affect cattle or other animals. They are contagious and caused by two distinct but closely related betaretroviruses. Both types of tumour arise from secretory epithelial cells that retain their secretory function after transformation. OPA is produced by a tumour in the lungs of sheep; the tumour has also been seen in goats and wild moufflon, but more rarely. The disease has been recognised in over 20 countries spanning Europe, Africa, the Americas and Asia, and in a wide variety of breeds. Studies in Britain and South Africa show that OPA accounts for almost 70 per cent of all sheep tumours. As discussed in this article, recent studies have thrown light on the aetiology, pathogenesis and epidemiology of OPA and this, in turn, has led to current research efforts to develop preclinical diagnostic tests for the disease.

CLINICAL SIGNS

OPA is generally accepted to have a long incubation period in naturally infected sheep because the disease is not obvious clinically until animals are about two to four years old. However, tumours have been seen exceptionally in lambs as young as a few weeks and in sheep as old as 11 years of age.

Clinical signs are those of an afebrile, progressive respiratory illness associated with loss of weight. The severity of the tachypnoea and dyspnoea, which is often associated with noticeable movement of the abdominal wall (abdominal lift), depends on the extent of the tumour and the loss of normal functional lung. A pathognomonic feature of OPA is the accumulation of fluid (lung fluid) within the respiratory tract, which may flow from the nostrils when the hindquarters are raised (wheelbarrow test). In advanced cases, high-pitched and moist sounds may be heard on auscultation or even by the unaided ear.

Appetite is maintained in affected animals, although loss of weight is obvious. Death inevitably occurs, often suddenly, from a complicating *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*) pneumonia.

(above and right) Frothy seromucoid fluid is discharged from the nostrils of sheep with OPA when the hindquarters are raised and the head lowered

Daniela Salvatori is a graduate of the University of Camerino, Italy. She is currently completing a PhD on the epidemiology and pathogenesis of OPA at the Moredun Research Institute, Edinburgh.

Marcelo de las Heras is chair of veterinary pathology at the University of Zaragoza, Spain, where his research focuses on contagious respiratory tumours of sheep and goats.

Mike Sharp is head of pathology at the Veterinary Laboratories Agency and has a particular interest in infectious diseases of farm ruminants.

History and geographical distribution of OPA

OPA was first recognised in South Africa in the 19th century as a cause of respiratory distress in sheep. The Afrikaans name 'jaagsiekte', derived from 'driving' (jaag) and 'sickness' (ziekte), reflected the tendency of diseased sheep to lag behind the flock during herding. Since then, OPA has been identified in a wide variety of breeds worldwide. A number of other names have been used to describe the disease, including sheep pulmonary adenomatosis and ovine pulmonary carcinoma, but at a workshop in Missilac, France, in 2001 it was agreed that the term OPA would be used to describe the disease in future.

To date, OPA has been identified in over 20 countries on the continents of Europe, Africa, the Americas and Asia; it appears to be absent from Australia and New Zealand. The disease occurred in Iceland (see below) but, following a successful eradication programme, no cases of OPA have been recorded since 1952.

OPA in Iceland

OPA was introduced into Iceland in the 1930s, along with scrapie, maedi-visna and paratuberculosis, by a small number of Karakul rams imported from Germany but originating from Bukhara in Russia. A year after their arrival, the first case of what was later identified as OPA appeared in one- to three-year-old sheep; during the next two to three years about 60 per cent of the breeding stock in affected flocks died of the disease. Subsequently, mortality due to OPA dropped to under 10 per cent in three years and, during this period, another respiratory disease, 'maedi' (Icelandic for dyspnoea), emerged. Maedi is caused by the lentivirus maedi-visna virus (MVV). It was later established that two of the imported Karakul rams were infected with MVV and one of them was carrying the jaagsiekte agent. Sigurdsson (1954) used the adjective 'slow' to describe the peculiar clinical evolution of this group of diseases.

In some countries, another form of the tumour (atypical OPA) has been reported, which is not associated with excess accumulation of fluid in the lung (as discussed below) and, therefore, generally presents as an incidental finding at necropsy or in the abattoir.

PATHOLOGY

OPA lesions are confined to the lungs, although occasionally the associated lymph nodes are involved. Commonly, OPA presents as extensive tumours involving the entire ventral half of the diaphragmatic and other lobes. Affected lungs are enlarged and heavier than normal.

Tumours are solid and grey-pink in colour, with a shiny translucent sheen; often they are separated from adjacent normal lung tissue by a narrow emphysematous zone. The presence of frothy white fluid exuding from cut surfaces or in the respiratory passages is a prominent feature and is obvious even with very small lesions.

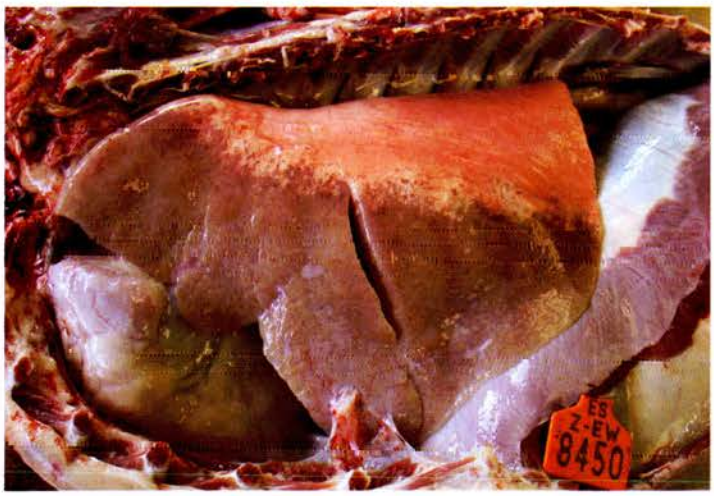
Histological examination of OPA lesions reveals the characteristic proliferation of cuboidal or columnar cells that replace the normal thin alveolar cells. Often these tumour cells form papilliform growths that project into the alveoli. Intrabronchiolar proliferation may also be present. Electron microscopy has revealed that the cells forming alveolar lesions are type II pneumocytes, while those forming bronchiolar tumours are Clara cells. Thus, OPA is a tumour of secretory epithelial cells in the lung, type II pneumocytes secreting lung surfactant and Clara cells secreting other proteins for the distal airways. Large macrophages frequently accumulate in the alveoli surrounding the tumours. A small proportion of tumours contain nodules of loose connective tissue in a mucopolysaccharide substance.

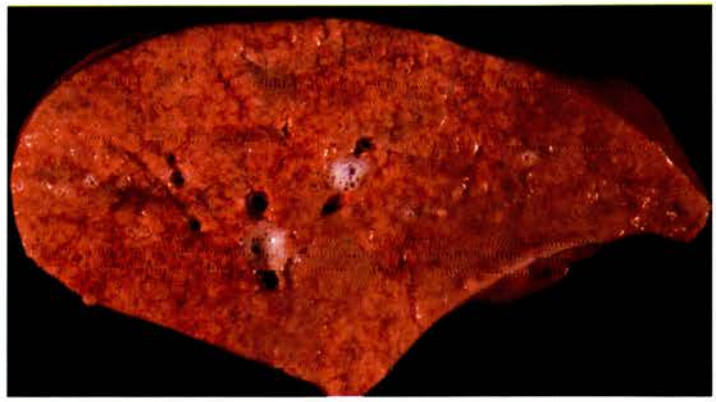
In contrast to this pathological picture for classical OPA, tumours in the atypical form of OPA comprise solitary or coalesced hard white nodules, which have a dry cut surface and show clear demarcation from surrounding tissues (see right); the presence of excess fluid is not a prominent feature. The histological appearance of these tumours is essentially the same as for classical OPA but with fibrosis and an exaggerated inflammatory response, mostly involving lymphocytes and plasma cells.

Secondary infections, particularly pneumonic pasteurellosis, are common in sheep with OPA, leading to pleurisy and abscessation. Flocks that experience recurring problems with pasteurellosis should consider OPA as an underlying factor, as lesions of OPA may be masked by the acute reaction to the bacteria.

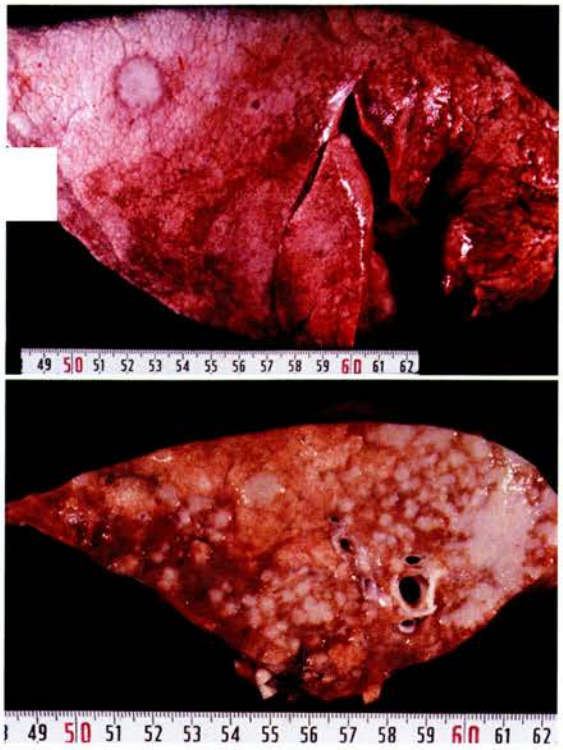
EVIDENCE THAT OPA IS CAUSED BY A RETROVIRUS

Transmission of OPA through inter-flock and international trade of live animals has clearly demonstrated that the disease is contagious and stimulated the search for the causal agent. A number of studies have consistently shown lung fluid and tumours from OPA-affected sheep to contain a retrovirus, known as jaagsiekte sheep retro-





Pathology findings in classical OPA. (left) Tumour appears as purple-coloured areas in the cranioventral part of the lung. (above) Section of the diaphragmatic lobe of a lung showing tumour. Affected areas have a 'meaty' appearance and frothy fluid is exuding from the bronchioles



Atypical OPA. (top) Well-demarcated, white, solitary tumour nodule in the dorsocaudal area of the right lung. (bottom) Section of a lung from a severe case, showing numerous disseminated dry confluent nodules

virus (JSRV). Although it was unclear at first that this virus had anything to do with the aetiology of OPA, it was found that successful transmission of OPA could be achieved only with inocula that contained JSRV. These observations made JSRV the best candidate for the aetiological agent of OPA.

However, confirmation of the pathogenic role of JSRV in OPA was complicated by the presence of 15 to 20 copies per sheep genome of JSRV-related endogenous sequences (see box on the right). These sequences increased the difficulty of detecting integrated exogenous JSRV, and raised the possibility that the expression of JSRV in OPA may represent the activation of endogenous virus(es) in the host cells as a consequence of carcinogenesis. The development of reagents and techniques to distinguish between exogenous and endogenous ovine retroviruses was, therefore, a critical step. Specifically, the availability of a genomic sequence for JSRV facilitated the development of recombinant JSRV capsid antigens (JSRV-CA). These were used to produce specific antisera and a blocking ELISA to detect JSRV and to determine the anatomical distribution of JSRV in sheep.

JSRV was detected only in lung fluid and tumours from affected animals and not in any other tissue from affected or unaffected sheep. Immunohistochemical examination of tumour tissues, using the same antiserum to JSRV-CA, detected JSRV protein in the cytoplasm of the transformed epithelial cells in the pulmonary alveoli of both naturally and experimentally OPA-affected sheep. Although JSRV-CA was observed in all OPA tumours, not all tumour cells were positive, even in the same nodule. There was no staining of non-transformed epithelial cells, nor of stromal cells, and specific staining was observed only rarely in the extracellular alveolar spaces. Specific staining was not demonstrated in any other tissue, with the exception of a few large lymphoblastoid cells in the paracortical zones and medullary sinuses of the tracheobronchial and mediastinal lymph nodes and some macrophages within the pulmonary alveoli. These observations demonstrated that JSRV appears to replicate principally in the transformed epithelial cells in OPA tumours as well as a minor subset of lymphoid cells.

