A STUDY OF THE SENSITIVITY AND SPECIFICITY OF AN IMMUNOPEROXIDASE TECHNIQUE FOR THE DIAGNOSIS OF MAEDI - VISNA VIRUS INFECTION IN SHEEP

By

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SUMMARY

In this study the sensitivity and specificity of an immunoperoxidase technique for the detection of lentiviral protein in formalin-fixed lung tissues of sheep were assessed. Formalin-fixed specimens of lungs of 52 sheep originating from two different infected flocks and formalin-fixed lung specimens of 20 sheep from a third, non-infected flock were selected. Multiple sections from each animal were stained with a monoclonal antibody against Maedi-Visna Virus (MVV) antigen. Control sections were stained with an irrelevant antibody, and without antibody, respectively in both the positive and negative groups. Histologic lesions of Maedi were graded according to severity and the number of sections staining positive. The average count of infected cells was determined for all animals which exhibited positive staining against viral protein. It was determined that the IMP technique had a sensitivity of 51,9% and a specificity of 100% if three sections of each animal were examined in both infected flocks. The sensitivity was greatly influenced by the presence or absence of typical histological lesions and the average cell counts seem to be correlated with the severity of the histological lesions. Animals without histological lesions were mostly negative with the IMP technique. The sensitivity was also influenced by the number of sections examined per animal and decreased if fewer sections were examined. Statistical analysis confirmed that the proportions of sections which were positive by means of IMP staining differed significantly between the four histological categories of normal, mild, moderate and severe. The more severe the lesions, the higher the proportion of sections were that could be diagnosed as infected by means of the IMP technique. It also confirmed that the ability to detect MVV infection in sections decreases significantly with a smaller sample size. In conclusion it would seem that this technique may be used in confirming infection in individual animals selected on the presence of typical pulmonary lesions. It is, however, not suitable as a routine screening test for the infection.

SAMEVATTING

Die sensitiwiteit en spesifisiteit van die immunoperoksidase tegniek (IMP) vir die diagnose van lentivirus in formalien-gefikseerde longweefsel in skape was ondersoek. Formalien-gefikseerde long monsters van 52 skape , afkomstig van twee verskillende besmette kuddes, en formalien-gefikseerde long monsters van 20 skape afkomstig van ‘n derde, nie-besmette kudde, was ondersoek. Veelvuldige histologiese snitte van elke dier was gekleur met ‘n monoklonale teenliggaam teen die Maedi-Visna Virus (MVV) antigeen. Kontrole snitte was gekleur met ‘n irrelevante teenliggaam, en sonder teenliggaam, in alle diere. Histologiese letsels was gegradeer ooreenkomstig die ergheidsgraad van die letsels; en die aantal snitte wat positief gekleur het per dier. Die gemiddelde aantal selle wat positief gekleur het vir antigeen was ook bepaal. Die IMP tegniek het ‘n sensitiwiteit van 51,9% en ‘n spesifisiteit van 100% indien drie long snitte per dier in beide die besmette kuddes ondersoek word. Die sensitiwiteit was grootliks beïnvloed deur die teenwoordigheid of afwesigheid van tipiese letsels en die aantal selle wat virus antigeen bevat blyk om te korreleer met die graad van die letsels. Diere sonder tipiese histologiese letsels was meestal negatief vir virus antigeen met die IMP tegniek. Die aantal snitte ondersoek het die sensitiwiteit beïnvloed en ‘n afname in die sensitiwiteit was opgemerk indien minder snitte per dier ondersoek was. Statistiese ontleding het getoon dat dat die aantal snitte wat positief toets met die IMP tegniek betekenisvol verskil tussen die histologiese kategorieë van erg, matig, lig en normaal. ‘n Groter persentasie van die snitte het positief gekleur in gevalle met meer gevorderde letsels. Dit het ook getoon dat die vermoë om MVV infeksie te diagnoseer betekenisvol afneem met vermindering in die aantal snitte wat ondersoek word. Die gevolgtrekking is dus dat die IMP tegniek van diagnostiese waarde is in individuele diere met tipiese histologiese letsels, en dat dit nie geskik is nie as ‘n roetine siftings-toets op kudde basis.