These results supported the notion that JSRV causes OPA, but did not rule out other possibilities. JSRV might be acting as a helper virus for some other replication defective, acutely transforming retrovirus; alternatively, it may represent an endogenous virus, reactivated as a consequence of neoplasia or, indeed, as a result of recombination with endogenous sequences, as has been reported in other species.

To investigate this relationship further, the distribution of JSRV transcripts was examined in various tissues from OPA-affected and unaffected animals. Transcripts were demonstrated in both OPA-affected and unaffected animals, providing clear evidence for transcription of JSRV-related endogenous retroviruses (enJSRVs). However, RNA transcripts in lung fluid (exogenous JSRV) were shown by molecular analysis to differ from the enJSRV transcripts obtained from non-tumour tissues. Furthermore, the exogenous form of JSRV was exclusively and consistently present in tumour tissues, draining lymph nodes and lung secretions of OPA-affected sheep. To rule out the possibility that the exogenous form of JSRV may have represented enJSRV that normally was transcriptionally silent but had reactivated as a downstream event of neoplasia, it was necessary to

Betaretroviruses: biology and clinical implications

Betaretrovirus is a genus of the family Retroviridae and consists of:

- Exogenous viruses:
  - Jaagsiekte sheep retrovirus (JSRV)
  - Enzootic nasal tumour virus (ENTV-1) associated with enzootic nasal adenocarcinoma of sheep
  - Enzootic nasal tumour virus (ENTV-2) associated with enzootic nasal adenocarcinoma of goats
- Endogenous viruses known as enJSRVs: this group of viruses is closely related to JSRV and ENTV; 15 to 20 copies of enJSRVs are present in the genome of sheep and goats

Features of endogenous retroviruses (enJSRVs)

- enJSRVs are integrated in the germ line of their host species and thus can be transmitted vertically
- Within the family Retroviridae, several exogenous retroviruses have endogenous counterparts (feline leukaemia virus, mouse mammary tumour virus, murine leukaemia virus and avian leukaemia virus)
- enJSRVs are highly expressed along the epithelium of the genital tract of the ewe and in the thymus of ovine fetuses

Biological significance of enJSRVs

- enJSRVs and the exogenous and pathogenic JSRV use the same receptors for entering into cells; thus enJSRVs can compete with JSRV at the level of receptor usage and interfere with JSRV at the level of virus entry (receptor interference)
- The expression of endogenous elements during fetal development can lead the immune system to recognise the viral elements as 'self' (tolerance phenomenon); this could explain why there is no antibody response to JSRV

Resemblance between OPA and human bronchoalveolar carcinoma

The histology and ultrastructure of human bronchoalveolar carcinoma (BAC) closely resembles OPA. BAC is a subset of pulmonary adenocarcinoma arising from type II pneumocytes or Clara cells. It is now defined as a type of adenocarcinoma with a pure bronchoalveolar carcinoma growth pattern and no evidence of stromal, vascular or pleural invasion; the neoplastic cells grow as a single layer along the walls of the terminal airways and alveoli.

Recently, evidence of a protein related immunologically to JSRV in some human lung tumours suggested a possible correlation between BAC and an endogenous retrovirus. Two hundred and seventy-four samples from patients with BAC, other pulmonary malignancies, non-malignant pulmonary lesions and normal lung were examined by immunohistochemistry using a rabbit antiserum towards the major capsid protein of JSRV. The tumours included 129 BACs, 65 adenocarcinomas and 41 squamous cell carcinomas. Thirty-nine of the 129 BAC samples (30·2 per cent) and 17 of the 65 adenocarcinoma samples (26·2 per cent) were positive (de las Heras and others 2000). No positive staining was observed in non-tumour lesions or normal lung tissues.

These results support the view that the antigen in the human tumours may be a retrovirus protein, although no other virological or molecular data was obtained to support these observations.

demonstrate exogenous JSRV proviral DNA only in OPA-affected sheep and not unaffected animals.

Using a PCR specific for exogenous JSRV, this virus was demonstrated in 100 per cent of tumours and draining lymph nodes, as well as in a high proportion of other lymphoreticular tissues and peripheral blood mononuclear cells of OPA-affected sheep, but not in unaffected sheep. More recently, in situ PCR was employed to demonstrate JSRV proviral DNA in transformed and a few untransformed type II pneumocytes, alveolar macrophages and cortical follicles and paracortex of the draining lymph nodes. The results showed that JSRV was expressed at much higher levels in the transformed epithelial cells than in the lymphoid tissues, particularly those at non-local sites.

These experiments confirmed that the exogenous form of JSRV was associated exclusively with OPA and that it could play a major role the aetiology of the disease. However, final confirmation that exogenous JSRV was the cause of OPA required the isolation of pathogenic molecular clones of JSRV. This has since been achieved. Full-length proviral clones of JSRV were obtained from an OPA tumour and an OPA cell line, and JSRV virions produced in vitro by transient transfection of a cell line with the JSRV plasmid. The JSRV virus particles induced OPA in experimentally inoculated lambs, some within four months, providing definitive evidence that JSRV is the aetiological agent of OPA.

IMMUNE AND INFLAMMATORY RESPONSES TO JSRV IN SHEEP

A unique feature of OPA is the absence of a specific antibody response to JSRV, despite the highly productive infection in the lungs and the disseminated lymphoid infection. The reactivity to recombinant JSRV-CA in sera from affected sheep, described in some accounts, was not specific and reflected the presence of antibodies to the fusion partner of the recombinant antigen used in the assays. However, serum antibodies can be induced readily in sheep by immunisation with recombinant JSRV protein in adjuvant. These results indicate that sheep are not inherently unresponsive to JSRV antigens. It is possible that the expression of endogenous JSRV during ontogeny or in neonatal life may interfere with the exogenous JSRV infection and disease outcome via the induction of immunological tolerance. To date, specific cellular immune responses have not been demonstrated.

Another prominent feature of naturally OPA-affected sheep is marked peripheral neutrophilia and lymphopenia, specifically affecting CD4+ T lymphocytes (other subsets remain unaffected).

The persistent and disseminated infection of the lymphoreticular system by JSRV and dysregulation of the immune and inflammatory responses of infected sheep suggest that JSRV may interfere with the host immune responses. This theory is supported by studies demonstrating JSRV infection of a wide range of lymphoid cells. Holland and others (1999) showed that, in naturally infected sheep, JSRV proviral burden was greatest in the macrophage/monocyte cell population (1/2500 cells), followed by B cells (1/3800 cells), CD4+ T lymphocytes (1/6800 cells) and CD8+ T lymphocytes (1/16,700 cells). Furthermore, dissemination of JSRV was an early event following experimental infection of young lambs. The virus was present in CD4+ and CD8+ T lymphocytes, B lymphocytes and adherent mononuclear cells in the pulmonary lymph nodes as early as seven days post-inoculation, and was detected in peripheral blood leucocytes (PBLs) by 14 days post-inoculation. These observations are particularly significant as they indicate that dissemination of JSRV precedes transformation and, for the first time, that infected animals might be detected in OPA-affected flocks before the appearance of clinical signs.

In contrast to the haematological changes observed in naturally occurring OPA, no significant alterations were identified in the peripheral blood during the first 20 weeks after experimental inoculation of young lambs with JSRV, compared to age- and breed-matched controls (Summers and others 2002). These observations indicate that the alterations in peripheral blood lymphoid cell subsets are not an early event and probably do not occur in direct response to JSRV infection, but are a consequence of the superimposed bacterial infections that are common in natural cases of OPA.

Functional defects were also demonstrated in the immune responses of JSRV-infected sheep (Summers and others 2002). Lymphocytes from both natural cases of OPA and lambs inoculated experimentally with JSRV showed a significantly reduced proliferative response to concanavalin-A, a cell mitogen. The reduced response was detected in the lambs as early as eight weeks after inoculation and prior to clinical disease, when the response was only 33 per cent that of the control lambs.

These observations have provided the first indications that JSRV may alter the functional activity of immune cells in infected animals, although the virus apparently does not alter the phenotypic profile of the peripheral blood.

EPIDEMIOLOGY

The prevalence of OPA appears to vary between countries. For example, the disease is endemic in Peru, Scotland and South Africa and, in these countries, it can cause between 2 and 10 per cent annual mortality in adult sheep in affected flocks. A five-year longitudinal survey of two OPA-affected flocks in Scotland revealed that about 30 per cent of the sheep had histologically confirmed lesions in their lungs, and between 12 and 21 per cent had subclinical tumours (Sharp and DeMartini 2003). In contrast, OPA is infrequently diagnosed in the USA or Canada, where only a few cases have been reported. These differences in prevalence and incidence of the disease may reflect variations in viral strains, host susceptibility or animal management systems.

Most estimations of the prevalence of OPA have been based on clinical observations and histopathological diagnosis. Information on the true prevalence of JSRV infection in OPA-affected flocks is currently not available and this represents a major gap in veterinary knowledge. The detection of JSRV in the peripheral blood of experimentally infected lambs, before the development of tumour or onset of clinical disease, was therefore an important finding that offers a means to investigate the extent of JSRV infection in OPA-affected flocks (González and others 2001).

These findings have been applied in studies involving sheep from OPA-affected commercial flocks in Spain (García-Goti 1999). PBLs and tissue samples from sheep were examined for JSRV using PCR techniques. Overall, JSRV was detected in all sheep with either the classical or atypical form of OPA, as well as in 80 per cent of in-contact sheep. None of 71 samples from control sheep were positive. Although an earlier report had indicated that as few as 1/250,000 PBLs might be positive for JSRV (Holland and others 1999), in this study JSRV was detected in PBLs of all sheep with classical OPA, 83 per cent of sheep with atypical OPA, as well as 40 per cent of lesion-free in-contact sheep. These results clearly demonstrated, for the first time, that JSRV can be detected in naturally infected live sheep before the onset of clinical disease and even in the absence of discernible lung tumours. Although only a small number of in-contact sheep were examined, it would appear that subclinical JSRV infection can reach a high level in OPA-affected flocks.

To investigate further the dynamics of JSRV infection in flocks with OPA, García-Goti and others (2000) conducted a small prospective longitudinal survey in an affected flock. Using PCR techniques, JSRV was detected in PBLs of 28 per cent of the sheep. Subsequently, PBLs of 15 animals testing positively and five testing negatively were examined by PCR at monthly intervals for four months. JSRV was detected in only nine

Enzootic nasal adenocarcinoma of sheep and goats

Clinical signs

Clinical signs of enzootic nasal adenocarcinoma include seromucous nasal discharge, stertorous breathing, coughing and dyspnoea. The continuous flow of nasal secretions causes a characteristic discoloration and hair loss around the nostrils. The growth of the tumour mass can cause skull deformation, softening of the cranial bones and exophthalmos.

The disease is afebrile. After several months the affected animal loses weight and dies as a result of complications.

Depilation around the nostrils is caused by the continuous flow of seromucous fluid produced by the tumour. Exophthalmos and tumefaction of the frontal areas of the skull are also typical signs of enzootic nasal adenocarcinoma

Gross pathology of enzootic nasal adenocarcinoma. The tumour is grey in colour with a nodular surface and a scanty mucous secretion. The tumour can fill the ethmoidal area and may occlude nasal passages

Pathology

Tumours arise uni- or bilaterally from the ethmoid turbinates. These masses are soft, grey or reddish-white with a granular surface covered by mucus. Sometimes they can fully occupy the nasal chambers and invade maxillary or frontal areas; this can cause compression of the surrounding structures. Histologically, these tumours are classified as low-grade adenocarcinomas. The transformed cells originate from the nasal mucosal glands. No metastases to regional lymph nodes or other organs have been found.

Future research priorities

Questions that need to be answered

- What is the prevalence of OPA?
- What proportion of sheep develop OPA?
- When does infection occur?
- What are the routes of transmission:
  - Vertical?
  - In utero?
  - Via milk?
- Familial susceptibility? High/low incidence line?

How information will be used

- To identify management approaches to reduce the risks of on-farm transmission of JSRV
- To identify familial trends and high/low incidence lines
- To identify a resistance gene that will allow:
  - Screening for resistant sheep
  - Breeding for resistant sheep
- Possible vaccination?

of the original 15 positive sheep during this period and in four of those that were negative. These results demonstrated the fluctuation in detection of JSRV in blood and, more importantly, confirmed the high prevalence of JSRV infection in OPA-affected flocks.

In the mid-1980s, a severe outbreak of a disease resembling OPA was described in a flock of captive moufflon (*Ovis musimon*), a species of wild sheep phylogenetically related to the domestic sheep, *Ovis aries*. The flock comprised over 60 moufflon which were kept on a game farm in Sardinia, Italy, with the aim of producing animals to be released later into their natural habitats. The moufflon and a flock of sheep in which OPA was endemic had repeated contacts (Ceretto and Deiana 1967). Studies on DNA from archival materials concluded that JSRV was the cause of OPA in the moufflon (Sanna and others 2001).

APPROACH TO DISEASE CONTROL

At present, the only means of control of OPA is regular inspection of adult sheep in affected flocks. Prompt culling of any suspicious animal, as well as the offspring of affected ewes (which frequently develop OPA), is advisable. These methods have not been shown to eradicate OPA from a flock in which the disease is endemic, but may bring about a reduction in the prevalence of infection. PCR techniques able to detect JSRV in the blood before the onset of clinical signs offer new opportunities that may lead to effective control. Currently, a large EU collaboration involving Scotland, Spain and Italy is undertaking research to improve diagnostic tests to detect JSRV during the preclinical stages of infection.

Meanwhile epidemiological studies are currently in progress in Scotland and Spain that will report the prevalence of JSRV infection in flocks with history of OPA (D. Salvatori, C. Cousens, L. Gonzalez, P. Dewar and J. M. Sharp, unpublished data). Further aims of these studies are to understand what proportion of infected sheep will develop OPA, when the infection is acquired and the routes of transmission (eg, vertical transmission, in utero, via milk). Investigations using PCR techniques have provided some preliminary evidence of the presence of JSRV in fetuses (D. Salvatori and M. de las Heras, unpublished data). This finding apparently conflicts with the findings of an earlier study by Parker and others (1998) which showed that embryo transfer can be used to obtain animals free of OPA. In that experiment, 215 embryos recovered from 76 donor ewes from flocks with endemic OPA and mated with rams that did not have any sign of OPA were transferred to 131 recipients. Thirty-eight of 51 progeny from OPA-positive donors and 55 of 74 progeny from donors in which no lesions of OPA were detected survived for at least five years. During this time, there was no evidence of OPA in the recipients or their progeny on the basis of clinical and pathological criteria. Further investigations are necessary to clarify whether vertical transmission of infection occurs.

References

CERETTO, F. & DEIANA, S. (1967) Adenomatosi polmonare enzootica degli ovini (jaagsiekte) in Sardegna (jaagsiekte in Sardinia). *Atti della Societa' Italiana di Scienze Veterinarie* **21**, 606-611

DE LAS HERAS, M., BARSKY, S. H., HASLETON, P., WAGNER, M., LARSON, E., EGAN, J., ORTIN, A., GIMENEZ-MAS, J. A., PALMARINI, M. & SHARP, J. M. (2000) Evidence for a protein related immunologically to the jaagsiekte sheep retrovirus in some human lung tumours. *European Respiratory Journal* **16**, 330-332

GARCÍA-GOTI, M. (1999) Estudios sobre la patogenia y diagnostico de la adenomatosis pulmonar ovina. PhD Thesis, University of Zaragoza, Spain

GARCÍA-GOTI, M., GONZÁLEZ, L., COUSENS, C., CORTABARRIA, N., EXTRAMIANA, A. B., MINGUIJON, E., ORTIN, A., DE LAS HERAS, M. & SHARP, J. M. (2000) Sheep pulmonary adenomatosis: characterization of two pathological forms associated with jaagsiekte retrovirus. *Journal of Comparative Pathology* **122**, 55-65

GONZÁLEZ, L., GARCÍA-GOTI, M., COUSENS, C., DEWAR, P., CORTABARRIA, N., EXTRAMIANA, B., ORTIN, A., DE LAS HERAS, M. & SHARP, J. M. (2001) Jaagsiekte sheep retrovirus can be detected in the peripheral blood during the preclinical period of sheep pulmonary adenomatosis. *Journal of General Virology* **82**, 1355-1358

HOLLAND, M., PALMARINI, M., GARCÍA-GOTI, M., GONZÁLES, L., McKENDRICK, L., DE LAS HERAS, M. & SHARP, J. M. (1999) Jaagsiekte retrovirus is widely distributed both in T and B lymphocytes and in mononuclear phagocytes of sheep with naturally and experimentally acquired pulmonary adenomatosis. *Journal of Virology* **73**, 4004-4008

PARKER, B. N. J., WRATHALL, A. E., SAUNDERS, R. W., DAWSON, M., DONE, S. H., FRANCIS, P. G., DEXTER, I. & BRADLEY, R. (1998) Prevention of transmission of sheep pulmonary adenomatosis by embryo transfer. *Veterinary Record* **142**, 687-689

SANNA, M. P., SANNA, E., DE LAS HERAS, M., LEONI, A., NIEDDU, A. M., PIRINO, S., SHARP, J. M. & PALMARINI, M. (2001) Jaagsiekte sheep retrovirus is associated with ovine pulmonary carcinoma in Sardinian moufflons. *Journal of Comparative Pathology* **125**, 145-152

SHARP, J. M. & DEMARTINI, J. C. (2003) Natural history of JSRV in sheep. *Current Topics in Microbiology and Immunology* **275**, 55-79

SIGURDSSON, B. (1954) Rida, a chronic encephalitis of sheep: with general remarks on infections which develop slowly and some of their special characteristics. *British Veterinary Journal* **110**, 287-302

SUMMERS, C., NEILL, W., DEWAR, P., GONZÁLES, L., VAN DER MOLEN, R., NORVAL, M. & SHARP, J. M. (2002) Systemic immune responses following infection with Jaagsiekte sheep retrovirus and in the terminal stages of ovine pulmonary adenocarcinoma. *Journal of General Virology* **83**, 1753-1757

Further reading

FAN, H. (Ed) (2002) Jaagsiekte Sheep Retrovirus and Lung Cancer. Current Topics in Microbiology and Immunology, Volume 275. Berlin, Springer-Verlag

PALMARINI, M. & FAN, H. (2001) Retrovirus-induced ovine pulmonary adenocarcinoma, an animal model for lung cancer. *Journal of the National Cancer Institute* **93**, 1603-1614

PALMARINI, M., MURA, M. & SPENCER, T. E. (2004) Endogenous betaretroviruses of sheep teaching new lessons in retroviral interference and adaptation. *Journal of General Virology* **85**, 1-13

SHARP, J. M. & DE LAS HERAS, M. (2000) Contagious respiratory tumours. In Diseases of Sheep, 3rd edn. Eds W. B. Martin and I. D. Aitken. Oxford, Blackwell Science. pp 181-186

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1. Dohmen, M J W et al (1995) Theriogenology 43, 1379-1388. 2. LeBlanc, S (2003) Cattle Practice 11, 255-261.
3. Esslemont R J and Kossaibati, M (2002) DAISY Research Report No 5. 4. McDougall, S (2003) Cattle Practice 11, 271-283.

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Trading structures for veterinary practices 1. The options

TRADITIONALLY, the options for structuring a veterinary practice were restricted to the sole principal set-up or the partnership and, although the RCVS relaxed the rules governing trading structures for practices some years ago, these remain the most common trading vehicles in the profession. In the first of two articles, Andy Moore looks at the different trading structures that are available for veterinary businesses and, in the light of changes in the tax treatment for some of these structures, outlines the various opportunities and drawbacks that come with each one.

ANDY MOORE

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THERE have been various developments over recent years regarding the available trading structures, and the taxation thereof, not only for veterinary practices but for businesses generally. This, combined with changes in the tax treatment for some of those structures, means that there is a need for businesses to review their position to establish whether there are potential benefits (or pitfalls) of a change in trading structure.

In order to consider the position fully, we need to look at three areas:

- The different trading structures that are currently available within the UK and the benefits and disadvantages of each of them.
- The potential tax savings of incorporation – if incorporation is the route selected, what are the potential financial benefits for the practice? This will be discussed in detail in a second article, to be published in the September issue of *In Practice*.
- The potential opportunities and pitfalls of any changes in trading structure.

THE ALTERNATIVE STRUCTURES

Broadly speaking, there are four potential structures: the sole principal set-up, the partnership, the limited company (that is, incorporation) and the limited liability partnership. A fifth, 'halfway house' option, that of the service company, is also briefly reviewed.

This article does not consider joint venture arrangements, nor the possibility of limited company partners, which has come back into fashion and is being suggested by some advisers at present. The former are relatively well developed arrangements evolved by specific corporate entities. Suffice to say, they are not perfect for everyone but, for some, they present the ideal route into veterinary practice. In the case of limited company partners, a limited company is formed and becomes a partner in an existing partnership. Funds are then diverted through to the company as profit share which, in turn, is drawn as dividends from the limited company. While this arrangement can produce some tax savings, these are at a lower level than the other options considered here.

FACTORS INFLUENCING CHOICE OF STRUCTURE

Until relatively recently, the RCVS would not permit a veterinary practice to trade in any vehicle other than a sole principal or partnership structure. These are, therefore, by far the most common trading structures within the profession. In the late 1990s, it became permissible for veterinary practices to trade through a limited company; the rules regarding non-veterinary ownership of veterinary practices were also relaxed.

Up to now, there has not been a great uptake by practices wishing to transfer ownership to a different structure. However, with effect from April 1, 2002, a change in the way in which limited companies are taxed has increased the attractiveness of incorporation. Specifically, the rates at which they pay tax were altered, allowing a company to earn up to £10,000 in a year and pay 0 per cent tax. At about the same time, the pensions legislation was reformed, with the result that it was no longer necessary to have earnings every year in order to be able to fund a pension contribution. This has allowed profits to be extracted by way of dividends as opposed to salary, and that has brought with it a National Insurance (NI) saving which grows as NI rates increase. The latest Budget countered this use of the rules but, in short, the changes only reduce the savings where total profits are under £50,000 per year.

The following sections will consider each of the potential trading structures in more detail.

SOLE PRINCIPAL AND PARTNERSHIP

These two trading structures can be considered together due to their very similar treatment as far as tax and legalities are concerned. As mentioned above, they are the most common structures that exist within the veterinary profession.

The structures of sole principal and partnership are the most flexi-

ble, and both are very easily formed. They are also the cheapest in terms of professional fees to set up and to run. In addition, one of the major attractions of these structures is the complete privacy of the individual's, or partners', financial affairs.

Most veterinary surgeons will be familiar with the overall financial workings of the sole principal/partnership structure in that the principal or partners take drawings from the practice. Hopefully, these drawings are less than the profits generated. The tax paid by the principal or partners is calculated on the profits generated by the practice, rather than the amounts withdrawn or introduced into the practice, and the tax liability is paid in three instalments during the course of the year, in January, July and a balancing payment in the next January. Tax returns have to be completed for the partnership and partners or for the sole principal.

Normal business regulations apply to these business structures. There are, of course, additional requirements for veterinary practices, such as those relating to the COSHH regulations and the Medicines Act, the need for professional indemnity insurance, and so on.

Legal aspects

If the practice trades as a partnership then it is normal (though not obligatory) to have a partnership agreement. If there is no partnership agreement then, in the event of a dispute or any decisions being required, one would revert to the Partnership Act 1890, although this is obviously not ideal and should be considered the last resort.

Finally, there is joint and several unlimited liability in both of these structures. If something happens to affect the financial affairs of the partnership adversely then the partners' or sole principal's personal financial position is also affected.

LIMITED COMPANY

The major difference between limited companies and any other structure is that there are completely separate legal identities for those owning the business (shareholders) and those running the business (directors). Directors and shareholders do not necessarily need to be the same individuals, although for many small privately owned companies this is often the case.

The roles of director and shareholder have distinct responsibilities, which will not be considered in depth in this article. Suffice to say, it is important not to lose sight of the distinction.

If we first consider the drawbacks to trading through a limited company we can then go on to consider the distinctions and the benefits.