1. INTRODUCTION

Maedi-Visna is an insidious and erosive viral disease of sheep resulting in losses in production and reproduction. Maedi-Visna is a composite Icelandic name referring to two clinical syndromes caused by the same slow virus, the maedi-visna virus (MVV) (Dawson, 1980; Palsson, 1990; Petursson, Georgsson & Palsson, 1990). Maedi (which means dyspnoea) is characterized by chronic progressive pneumonia, and Visna (which means wasting) by meningoencephalitis of adult sheep leading to weakness and progressive paresis of especially the hind legs (Dawson, 1980; Palsson, 1990; Petursson, Georgsson & Palsson, 1990).

1.1 Historical perspective

The histological lesions now considered to be characteristic of Maedi-Visna were described in South Africa in 1915 by Mitchell and the disease came to be called "Graaff-Reinet disease" (Palsson, 1990; Verwoerd, Tustin & Williamson, 1994). Although reports of similar conditions followed from several countries, MVV was only established as the causative agent in Iceland a few decades later, when it was isolated from affected sheep for the first time (Palsson, 1990). It is now known to have a worldwide distribution (Houwers, 1990; Petursson, Georgsson & Palsson, 1990).

Several different strains of MVV with differing degrees of virulence have been isolated. Depending on the reported differences in breed susceptibility, differing management systems, and climatic conditions, infection may result in varying expression of clinical disease and lesions in different geographical regions (Houwers, 1990; Palsson , 1990; Petursson, Georgsson & Palsson, 1990). Infection may cause multisystemic lesions such as chronic, progressive, interstitial pneumonia; chronic, demyelinating, encephalomyelitis; chronic, indurative mastitis and chronic, proliferative arthritis; alone or in various combinations (Dungworth, 1993; Palsson, 1990; Petursson, Georgsson & Palsson, 1990). As a result a variety of regional names have been given

to these related diseases including: "Graaff-Reinett disease" in SA, "Montana sheep disease" and "Ovine progressive pneumonia" in the United States of America, "Zwoegersiekte" in the Netherlands, "La Bouhite" in France and the more often used "Maedi-Visna" in Iceland (Palsson, 1990; Petursson, Georgsson & Palsson, 1990).

Following the original description by Mitchell, the disease attracted almost no attention and was not considered to be present in South Africa until 1986, when MVV was isolated from an experimental animal suffering from jaagsiekte (ovine pulmonary adenomatosis) (Payne, *et al.,* 1986; Verwoerd, Tustin & Williamson, 1994). This virus was designated the South African Ovine Maedi-Visna isolate (SA-OMV virus) (Payne, *et al.*, 1993; Verwoerd, Tustin & Williamson, 1994). Maedi-Visna was diagnosed in 1993 in a flock of sheep in South Africa by post mortal examination and serological screening (Vorster, *et al.,* 1994). The current extent and geographical distribution of infected flocks (and the economic impact thereof) in South Africa are, however, unknown.

1.2 Viral characteristics

The virus is a member of the lentivirus subgroup of the retrovirus family that includes the human immunodeficiency viruses, HIV 1 and 2, and the caprine arthritis-encephalitis virus (CAEV) (Petursson, 1990). Several different, but related, strains of ovine lentiviruses, of which MVV is the prototype, have been isolated in various parts of the world (Petursson, 1990; Petursson, Georgsson & Palsson, 1990; Verwoerd, Tustin & Williamson, 1994). The South African strain is known as the South African Ovine Maedi-Visna virus (SA-OMV) (Payne, *et al.,* 1986; Verwoerd, Tustin & Williamson, 1994).