Drawbacks

The main drawbacks to trading through a limited company can be summarised as follows:

- The accounts have to be filed with the Registrar of Companies and are available for inspection by the public. For small and medium-sized companies these can be lodged in abbreviated form and, therefore, the amount of financial information available to the public will be reduced. The Companies Act is currently in the process of being reviewed. One proposal contained within the revised Companies Act is that there is full financial disclosure of the profit and loss account for the limited company. However, even if this is applied, it is likely that the figures disclosed will be in a reduced format when compared with normal partnership accounts.
- Following on from the above, accounts have to be prepared in a format specified by the Companies Act 1985. Additionally, if the turnover of the practice is in excess of £5·6 million (from March 30, 2004), there is a requirement to undergo an audit. This means an increase in the professional fees payable by the company. Where the shareholders and directors are the same individuals, some believe there is little added value.
- There are additional deadlines relating to filing of the accounts and the annual return at Companies House. Failure to meet these deadlines results in an automatic fine. It is therefore a regulated environment, and one in which good business organisation is essential.
- Corporation tax, which is based on profits retained (before dividends) by the company, is usually paid in one instalment, nine months after the company's year end. This can have an impact on cash flow.
- The directors of the limited company, who are equivalent to the partners in a partnership, are employees of the company and, as such, are subject to the employment tax regime; for example, if they are provided with a car by the company then that is treated as a company car and taxed as a benefit in kind.
- There will be larger professional fees incurred in running a company, mainly due to the increased work required on the annual accounts.

Distinctions

The main distinctions between a limited company and other structures can be summarised as follows:

- As mentioned above, the company and the individuals involved with the company have separate legal identities. The shareholders own the company and the directors run it. For veterinary practices, the clinical directors of the company, who control the clinical decision-making, must all be members of the RCVS; care needs to be taken in this area to ensure control procedures are in place to avoid any (say financial or income-generating) decisions by a non-MRCVS impacting on the clinical decision-making.
- The profits (which, in a partnership, are taken as drawings by the partners) are taken by way of salary for the directors, in respect of the work that they undertake; and as dividends by the shareholders, in respect of the return on the capital they have invested.

Benefits of incorporation

So, having considered the drawbacks and differences, why might

Forming a limited company

Limited companies are very straightforward to set up in that they can be purchased 'off the shelf' for approximately £100. If the word 'veterinary' is used within the name of the company then approval has to be sought from the RCVS before incorporation; indeed, the Registrar of Companies will not permit the use of the word without this approval. To an extent, the simplicity ends there.

Limited companies are governed and subject to additional regulatory requirements over and above those applied to a normal trading business. The Registrar of Companies is concerned with the filing of various documents, such as accounts and annual returns. The company must have a Memorandum and Articles of Association, which are its 'rule book'; these documents set out what the company can and cannot do (as it has no mind of its own). The directors must run the company in accordance with this rule book. In addition, they have to produce financial statements in a format laid down by the Companies Act 1985 and in accordance with financial reporting standards currently in force. On top of this, there is a considerable amount of additional regulation involved.

Service company

The final option to consider – the service company – is really a 'halfway house' between a partnership and a limited company. In this structure, in outline, a limited company is set up to run alongside the partnership. The limited company employs all the members of staff and buys in the supplies. The company then sells those supplies and staff to the partnership at a mark-up. The level of mark-up is quite closely regulated, as there is scope to manipulate the figures – which the Inland Revenue is keen to stamp out.

The effect of this arrangement is that profits are held within the limited company, where they are taxed at a lower rate than would apply within the partnership – where profits would be taxed at the highest rate of tax, currently 41 per cent. This vehicle therefore provides potential savings, albeit less than full incorporation of the entire practice, but still an improvement on the position as compared with a partnership.

This does necessitate running a second business, with a second VAT registration, a second bank account – and incurring a second set of accountancy fees! However, the transactions are limited and costs should not be that great.

The savings across the practice are potentially 41 per cent on the first £10,000 of profits if retained (15 per cent if paid out), and then roughly 20 per cent on the next £40,000 of profits if retained (or 1·75 per cent if paid out), and approximately 20 per cent on the balance of profits included within the limited company. This is a saving across the entire practice, rather than per partner. Therefore, practices with more partners will make a smaller saving per partner.

practices consider incorporation? Some reasons are outlined below, and there will be further discussion on this in the next article.

- A starting corporation tax rate of 0 per cent, rising to 19 per cent for profits up to £300,000, and combined with the reform of the pensions legislation, has increased the incentive for practices to look at this alternative. Even after the latest Budget changes (Gunn 2004), savings could be made by many practices.
- The potential tax and NI savings are at a reasonable level and warrant practices giving further consideration to incorporation.
- The main reason for limited companies being developed, many years ago, was to limit the liability of those starting in business or going into business. This benefit still attaches to the limited company structure, but many are unaware of it. The limitation of liability applies assuming the governing regulations and legislation have been compiled with.
- A corporate structure can help with succession planning and in planning exit strategies for senior partners.

LIMITED LIABILITY PARTNERSHIP

Limited liability partnerships were created to a large extent by the big accountancy practices, prompted by concern at the increase in litigation, in terms of both the frequency and the size of claims. Professional partnerships were keen to find ways in which they might be able to protect the family assets. The result was a hybrid arrangement, a limited liability partnership. This is formed, regulated and governed in a very similar manner to a limited company. The liabilities of individual partners are restricted to an extent, although there is not a complete limitation of liability.

Limited liability partnerships are very similar to a partnership as far as tax and internal running are concerned. The partners within a limited liability partnership are called members; rather than having a partnership agreement, they have a members' agreement.

This structure has been in force for a relatively short period of time and, initially, commentators felt it would be the vehicle of choice. However, there has been very little take-up of trading through this structure. Essentially, limited liability partnerships appear to suffer the heavier regulation of a limited company without any of the associated tax benefits. The tax is calculated as though for a partnership and, therefore, unlike a limited company, there is no tax advantage to changing to a limited liability partnership.

In general, this structure should probably be avoided by veterinary practices unless a real and substantial uninsured risk may exist and a limited company arrangement is not appropriate. At the end of the day, this structure was designed by accountants for accountants, auditors and lawyers; in general, anything designed for those sectors should be avoided, due to complexity and expense.

TIME TO TAKE STOCK

As discussed above, recent changes in legislation have forced businesses generally to consider incorporation. Within the professions there has generally been a very slow uptake of incorporation. Taking the accountancy profession as an example, it has been possible to trade through a limited company since 1989 but, until recently, there had been relatively few incorporations. Within the past year, however, there has been a rush of incorporations, driven by businesses looking to maximise their tax benefits.

Historically, one of the reservations held by the professions with regard to incorporation was the lack of financial privacy. However, given the limited nature of the information held on public record regarding finances, an individual would need a certain degree of financial knowledge and ability to be able to interpret the figures and ascertain likely earnings in a year. Of course, they would also have to have the desire to do so. We always remind practices of the fact that if clients are happy with the service they have been provided with, and they feel they have received value for money, then they will have no desire to search out how much money the local vet has made but, rather, will sing the vet's praises to their acquaintances. The situation may be different with regard to competitors, but this type of information is available in the general commercial world for many other types of business.

There is always a concern that the Government may change the legislation so that the position becomes less advantageous. If that is the case, it is possible under the current legislation to transfer back to a partnership relatively easily, but it does require the practice to be 'on the ball' in order to react to changes.

Incorporation may not be the right move for every practice, but I would certainly advocate that practices review their own position to decide one way or another, rather than simply continue as they are through apathy or inertia.

Reference
GUNN, D. (2004) Budget 2004 – implications for veterinary practice. *In Practice* **26**, 282-283

Practice microscope – toy or tool?

IN this article, Donald Mactaggart argues that the microscope is possibly the most important tool in a veterinary practice. If used to its full potential it is also one of the most cost-efficient. Treat it with the respect that it deserves and the practice will be amply repaid.

DONALD MACTAGGART

REQUIREMENTS OF A PRACTICE MICROSCOPE

The minimum requirements for the practice setting are a binocular microscope with four objective lenses – x4, x10, x40 and x100 (oil immersion). The eyepieces contain x10 lenses, thus giving final magnifications of x40, x100, x400 and x1000. Older, monocular microscopes without an integral light source are not adequate for use in the practice.

The choice of magnification depends on the specimen to be examined: x40 (ie, using the x4 objective) is mainly used for examination of large ectoparasites such as fleas, ticks, lice and *Trombicula* species mites; x100 is more useful for identifying smaller mites, such as *Demodex* and *Sarcoptes* species, or for examination of samples of urine for crystals or faeces for worm eggs; x400 is mainly used for high-power scanning cytology of tissue aspirates and blood smears; and x1000 (oil immersion) is used for detailed cytology and microbiology (eg, examination of bacteria or yeasts).

The microscope settings will need to be adjusted depending on the material being examined. It is these movable parts that are the most likely to wear out or be damaged in old or cheaper instruments. The quality of these movable parts should be checked before purchase. The controls for the diaphragm, condenser, stage and focus should be smooth to use and hard-wearing. The iris diaphragm and condenser knob are adjusted to vary the amount of light and contrast within the sample. When performing cytology, the condenser and diaphragm should be opened up, allowing maximum light onto the slide. When examining skin scrapings or urine crystals, the condenser and diaphragm should be turned down to reduce the intensity of light. This will increase the contrast, making the sample less transparent and easier to see.

(left) A good quality binocular microscope, suitable for practice use. An old, monocular microscope with no integral light source (pictured right) would not be adequate in the practice setting

CARE OF THE MICROSCOPE

A microscope is a precision instrument and should be treated with respect. It should be kept in a convenient area, away from vibration, with a comfortable seat provided to avoid operator discomfort after frequent use. The microscope should not be moved unnecessarily. The lenses should be cleaned on a daily basis, using lens tissue wipes to avoid scratching them. The oil immersion lens should be wiped with spirit and dried after each use. The microscope should be protected with a dust cover when not in use.

It may be useful to have a full service carried out every five to 10 years, but with good routine maintenance this may not be necessary.

PRACTICAL MICROSCOPY

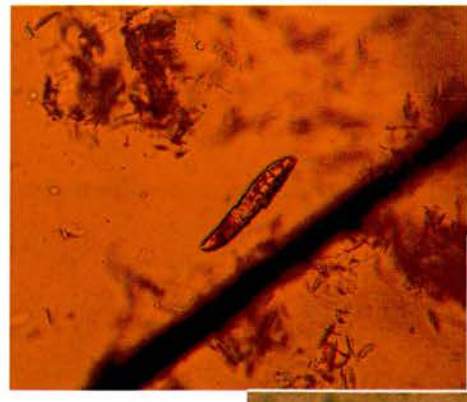
A microscope will be principally used in the practice for examination

Donald Mactaggart qualified from Edinburgh in 1988. He is a partner at the Thistle Veterinary Health Centre in Edinburgh, a four-vet small animal practice which receives referrals in dermatology and dentistry. He gained the RCVS certificate in small animal dermatology in 1991 and has been close to his microscope ever since.

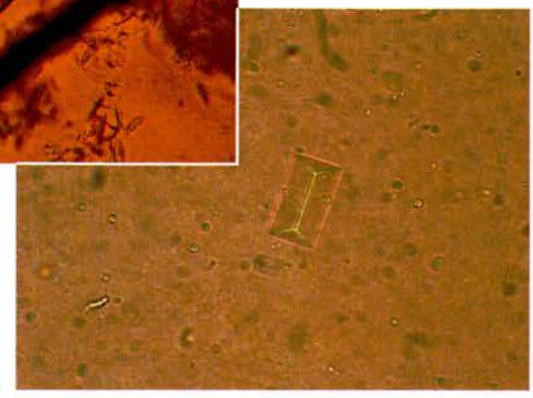
Successful microscopy

Successful microscopy depends upon good sample collection, a good quality microscope and good interpretation/diagnostic skills.

One of the main advantages of examining samples microscopically yourself is the rapid result. Another advantage is that one knows if the sample collected is of good quality straight away and, if it is not, it is simple to collect another one. The main limiting factor is your own diagnostic skills, but these will improve with experience. If in doubt, send the sample off to a more experienced clinician for assessment.



An adult *Demodex canis* mite (original magnification x100). Skin scrapings should be taken and examined microscopically in any case of suspected skin disease



A struvite urinary crystal (original magnification x400)

of skin scrapings, for cytology, and for examining samples of urine, faeces and blood.

Skin scrapings

Skin scrapings are indicated in any animal with skin disease. Taking them involves minimal time and cost and can give that rare thing in dermatology, an instant diagnosis! If you are struggling to differentiate *Demodex* mites from hair, it is probably time to buy a new microscope.

Cytology

Cytology has many and varied applications (see box below), yet is underused in practice. Again, it can provide rapid results at minimal cost.

The most common indications for cytology in small animal practice are microbial examination of the ears or skin, and examination of cutaneous 'lumps'. Cytology can guide us on what therapy to use for the infection or what action should be taken with the 'lump'.

With experience, the veterinary practitioner can soon become familiar with basic cytology, for example, differentiating inflammation from neoplasia or bacterial from yeast infection. However, detailed cytological interpretation, such as the classification of neoplasia, requires the experience of a trained cytopathologist.

When evaluating cytological samples, it is a good idea to take two samples, examine one yourself and send the other to a trained cytologist. This allows you to assess whether your sample collection technique is good before sending the sample off to the lab; in addition, your learning curve will increase dramatically if you can compare your interpretation with that of the expert.

Urine samples

Urine samples are examined microscopically mainly for the presence of crystals. Cytological examination is also useful in checking for infection and neoplasia in the urinary tract.

Faecal examination

With faecal samples, microscopy is mainly carried out to assess the presence of endoparasites and their ova, but special stains can also be used in investigating malabsorption/maldigestion.

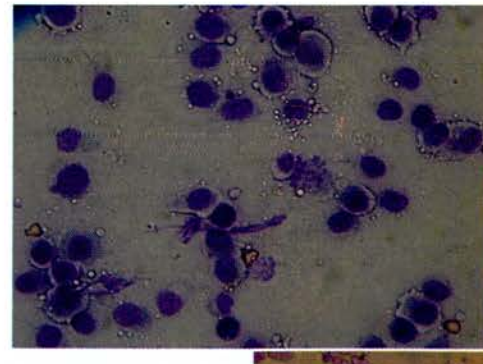
Blood smears

A blood smear should always be examined microscopically when a blood sample is taken for haematology.

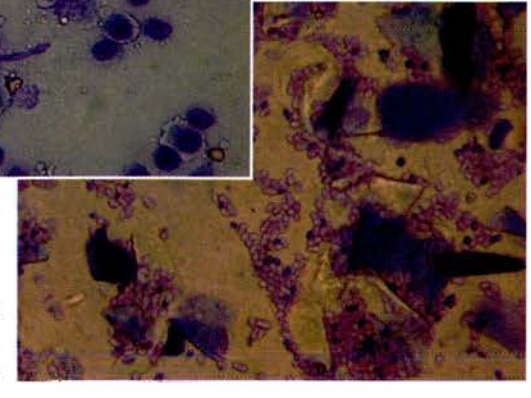
General indications for the use of cytology

Examination/assessment of:

- Thoracic or abdominal effusions
- Respiratory aspirates/washes
- Organomegaly – liver, kidney, prostate, spleen
- Unidentified abdominal masses
- Lymphadenopathy
- Urine sediment
- Cutaneous masses/ulcerative lesions
- Disease of the gastrointestinal tract
- Reproductive system



Cytological examination of a fine needle aspirate from a histiocytoma (x1000, oil immersion)



Cytological preparation showing *Malassezia* yeast in canine skin (original magnification x1000)

Making the microscope work for you: costs and fees relating to microscopy

1. Cost of consumables required for microscopy (based on 2004 list price, excluding VAT)

	Cost per pack (£)	Number in pack/quantity	Cost per preparation (£)
Skin scrapes			
Microscope slides	5.36	50	0.11
Coverslips	2.25	100	0.02
Scalpels	12.00	100	0.12
Liquid paraffin	5.17	2000 ml	0.005*
Total per preparation			0.25
Cytology			
5 ml syringes	4.19	100	0.04
23 G needles	4.25	100	0.04
Microscope slides	5.36	50	0.11
RapiDiff stain (Triangle Biomedical Sciences)	40.00	500 ml	0.08*
Coverslips	2.25	100	0.02
Microscope oil	7.45	100 ml	0.01*
Lens tissue wipes	2.73	25	0.11
Total per preparation			0.41
Examination of urine samples for crystals/faecal samples for eggs			
Centrifuge tubes	Free from laboratory		0.00
Disposable pipettes	2.85	50	0.06
Microscope slides	5.36	50	0.11
Coverslips	2.25	100	0.02
Total per preparation			0.19

*Approximate costs, based on the estimated volume used per slide

2. Fees generated by microscopy

The following costings are based on the work carried out in a three-man small animal practice over one month (February 2004). No referral work has been included; haematology is outsourced, so no blood smears are examined. All prices are exclusive of VAT

	Fees charged per sample (£)	Consumable costs per sample (£)	Fees – costs (£)	Samples examined during the month	Income generated during the month (£)	Approximate income per year (£)†
Cytology	11.70	0.41	11.29	23	259.67	3277
Skin scrapes	16.25	0.25	16.00	17	272.00	3433
Urine analysis	16.25	0.19	16.06	6	96.36	1216
Faecal analysis	16.25	0.19	16.06	1	16.06	203
Total						8129

†Calculated for a leap year as (monthly income ÷ 29) x 366 and rounded to the nearest £

Predicted fees generated minus consumable costs, per full-time veterinary surgeon, per year **£2710**

FINANCIAL AND OTHER BENEFITS

A good basic microscope can be bought for under £500 but, as with most equipment, you get what you pay for. If you have a particular interest in microscopy then the extra investment in a superior instrument is well worthwhile. Even if you choose a top quality microscope, costing £1500, your costs should soon be recovered.

The analysis presented in the box above suggests that a full-time veterinary surgeon should be able to generate approximately £2700 per annum through microscopy work. This could easily triple if there was someone in the practice with a specific interest in dermatology or cytology.

Non-financial benefits

In addition to the large amount of revenue generated for a relatively small outlay, microscopy can provide other, non-financial benefits to the practice. Most of all, the clinical interest of the veterinary surgeon is maintained; veterinary nurses, too, are often interested in laboratory work and, by extension, may also enjoy microscopy. It is very satisfying to be able to collect a sample, analyse it and give your client the result within minutes. Clients appreciate this rapid turnaround, and the microscope is also useful for client edu-cation: it is always easier to convince clients that their pet has an ectoparasite if they can see the culprit themselves!

RESPECT YOUR MICROSCOPE

A microscope should last at least 10 years; a good quality one should last twice this if it is well looked after. I cannot think of another piece of equipment in veterinary practice that costs so little, generates so much income and has such a long lifespan.

Take care of your microscope and it will take care of you – as well as your patients.

Further reading

HILL, P. B. (2002) Performing and interpreting diagnostic tests. In Small Animal Dermatology. Oxford, Butterworth-Heinemann. pp 148–230
RASKIN, R. E. & MEYER, D. J. (2001) Atlas of Canine and Feline Cytology. Philadelphia, W. B. Saunders

Visiting lectureship: providing lessons in the art of veterinary practice

AS the veterinary curriculum gets increasingly crowded, opportunities for general practitioners to show students something of the art of the profession are becoming fewer and need to be grasped. For Alan Leyland, an invitation to talk to third-year veterinary students about practical aspects of antibiotic use proved a good way for him to share his knowledge and give something back to his profession. There were other benefits, too. The experience provided an interesting diversion from practice life – and a chance to learn something himself.

ALAN LEYLAND

Alan Leyland qualified from Liverpool in 1968 and works in small animal practice in Wallasey, Merseyside. He was a visiting lecturer to the University of Liverpool between 1998 and 2002. He was president of the Society of Practising Veterinary Surgeons in 1998/99.

OVER a period of four years, I was asked to spend an afternoon each year with the third-year veterinary students at the University of Liverpool, talking about 'the use of antibiotics in practice' as part of their infectious diseases course.

The invitation originally came out of the blue from the lecturer who was in charge of the course at the time. Not being a veterinary surgeon herself, she saw the benefit of inviting an ordinary general practitioner to talk about practical aspects of the topic. Although in former days I had lectured at the university in clinical pathology, I am fairly sure that the invitation was extended to me as a local vet in practice rather than as a former lecturer.

I had no specialised knowledge of the pharmacology or esoterics of antibiotics. So, could I provide something of benefit to the students and, in so doing, enhance my own interest in the subject?

PREPARING FOR THE TASK

As I was not expected to do much more than tickle the students' interest and make the academic studies they had been doing relevant to life in practice, you might think that the necessary preparation was minimal, but I did spend a fair amount of time considering my approach and then checking many of the points I wished to make in textbooks and journals. The only guidance I had been given when I was invited to contribute was 'tell the students how you make an informed choice of antibiotics for particular cases' – which was, of course, a deceptively simple sounding request!

I believe that the best possible way to undertake personal CPD is through preparing a presentation, and, even though I had no intention of lecturing on the scientific minutiae, I was certainly stimulated to improve my own knowledge of the subject.

Bridging the gap between academia and practice

The concept was to stimulate the interest of the students, at a time when the enthusiasm with which they had ventured into the veterinary faculty in their first year was waning to a low point before commencment of their clinical studies, as well as to make the course content relevant to the job for which they were all, in the main, aiming.

Pressures on university lecturers to gain academic experience, and discouragement from undertaking locums, as had been more common in former times, means that there is an increasing dearth of staff with real practice experience. I therefore viewed becoming involved with lecturing the students as a very small campaign on my part to redress the balance, and to give them the benefit of a little bit of practical know-how.

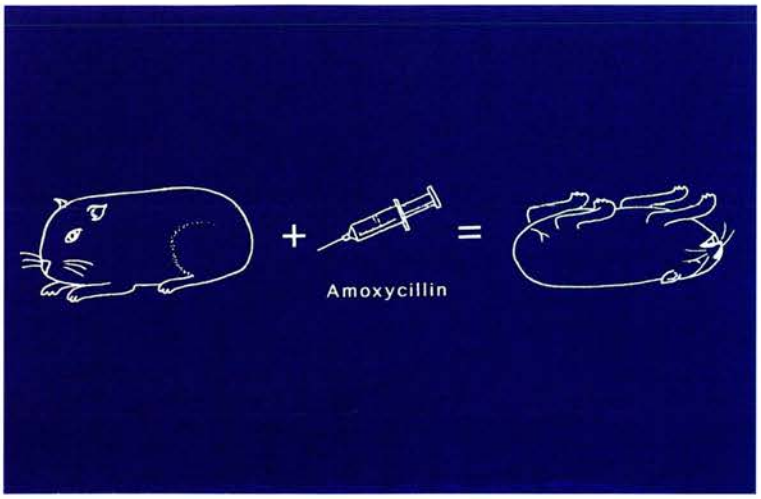
AN INTERACTIVE APPROACH

My approach was to pose a series of questions and statements to the students and encourage them to respond and interact. This worked well, with the students entering into the spirit of a very 'knockabout' session. My first assertion was that 'antibiotics are the most abused and overused drugs in our armamentarium'; secondly, I made the cynical, but true, observation that 'therapy works best where there is a natural

A job for everyone?

Many vets might be daunted by the prospect of speaking to a group of students for an afternoon, but if they were prepared to have a go many would enjoy it.

The students relate very well to anyone in practice. Clearly they see themselves as being there soon and realise they might not be ready, having concentrated on academic and practical knowledge. I recently spent a full day taking part in a communication training day for fourth-year students. The day involved having to play out scenarios with trained actors. I found the students' reaction to me, as one at the 'coal face', to be very positive.



A cartoon used by the author to encourage students to think about the importance of making the correct choice of antibiotic drug

tendency for resolution' and that, therefore, it is important to 'do no harm'.

I chose not to include anything about, for example, 'macrolide' antibiotics or bactericidal versus bacteriostatic modes of action, as I presumed the students had already learned those sorts of things as part of the formal lecturing they had received. Rather, I asked, in a given situation, 'should an antibiotic be used at all?'

Practical emphasis

To make the point that I was giving an essentially practical discourse, I started by asking the students to compare the costs of a 10-day course of four different antibiotics for a 20 kg collie: oxytetracycline, combined trimethoprim and sulphadiazine, potentiated amoxycillin and enrofloxacin. To encourage them to think about the impact of these costs on the client, I also asked them what the current old age pension was worth, which most students knew!