The Maedi-Visna virus genome, like other lentiviruses, consists of a single-stranded RNA molecule containing three major genes that encode the major structural proteins of the virus: the viral glycoproteins are encoded by the *env* gene, viral core proteins are encoded by the *gag* gene, and viral RNA-dependent DNA polymerase (RTase) is encoded by the *pol* gene (Petursson,

1990). The number of polypeptides in mature virions has been reported to vary from 10 to 25 and four of those, i.e. p25, p16, p14 and gp135, comprise about 80% of the protein mass of the virion (Petursson, 1990). The p25 polypeptide is the main component of the virus core and comprises about 40% of the protein mass of the virion (Petursson, 1990). Lentiviruses share nucleotide homology and serological properties: the ovine Maedi-Visna virus and the caprine arthritis-encephalitis virus show extensive homology in their *gag-pol* genes and gene products (Verwoerd, Tustin & Williamson, 1994). Genetic divergence exists between the MVV, CAEV and SA-OMV viruses, and MVV and SA-OMV are now considered to be distinct subgroups (Payne, *et al.,* 1986; Verwoerd, Tustin & Williamson, 1994). SA-OMV is more closely related to MVV than it is to CAEV (Verwoerd, Tustin & Williamson, 1994).

1.3 Clinical signs

Lentiviruses usually cause persistent, lifelong infections and diseases that are characterized by a long incubation period and a chronic debilitating clinical course (Dawson, 1980; Palsson, 1990; Petursson, Georgsson & Palsson, 1990). Following MVV infection, the incubation period usually varies from 3-4 years and clinical signs are therefore seen mainly in adult sheep (Dawson, 1980; Palsson, 1990; Petursson, Georgsson & Palsson, 1990; Verwoerd, Tustin & Williamson, 1994). Infection with MVV primarily affects the lungs and udder, while the central nervous system and joints are less commonly affected (Houwers, 1990; Palsson, 1990; Petursson, Georgsson & Palsson, 1990; Verwoerd, Tustin & Williamson, 1994). Emaciation and respiratory distress (dyspnoea), the usual clinical signs, are present for several months before affected sheep eventually die (Dawson, 1980; Houwers, 1990; Palsson, 1990; Petursson, Georgsson & Palsson, 1990; Verwoerd, Tustin & Williamson, 1994).

1.4 Pathology

Pulmonary lesions in sheep suffering from Maedi are characterized by the development of a

chronic, progressively worsening, interstitial pneumonia. The lungs of affected sheep that die or are slaughtered, do not collapse when removed from the thorax and often retain the impression marks of the ribs (Dungworth, 1993; Petursson, Georgsson & Palsson, 1990). Both the lungs and their associated lymph nodes may be markedly enlarged and increased in weight (Dungworth, 1993; Petursson, Georgsson & Palsson, 1990). The lesions are distributed throughout the lungs imparting either a uniform greyish or a mottled greyish-brown colour and the lungs are firm (Dungworth, 1993; Petursson, Georgsson & Palsson, 1990). The characteristic histological features include thickening of the alveolar septae due to infiltration of mononuclear cells (mainly macrophages and lymphocytes), and the development of lymphoid follicles in intra-alveolar, peribronchial and perivascular locations (Dungworth, 1993; Petursson, Georgsson & Palsson, 1990). Smooth muscle hypertrophy and fibrosis of alveolar walls may also be present (Dungworth, 1993; Petursson, Georgsson & Palsson, 1990).

1.5 Diagnosis

The clinical signs of MVV infection are not specific enough to enable a reliable diagnosis of the disease to be made and confirmation of the infection must be attempted by laboratory procedures such as serology, viral isolation and histopathological examination (Hoff-Jorgensen, 1990; Petursson, Georgsson & Palsson, 1990; Verwoerd, Tustin & Williamson, 1994).

The use of serological techniques have the advantage that large numbers of animals can be screened fairly rapidly for the presence of the infection. These findings will then indicate the necessity of further investigations such as comprehensive pathological investigations to confirm the presence of overt disease. Antibodies to the infection with MVV can be detected using different techniques: virus neutralization, complement fixation, immunofluorescence, agar gel immunodiffusion (AGID), passive haemagglutination and the enzyme-linked immunosorbent assay (ELISA) (Hoff-Jorgensen ,1990; Houwers, Gielkens & Schaake, 1982; Petursson, Georgsson & Palsson, 1990). An ELISA test has recently been developed at the Onderstepoort Veterinary Institute for the diagnosis of MVV infections in sheep with the transmembrane and p24 proteins

of SA-OMV as antigens in the form of a fusion protein (Boshoff, *et al.,* 1997).