Next, consideration was given to the activity of antibiotics against specific organisms, and when it was appropriate to carry out sensitivity and resistance testing. A discussion of routes of administration raised many interesting concerns regarding, for example, client compliance, and achieving suitable blood levels in some compromised animals with poor ability to absorb drugs from the alimentary tract.

Making the correct choice of antibiotics for various sites in the body was discussed in respect of issues such as the acid pH of the prostate affecting the in vivo activity compared to in vitro activity, and efficacy in the presence of pus or fibrosis, or at a site in bone. Adverse reactions and their reporting, compatibility with other medications, legislation, particularly the 'cascade', and suitability for age and species were all of great interest to the students.

Encouraging debate

Finally, by working through a series of hypothetical cases, I attempted to persuade my audience that although antibiotics are essential for some conditions and surgery, their use should be unnecessary for cases such as lump removals, spaying and neutering, or, for example, in the mildly ill dog with 'gastritis', unless there is blood or pyrexia. This caused some disquiet and debate among the students about sterile technique, and so on, as many of them had seen antibiotics used in these situations in practice.

POSITIVE REACTIONS

The first year I spoke to the students I was one of three vets who had been invited, but in following years I was invited to lead the discussions for the whole afternoon – so I suppose I must have been OK at it!

Each year, I would change my presentation to make it 'fresher', both for the benefit of the students and for myself. I always had a great reaction from the students, who would queue up after the session to ask further questions or arrange EMS at my practice.

On a nostalgic note, returning to the same lecture theatre where, more than 30 years ago, I sat with 40 of my peers, now stuffed with 100 students, was a strange experience.

OTHER OPPORTUNITIES

Over time there were changes to the personnel at the university and, I believe, it was an oversight one year for me not to be asked to speak again. Although there was some sympathy for my lecture to continue, I think pressures on the syllabus overrode this.

However, since then, I have been given other opportunities to speak to groups of students and vets in practice. I used the same material on the use of antibiotics when sent by the Society of Practising Veterinary Surgeons (SPVS) to speak to the Bristol students' clinical club. I also get to make presentations to other groups – twice so far at BSAVA satellite management days, last year and this, and the occasional clinical seminar.

I am not sure you can go looking for these opportunities so much as accept them if they come along, but, as some vets would run a mile if invited to make this kind of presentation, perhaps it helps to be known as someone who is keen to become involved. Many of my opportunities to speak have sprung from being an ex-president of SPVS, as well as being known to have strong views on some issues!

TIME WELL SPENT

I enjoyed the experience of speaking to the students immensely. At the time I was invited to speak I had not thought about remuneration, and was surprised to receive a modest fee – although the amount would certainly not count as a 'practice builder' and would not in itself be a reason for doing the sessions.

Overall, would I recommend being a visiting lecturer as a way of adding interest to a regular job in practice? Most definitely: it was a good way to do my own learning and, of course, provided an opportunity to give something back to the profession from which I have profited. In addition, once you have been in practice for many years, anything which breaks up the week is a good thing!

A partner ponders

ALMOST daily, the cheerful, cherubic figure of Declan Curry waxes lyrical on breakfast TV about the financial markets here and abroad. Apparently innocently, he prefaces his prediction for the opening moves of the London financial market with the depressing phrase 'and the bookies predict'. His clear declaration that the performance of my hard-earned pension pot is part dependent on professional gamblers does not inspire confidence. Nor does the performance of my managed pension funds inspire me, as it seems that without inside information, luck or exceptional expertise it is impossible for fund managers to consistently outperform the indices. The old axiom 'You can't beat the market' appears a truism.

These financially morbid thoughts were well implanted in my psyche when, three years ago, Her Majesty's Government launched the 'stakeholder pension' scheme. As a non-intervener in the financial affairs of others, I was obligated to select a stakeholder scheme for our staff. Conscious then of the pensions misselling scandal of a previous generation, I elected to adopt safety first mode and go with a major pensions company then confidently described as the largest mutually owned insurance company in Europe and with multibillion pound reserves.

Thankfully, my negative expectations of the stakeholder scheme seem to have been contagious and none of our staff has taken out such a scheme through the practice. In the intervening period, the index of top 100 UK companies has declined by 25 per cent (from 6000 to 4500 as I write), the insurance company then defiantly mutual is in the throes of demutualisation and the multibillion pound reserves, yes you've guessed it, all but gone as a result of the company's decision to retain high exposure to equities when others moved into bonds. Our stakeholder scheme has been a compliance paper exercise, apparently like 80 per cent of all such schemes set up by employers; it is an empty shell with no members and seems destined to remain so unless, or rather until, employer contributions become mandatory.

Only recently did I realise that our staff had launched their own investment scheme which involved weekly contributions to a mutually owned, low-cost fund with the potential for almost unlimited returns. Investing in the National Lottery via a practice syndicate is admittedly a high-risk investment strategy but does benefit from transparency – most weeks you are almost guaranteed to lose, but the adrenaline rush more than compensates for this when one or two numbers come up.

Most financial articles conclude with a confident tip, and this is no exception. I suggest that all partners participate in practice lottery syndicates – for when such a syndicate enjoys a multimillion pound win, the beneficiaries may opt to desert the practice 'en masse' and, by sharing in the win, you will have the option of doing likewise!

The monthly CPD guide

IN each issue, *In Practice* publishes information for veterinary surgeons about courses and meetings in the months ahead. Details of forthcoming events which might be included in this CPD guide should be sent to 'Coming Up', *In Practice*, 7 Mansfield Street, London W1G 9NQ, fax 020 7637 0620, at least two months before the event is due to take place. A more extensive listing of courses and events, which is updated weekly, appears in the CPD calendar on *The Veterinary Record/In Practice* website at www.vetrecord.co.uk

● denotes new entry

AUGUST

3

● **Introduction to small animal ultrasonography**
Course, to be held in Newmarket. Details from Improve International, *telephone 01793 759159, fax 01793 759259, e-mail: enquiries@improve-international.com*

5

● **Exotics: rabbits and rodents**
Course, to be held in Leeds. Details from Improve International, *telephone 01793 759159, fax 01793 759259, e-mail: enquiries@improve-international.com*

10

● **Ophthalmology: examination of the canine and feline eye**
Course, to be held in Dublin. Details from Improve International, *telephone 01793 759159, fax 01793 759259, e-mail: enquiries@improve-international.com*

11

● **Small animal medicine 2: the vomiting cat and dog**
Course, to be held in London. Details from Improve International, *telephone 01793 759159, fax 01793 759259, e-mail: enquiries@improve-international.com*

12

● **Emergency medicine: emergency and critical care techniques**
Course, to be held in Cricklade, Wiltshire. Details from Improve International, *telephone 01793 759159, fax 01793 759259, e-mail: enquiries@improve-international.com*

13 to 14

Soft tissue surgery
Workshop, organised by Glasgow University, to be held at the veterinary school. Details from the CPD Unit, *telephone 0141 330 4995, fax 0141 330 2256, e-mail: cpd@vet.gla.ac.uk*

13 to 14

● **Surgery of the upper airway: how to diagnose and deal with upper airway disease in dogs**
Course, to be held in Newmarket. Details from Improve International, *telephone 01793 759159, fax 01793 759259, e-mail: enquiries@improve-international.com*

16 to 20

Association of Avian Veterinarians
Conference, to be held in New Orleans, USA. Details from Mr J. Chitty, *telephone 01264 352323, fax 01264 336296, e-mail: strathmoreveterinaryclinic@btopenworld.com*

17

● **Cytology: applications and techniques for cytological investigation in small animal practice**
Course, to be held in Leeds. Details from Improve International, *telephone 01793 759159, fax 01793 759259, e-mail: enquiries@improve-international.com*

19 to 20

● **Orthopaedics level 1 – beginners**
Course, to be held in Cricklade, Wiltshire. Details from Improve International, *telephone 01793 759159, e-mail: enquiries@improve-international.com*

23 to September 3

● **OVS training**
Course, organised by Glasgow University, to be held at the veterinary school. Details from the CPD Unit, *telephone 0141 330 4995, fax 0141 330 2256, e-mail: cpd@vet.gla.ac.uk*

24

● **Endoscopy: rigid endoscopy in small animal practice**
Course, to be held in Cricklade, Wiltshire. Details from Improve International, *telephone 01793 759159, fax 01793 759259, e-mail: enquiries@improve-international.com*

25

Small animal ultrasonography
Course, organised by BCF Technology, to be held in Huntingdon. Details from Miss T. Lindsay, *telephone 01506 460023, fax 01506 460045, e-mail: office@bcftechnology.com*

25

● **An update on aspects of feline medicine**
Course, to be held in London. Details from Improve International, *telephone 01793 759159, fax 01793 759259, e-mail: enquiries@improve-international.com*

25 to 28

World Congress of Veterinary Dermatology
To be held at the Hofburg Conference Centre, Vienna, Austria. Details from Ärztezentrale Med Info, *telephone 00 43 1 531 1671, fax 00 43 1 531 1661, e-mail: azmedinfo@media.co.at*

27 to 28

Advanced equine dental technicians
Course, organised by the British Equine Veterinary Association, to be held in Hartpury. Details from the Administrative Office, *telephone 01223 836970, fax 01223 835287, e-mail: arabella@beva.org.uk*

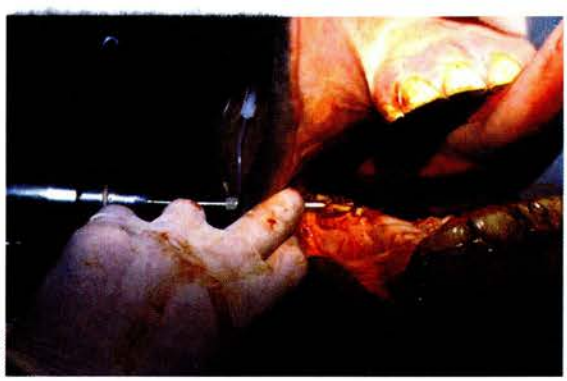
SEPTEMBER

1

Cattle fertility
Conference, organised by the British Cattle Veterinary Association and Nottingham University, to be held at Sutton Bonington. Details from the BCVA Office, *telephone 01452 740816, fax 01452 741117, e-mail: office@cattlevet.co.uk*

3 to 4

Abdominal surgery
Course, to be held in Ellesmere Port and Liverpool. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555 e-mail: info@cpd-solutions.com*



BEVA

Advanced equine dental technicians course, August 27 to 28

5

Veterinary education and other health professions: what can we learn from each other?
Workshop, part of the Association for Medical Education in Europe Conference, to be held in Edinburgh. Details from Ms C. Bell, *e-mail: C.Bell@vet.gla.ac.uk*

6

Equine dentistry for veterinary surgeons
Course, organised by the Royal (Dick) School of Veterinary Studies, to be held at Easter Bush. Details from the CPD Unit, *telephone 0131 651 1180, e-mail: cpd@ed.ac.uk*

7

Introduction to abdominal ultrasound
Workshop, to be held in Cheltenham. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

7 to 8

Diagnostic techniques in equine practice
Course, organised by the Royal (Dick) School of Veterinary Studies, to be held at Easter Bush. Details from the CPD Unit, *telephone 0131 651 1180.*

7 to 9

OVS welfare training (red and white meat)
Course, organised by Bristol University, to be held at Langford. Details from Dr L. Hewitt, *telephone 0117 928 9295, fax 0117 928 9324, e-mail: Leisha.Hewitt@bristol.ac.uk*

8

Ocular ultrasonography
Course, organised by BCF Technology, to be held in Leominster. Details from Miss T. Lindsay, *telephone 01506 460023, fax 01506 460045, e-mail: office@bcftechnology.com*

8

Pet bereavement
Workshop, organised by the Cambridge Pet Crematorium and the Pet Bereavement Support Service, to be held in Birmingham. Details from CPC, *telephone 01763 208295, e-mail: mail@cpc-net.co.uk*

8

Getting to grips with rodents
Course, organised by the BSAVA, to be held in Quedgeley. Details from the BSAVA Administration Office, *telephone 01452 726700, fax 01452 726701, e-mail: courses@bsava.com*

8

Wound management and reconstruction for veterinary surgeons
Course, organised by Bristol University, to be held at Langford. Details from the Langford Continuing Education Unit, *telephone 0117 928 9502, fax 01934 852170, e-mail: Langford-CE@bristol.ac.uk*

8

Veterinary Practice Management Association
Meeting, organised by the Yorkshire Region, to be held at Torrington Orthopaedic Referrals, Brighouse, West Yorkshire. Details from Ms S. Hibbert, *telephone 01422 320008, e-mail: shibbert@vets82.fsnet.co.uk*

8 to 9

Veterinary role in nutrition and fertility management in dairy cows
Course, organised by the Royal (Dick) School of Veterinary Studies, to be held at Easter Bush. Details from the CPD Unit, *telephone 0131 651 1180.*

8 to 9

Pharmacy
Course, to be held at the Holiday Inn, Reading. Details from the BCVA Office, *telephone 01452 740816, fax 01452 741117, e-mail: office@cattlevet.co.uk*

8 to 11

World Association for the History of Veterinary Medicine
Congress, to be held in Turin, Italy. Details from Professor M. Galloni, *telephone 00 39 11 670 9125, e-mail: marco.galloni@unito.it*

10

Bovine ultrasonography
Course, organised by BCF Technology, to be held in Cheshire. Details from Miss T. Lindsay, *telephone 01506 460023, fax 01506 460045, e-mail: office@bcftechnology.com*

10 to 12

Demystifying rabbit medicine and surgery
Course, organised by the BSAVA, to be held at the Bellhouse De Vere Hotel, Beaconsfield. Details from the BSAVA Administration Office, *telephone 01452 726700, fax 01452 726701, e-mail: courses@bsava.com*

11

Orthopaedics – thoracic limb lameness 2
Course, organised by Liverpool University, to be held at the Faculty of Veterinary Science, Liverpool. Details from CPD Vets, *telephone/fax 0151 794 6016, e-mail: cpdvets@liv.ac.uk*

11 to 12

Veterinary physiotherapy
Second annual conference, organised by the Royal Veterinary College, to be held at the Hawkshead campus. Details from the CPD Unit, *telephone 01707 666541, fax 01707 666877, e-mail: cpd@rvc.ac.uk*

13 to 17

Bovine reproduction – non-pregnant female
Course, organised by Liverpool University, to be held at Leahurst. Details from the DBR Coordinator, *telephone 0151 794 6081, fax 0151 794 6082.*

14

Small animal ultrasonography
Module 1 of a three-part course, organised by the Royal Veterinary College, to be held at the Hawkshead campus. Details from the CPD Unit, *telephone 01707 666541, fax 01707 666877, e-mail: cpd@rvc.ac.uk*

14 to 15

Practical ophthalmology
Course, to be held at the Wood Green Animal Shelters, Huntingdon. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

15

Feline gastrointestinal disease: below the diaphragm
Part 5 of a modular course, organised by Bristol University, to be held at Langford. Details from the Langford Continuing Education Unit, *telephone 0117 928 9502, fax 01934 852170, e-mail: Langford-CE@bristol.ac.uk*

15 to 18

British Equine Veterinary Association
Congress, to be held in Birmingham. Details from the Administrative Office, *telephone 01223 836970, fax 01223 835287, e-mail: arabella@beva.org.uk*

16

Feline cardiology
Refresher course, organised by Liverpool University, to be held at Leahurst. Details from CPD Vets, *telephone/fax 0151 794 6016, e-mail: cpdvets@liv.ac.uk*

17 to 18

Luxations and arthrodesis
Course, to be held in Ellesmere Port and Liverpool. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

18 to 19

Radiology of the thorax
Course, organised by the Royal Veterinary College, to be held at the Hawkshead campus. Details from the CPD Unit, *telephone 01707 666541,*

World Association for the History of Veterinary Medicine congress, September 8 to 11

fax 01707 666877,
e-mail: cpd@rvc.ac.uk

20 to 26
Annual SPVS sailing and CPD week
To be held off Largs, Scotland. Details from the SPVS Secretariat, *telephone 01926 410454, e-mail: office@spvs.org.uk*

22
Cattle fertility
Course, organised by the Royal Veterinary College, to be held at the Hawkshead campus. Details from the CPD Unit, *telephone 01707 666541, fax 01707 666877, e-mail: cpd@rvc.ac.uk*

22 to 23
Integrated diagnostic imaging: the thorax
Module 1 of a three-part course, to be held in Cheltenham. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

23
Clinical pathology
Workshop, to be held in Milton Keynes. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555.*

23
Small animal ultrasonography
Course, organised by BCF Technology, to be held in Huntingdon. Details from Miss T. Lindsay, *telephone 01506 460023, fax 01506 460045, e-mail: office@bcftechnology.com*

24
Plates and screws
Course, to be held in Milton Keynes. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

24
Introduction to homeopathic medicine
Module 1 of a five-part course, to be held at Eastwood Park, Falfield, Gloucester. Details from the William Kadleigh Academic Department, Bristol Homeopathic Hospital, *telephone 0117 946 6087, fax 0117 923 8759, e-mail: Caroline.Mawer@ubht.swest.nhs.uk*

24 to 25
Surgery of the canine hindlimb
Course, organised by the Royal Veterinary College, to be held at the Hawkshead campus. Details from the CPD Unit, *telephone 01707 666541, fax 01707 666877, e-mail: cpd@rvc.ac.uk*

24 to 26
Feline infectious diseases and cardiology
Congress of the European Society of Feline Medicine, to be held at the Burlington Hotel, Dublin, Ireland. Details from the ESFM, *telephone 0870 742 2278, fax 01747 871873, e-mail: conferences@fabcats.org*

25
Recent graduate reunion seminar
Organised by the Veterinary Defence Society, to be held at Leahurst. Details from the VDS, *telephone 01565 652737, fax 01565 751079.*

28 to 29
How we manage surgical emergencies
Course, organised by the BSAVA, to be held in Quedgeley. Details from the BSAVA Administration Office, *telephone 01452 726700, fax 01452 726701, e-mail: courses@bsava.com*

28 to 29
Mastitis – a business opportunity?
Meeting, organised by the British Cattle Veterinary Association and Intervet, to be held at the Hilton Hotel, Warwick. Details from the BCVA Office, *telephone 01452 740816, fax 01452 741117, e-mail: office@cattlevet.co.uk*

29
Basic small animal endoscopy
Course, organised by Liverpool University, to be held at the Faculty of Veterinary Science Building, Liverpool. Details from CPD Vets, *telephone/fax 0151 794 6016, e-mail: cpdvets@liv.ac.uk*

29 to 30
Small animal medicine
Module 1 of a three-part course, to be held in Milton Keynes. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

30
Basic small animal arthroscopy
Course, organised by Liverpool University, to be held at the Faculty of Veterinary Science Building, Liverpool. Details from CPD Vets, *telephone/fax 0151 794 6016, e-mail: cpdvets@liv.ac.uk*

30
Equine lameness
Workshop, organised by the British Equine Veterinary Association, to be held in Bristol. Details from the Administrative Office, *telephone 01223 836970, fax 01223 835287, e-mail: arabella@beva.org.uk*

30
Small animal pharmacy
Course, organised by the BSAVA Practice Support Group, to be held at the Nottingham Gateway Hotel, Nottingham. Details from Ms C. Ractliffe, *telephone 01452 726707, e-mail: practicestandards@bsava.com*

OCTOBER

1
Advanced small animal arthroscopy
Course, organised by Liverpool University, to be held at the Faculty of Veterinary Science Building, Liverpool. Details from CPD Vets, *telephone/fax 0151 794 6016, e-mail: cpdvets@liv.ac.uk*

1
Ophthalmology skills for general practice
Course, organised by Glasgow University, to be held at the veterinary school. Details from the CPD Unit, *telephone 0141 330 4995, fax 0141 330 2256, e-mail: cpd@vet.gla.ac.uk*

1 to 2
Soft tissue surgery: wound management and oncology
Module 1 of a four-part course, to be held in Ellesmere Port and at Liverpool University. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

1 to 3
BVA Congress
To be held at the Cavendish Conference Centre, London. Details from the BVA, *telephone 020 7636 6541, fax 020 7436 2970, e-mail: bvahq@bva.co.uk*

1 to 3
Medicine and surgery of the GI tract
Course, organised by the BSAVA, to be held at the Hilton Leeds Garforth Hotel, Leeds. Details from the BSAVA Administration Office, *telephone 01452 726700, fax 01452 726701, e-mail: courses@bsava.com*

2 to 3
Equine dentistry for new graduates
Course, organised by the British Equine Veterinary Association Trust, to be held in Newmarket. Details from the Administrative Office, *telephone 01223 836970, fax 01223 835287, e-mail: arabella@beva.org.uk*

5
Introduction to abdominal ultrasound
Workshop, to be held at the Wood Green Animal Shelters, Huntingdon. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

6
Seizures
Course, to be held in Milton Keynes. Details

from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

6

Advances in the science and application of animal training
Meeting, to be held at the Scottish Exhibition and Conference Centre, Glasgow. Details from Dr S. Wickens, *telephone/fax 01276 500880, e-mail: wickens@ufaw.org.uk*

6

Human-animal bond
Undergraduate training day, sponsored by Intervet, to be held at the Scottish Exhibition and Conference Centre, Glasgow, as part of the 10th International Conference on Human-Animal Interactions. Details from the IAHAIO, *telephone 07000 627123, e-mail: organiser@glasgow2004ad.com*

6 TO 7

Conditions of the geriatric horse and pony
Course, to be held at Chester Zoo. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

6 TO 9

People and animals: a timeless relationship
Conference, organised by the International Association of Human-Animal Interaction Organisations, to be held in Glasgow. Details from the IAHAIO, *telephone 07000 627123, fax 01698 738044, e-mail: organiser@glasgow2004ad.com*

7 TO 8

Anaesthesia
Module 1 of a three-part course, to be held in Ellesmere Port. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

8

Feline behaviour
Course, organised by the Blue Cross, to be held at the Humber Business Centre, Grimsby College, Grimsby. Details from Ms C. Breckenridge, *telephone 01472 343278, fax 01472 269770, e-mail: info@bluecross.org.uk*

8 TO 9

Advances in feline infectious diseases
Course, organised by the BSAVA, to be held at the Hilton Cobham Hotel, Cobham. Details from the BSAVA Administration Office, *telephone 01452 726700, fax 01452 726701, e-mail: courses@bsava.com*

9 TO 10

Optimising management of common emergency and critical care cases
Course, organised by the Royal Veterinary College, to be held at the Hawkshead campus. Details from the CPD Unit, *telephone 01707 666541, fax 01707 666877, e-mail: cpd@rvc.ac.uk*

9 TO 10

Equine dermatological syndromes and case studies
Course, organised by Liverpool University, to be held at Leahurst. Details from CPD Vets, *telephone/fax 0151 794 6016, e-mail: cpdvets@liv.ac.uk*

13

Problem-solving medicine
Course, to be held in Milton Keynes. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

13

Small animal oncology
Workshop, to be held in Milton Keynes. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

14

Fix that itch: Tim and Peter's top tips
Course, organised by the BSAVA, to be held at the Hilton Manchester Airport Hotel, Manchester. Details from the BSAVA Administration Office, *telephone 01452 726700, fax 01452 726701, e-mail: courses@bsava.com*

14

Update on the scourges of canine and feline periodontal diseases and resorptive lesions
Course, to be held in Newmarket. Details from Lifelearn, *telephone 01638 577822, fax 01638 577975, e-mail: info@lifelearn.co.uk*

14

Small animal ultrasonography
Course, organised by BCF Technology, to be held in Huntingdon. Details from Miss T. Lindsay, *telephone 01506 460023, fax 01506 460045, e-mail: office@bcftechnology.com*

15

Building the future
Management day, organised by the Veterinary Practice Management Association, to be held at the Hanover International Hotel, Hinckley. Details from the VPMA Secretariat, *telephone 07000 782324, e-mail: secretariat@vpma.co.uk*

15 TO 16

Orthopaedic surgery: fracture surgery, tools and techniques 1
Module 1 of a four-part course, to be held in Ellesmere Port and at Liverpool University. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

16

Practical orthopaedics: using bone plates in practice
Course, organised by Glasgow University, to be held at the veterinary school. Details from the CPD Unit, *telephone 0141 330 4995, fax 0141 330 2256, e-mail: cpd@vet.gla.ac.uk*

16, 23 AND 30

• Recent graduate reunion seminars
Organised by the Veterinary Defence Society, to be held at Langford, October 16; Garscube, October 23; and the Royal Veterinary College, October 30. Details from the VDS, *telephone 01565 652737, fax 01565 751079.*

16 TO 17

Plastic reconstructive surgery
Course, organised by the Royal Veterinary College, to be held at the Hawkshead campus. Details from the CPD Unit, *telephone 01707 666541, fax 01707 666877, e-mail: cpd@rvc.ac.uk*

19

Small animal ultrasonography
Module 2 of a three-part course, organised by the Royal Veterinary College, to be held at the Hawkshead campus. Details from the CPD Unit, *telephone 01707 666541, fax 01707 666877, e-mail: cpd@rvc.ac.uk*

20

Cardiorespiratory medicine
Course, to be held in Milton Keynes. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

20

Pet bereavement
Workshop, organised by the Cambridge Pet Crematorium and the Pet Bereavement Support Service, to be held in Bristol. Details from CPC, *telephone 01763 208295, e-mail: mail@cpc-net.co.uk*

21

Thoracic radiology
Workshop, to be held in Milton Keynes. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

22 TO 24

• Practical veterinary dentistry
Course, to be held at the Vetdent Training Centre, Studley. Details

Cambridge Pet Crematorium

Pet bereavement, October 20

from Ms J. Lindsay, *telephone 01527 853022*

23

Soft tissue surgery – respiratory tract and ear

Course, organised by Liverpool University, to be held at the Faculty of Veterinary Science, Liverpool. Details from the CPD Office, *telephone/fax 0151 794 6016, e-mail: cpdvets@liv.ac.uk*

27

Basics in small animal ultrasound

Course, organised by Glasgow University, to be held at the veterinary school. Details from the CPD Unit, *telephone 0141 330 4995, fax 0141 330 2256, e-mail: cpd@vet.gla.ac.uk*

28

• **Addressing disease control in livestock health and welfare plans**

Course, organised by the SAC, to be held at Crichton Royal Farm, Visitors' Centre, Bankend Road, Dumfries. Details from Mrs I. Smith, *telephone 01292 520318, fax 01292 521069, e-mail: vcayr@ed.sac.ac.uk*

29

Equine anaesthesia

Basic course, organised by the Animal Health Trust, to be held at Lanwades Park, Kentford, Newmarket. Details from Ms K. Bond, *telephone 08700 502540, fax 08700 502541, e-mail: info@aht.org.uk*

29 TO 30

Basic equine dentistry for veterinary surgeons

Course, organised by the British Equine Veterinary Association, to be held in Hartpury. Details from the Administrative Office, *telephone 01223 836970, fax 01223 835287, e-mail: arabella@beva.org.uk*

29 TO 30

• **The cat's third life**

Course, organised by Glasgow University, to be held at the veterinary school. Details from the CPD Unit, *telephone 0141 330 4995, fax 0141 330 2256, e-mail: cpd@vet.gla.ac.uk*

29 TO 31

BCVA autumn meeting

To be held at the Hilton Hotel, Blackpool. Details from the BCVA Office, *telephone 01452 740816, fax 01452 741117, e-mail: office@cattlevet.co.uk*

NOVEMBER

1 TO 2

Equine prepurchase workshop

Organised by the British Equine Veterinary Association, to be held in Leahurst. Details from the Administrative Office, *telephone 01223 836970, fax 01223 835287, e-mail: arabella@beva.org.uk*

1 TO 2

Colic in horses: prevention, assessment and management in practice

Course, organised by the British Equine Veterinary Association, to be held in Newmarket. Details from the Administrative Office, *telephone 01223 836970, fax 01223 835287, e-mail: arabella@beva.org.uk*

3

How we tackle dyspnoea

Course, organised by the BSAVA, to be held in Quedgeley. Details from the BSAVA Administration Office, *telephone 01452 726700, fax 01452 726701, e-mail: courses@bsava.com*

3

Problem-solving medicine

Workshop, to be held in Ellesmere Port. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

5 TO 6

Investigating and treating heart disease in practice: how we do it

Course, organised by the BSAVA, to be held at the Hilton Warwick Hotel, Warwick. Details from the BSAVA Administration Office, *telephone 01452 726700, fax 01452 726701, e-mail: courses@bsava.com*

5 TO 6

• **Practical orthopaedics: surgery of the stifle and hock**

Course, organised by Glasgow University, to be held at the veterinary school. Details from the CPD Unit, *telephone 0141 330 4995, fax 0141 330 2256, e-mail: cpd@vet.gla.ac.uk*

6, 13 AND 27

• **Recent graduate reunion seminars**

Organised by the Veterinary Defence Society, to be held at University College Dublin, Dublin, November 6; Cambridge, November 13; and Edinburgh, November 27. Details from the VDS, *telephone 01565 652737, fax 01565 751079.*

6 TO 7

Diagnostic dilemmas and therapeutic solutions

Course, organised by the Royal Veterinary College, to be held at the Hawkshead campus. Details from the CPD Unit, *telephone 01707 666541, fax 01707 666877, e-mail: cpd@rvc.ac.uk*

6 TO 7

Critical care/soft tissue surgery

Meeting, organised by the Association of Veterinary Soft Tissue Surgeons and the British Association of Veterinary Emergency Care, to be held at Burleigh Court, Loughborough. Details from Mr R. Doust, *e-mail: R.Doust@vet.gla.ac.uk*

8 TO 12

OVS red and poultry meat hygiene

Part 1 of a two-part course, organised by Bristol University, aimed at veterinary surgeons wishing to apply for Official Veterinary Surgeon designation, to be held at Langford. Details from the Langford Continuing Education Unit, *telephone 0117 928 9502, fax 01934 852170, e-mail: Langford-CE@bristol.ac.uk*

9

External fixation

Workshop, to be held in Milton Keynes. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

10 TO 11

Cattle fertility

Meeting, organised by the British Cattle Veterinary Association and Intervet, to be held at the Hilton Hotel, Warwick. Details from the BCVA Office, *telephone 01452 740816, fax 01452 741117, e-mail: office@cattlevet.co.uk*

11

Cytology

Workshop, to be held in Milton Keynes. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

11

Emerging and re-emerging zoonoses: a medical crisis and veterinary perspective

Conference, organised by the Royal College of Pathologists, to be held in London. Details from Miss M. Casey, *telephone 020 7451 6740, e-mail: michelle.casey@rcpath.org*

12

Canine behaviour

Course, organised by the Blue Cross, to be held at the Humber Business Centre, Grimsby College, Grimsby. Details from Ms C. Breckenridge, *telephone 01472 343278, fax 01472 269770, e-mail: info@bluecross.org.uk*

12

Bovine ultrasonography

Course, organised by BCF Technology, to be held in Cheshire. Details from Miss T. Lindsay, *telephone 01506 460023, fax 01506 460045, e-mail: office@bcftechnology.com*

12 TO 13
Spinal surgery
Course, to be held in Ellesmere Port and at Liverpool University. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

12 TO 14
Advances in exotic, zoo and wild animal medicine
Meeting, to be held at the Zoological Society of London. Details from Mr A. Sainsbury, *telephone 020 7449 6671, e-mail: tony.sainsbury@ioz.ac.uk*

13
Rabbit health in the 21st century
Conference, to be held at the School of Chemistry, Bristol University. Details from Ms C. King, *telephone 01525 717041, e-mail: cking_bunnymad@hotmail.com*

13 TO 14
Small animal abdominal ultrasound
Course, organised by the Royal Veterinary College, to be held at the Hawkshead campus. Details from the CPD Unit, *telephone 01707 666541, fax 01707 666877, e-mail: cpd@rvc.ac.uk*

17
Feline cardiorespiratory disease: above the diaphragm
Part 6 of a modular course, organised by Bristol University, to be held at Langford. Details from the Langford Continuing Education Unit, *telephone 0117 928 9502, fax 01934 852170, e-mail: Langford-CE@bristol.ac.uk*

17
ECG workshop
To be held in Milton Keynes. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

17 TO 18
Foot problems in the horse
Course, to be held at Chester Zoo. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

17 TO 18
Integrated diagnostic imaging: the abdomen
Module 2 of a three-part course, to be held in Cheltenham. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555, e-mail: info@cpd-solutions.com*

17 TO 18
Equine lameness and farriery
Course, to be held in Newmarket. Details from Lifelearn, *telephone 01638 577822, fax 01638 577975, e-mail: info@lifelearn.co.uk*

18
Computers and information technology
Seminar, organised by the Society of Practising Veterinary Surgeons, to be held at the Holiday Inn, Coventry. Details from the SPVS Secretariat, *telephone 01926 410454, fax 01926 411350, e-mail: office@spvs.org.uk*

19
• **Glancing back and looking forward**
Course, part of the Glasgow Alumni Homecoming Weekend from November 19 to 21, organised by Glasgow University, to be held at the veterinary school. Details from the CPD Unit, *telephone 0141 330 4995, fax 0141 330 2256, e-mail: cpd@vet.gla.ac.uk*

19 TO 21
Challenges in internal medicine: how to solve those tricky cases
Course, organised by the BSAVA, to be held at the Palace Hotel, Buxton. Details from the BSAVA Administration Office, *telephone 01452 726700, fax 01452 726701, e-mail: courses@bsava.com*

20 TO 21
Haematology/ transfusion medicine
Course, organised by the Royal Veterinary College, to be held at the Hawkshead campus. Details from the CPD Unit, *telephone 01707 666541, fax 01707 666877, e-mail: cpd@rvc.ac.uk*

20 TO 21
Basic exotic animal endoscopy
Symposium, to be held at the University of Georgia, Athens, USA Details from Ms S. Kilgo, *telephone 00 1 706 542 1451, e-mail: skilgo@vet.uga.edu*

22 TO 26
• **OVS red and poultry meat hygiene**
Part 2 of a two-part course, organised by Bristol University, aimed at veterinary surgeons wishing to apply for Official Veterinary Surgeon designation, to be held at Langford. Details from the Langford Continuing Education Unit, *telephone 0117 928 9502, fax 01934 852170, e-mail: Langford-CE@bristol.ac.uk*

23 TO 24
• **Dairy cow nutrition**
Course, organised by the British Cattle Veterinary Association, to be held at the Hilton Hotel, Warwick. Details from the BCVA Office, *telephone 01452 740816, fax 01452 741117, e-mail: office@cattlevet.co.uk*

24
• **Small animal ultrasound: level II**
Course, organised by Glasgow University, to be held at the veterinary school. Details from the CPD Unit, *telephone 0141 330 4995, fax 0141 330 2256, e-mail: cpd@vet.gla.ac.uk*

24
• **Small animal ultrasonography**
Course, organised by BCF Technology, to be held in Huntingdon. Details from Miss T. Lindsay, *telephone 01506 460023, fax 01506 460045, e-mail: office@bcftechnology.com*

24 TO 28
• **Veterinary chiropractic: equine and canine**
Module I of a five-part course, organised by the International Institute of Veterinary Chiropractic, to be held in East Leake, Loughborough. Details from the IIVC, *telephone 00 49 4282 590099, fax 00 49 4282 591852, e-mail: IIVC2004@hotmail.com*

26
• **Cruciate workshop**
To be held in Ellesmere Port. Details from CPD Solutions, *telephone 0151 328 0444, e-mail: info@cpd-solutions.com*

26 TO 27
• **Soft tissue surgery: head, neck and thoracic conditions**
Module 2 of a four-part course, to be held in Ellesmere Port and Liverpool University. Details from CPD Solutions, *telephone 0151 328 0444, fax 0151 328 0555.*

27
• **Practical orthopaedics: the hip**
Course, organised by Glasgow University, to be held at the veterinary school. Details from the CPD Unit, *telephone 0141 330 4995, fax 0141 330 2256, e-mail: cpd@vet.gla.ac.uk*

27 TO 28
• **Basic echocardiography of the dog and cat**
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