In the majority of cases, the diagnosis of Maedi-Visna is based on a combination of the results of serological testing and detecting the presence of lesions by histopathological examination. Although viral isolation can be performed, it is expensive, labour-intensive, and therefore not always practical.

Diagnosing MVV infection by a combination of serology and histopathology has a few drawbacks:
-a positive serological result does not always mean that the particular infected animal will have typical histological lesions of MVV infection; and
-frequently an animal with or without exhibiting typical histological lesions may be seronegative for MVV infection (Hoff-Jorgensen, 1990; Johnson, Meyer & Zink, 1992)

Similar difficulties are experienced in cases where only formalin-fixed specimens are received for diagnostic purposes. The presence of specific histological lesions is not sufficient to make a diagnosis as the lesions of MVV, and although fairly typical, may be confused with lesions caused by infection with other microbial agents such as *Mycoplasma ovipneumonia* or infestation by lungworm.

The immunoperoxidase technique applied to tissue sections of formalin-fixed specimens provides pathologists with an additional diagnostic technique for MVV infection that may be useful when it is not possible to perform serology or viral isolation to make a specific diagnosis.

1.6 The immunoperoxidase test

In studies the immunoperoxidase (IMP) technique has been used successfully to investigate certain aspects of the pathogenesis of lentiviral infection. An immunoperoxidase test employing a monoclonal antibody (3F) was used by Brodie *et al.* (1992), amongst other tests, to determine the proportion of infected cells in affected lungs of experimentally infected animals. It was found that by applying this technique virus load was directly proportional to the degree of development of lesions in lungs (Brodie, *et al.,* 1992). In another study the technique was used to study replication of MVV in specimens obtained by broncho-alveolar lavage in naturally infected animals (Lujan, *et al.,* 1994). It was determined that viral replication occurred only in alveolar macrophages (Brodie, *et al.,* 1992; Lujan, *et al.,* 1994). At necropsy of some animals in this study it was found that animals with severe to moderate lesions in their lungs, had the highest number of positive cells; although sheep with similar lesions also contained fewer or no positive cells at all (Lujan, *et al.,* 1994). Broncho-alveolar lavage specimens of only eight out of the twenty sheep sampled tested positive; the percentage positive cells per animal ranged from 1% to 12% (Lujan, *et al.,* 1994). It is, however, not known how sensitive or specific the immunoperoxidase technique is for diagnosing natural MVV infection utilizing histopathological examination of formalin-fixed tissues.

2. HYPOTHESES

2.1 The immunoperoxidase technique will be a sensitive and specific test for diagnosing MVV infection in sheep that exhibit typical macroscopical and histological lesions. In these cases, the immunoperoxidase technique will allow an accurate diagnosis of MVV infection where no other diagnostic techniques are available or could be performed.

2.2 The immunoperoxidase technique will be far less specific and sensitive for diagnosing MVV infection in sheep which exhibit no typical macroscopical and histological lesions. In these cases

an additional diagnostic procedure such as serology will be necessary to diagnose MVV infection.

3. MATERIALS AND METHODS

3. 1 Naturally infected sheep (Group A)

The Goedemoed flock is seropositive for MVV infection and macroscopical and histopathological lesions, typical for MVV infection, have been detected in these animals. During slaughter all animals were inspected for the presence of lung lesions consistent with those of Maedi.

For the purpose of this study specimens for serological and pathological investigation were obtained from 40 animals with macroscopically visible lung lesions. Ten animals with severe lesions and 10 animals with mild lesions, all 20 being seropositive at slaughter, were selected for this investigation. The other 20 sheep consisted of 10 that were seronegative but with lesions present, and 10 seropositive animals without any macroscopically visible lung lesions. Selection of cases were on a random basis.

3.2 Positive control sheep (Group B)

Material from six sheep infected with MVV without typical lesions, and from six animals with typical lesions were used as positive controls. Samples were obtained from previous studies by Prof J.C. de Martini and others (Department of Pathobiology, Colorado State University, USA) .

3.3 Negative control sheep (Group C)

Negative control sheep compromised animals examined at a different abattoir, originating from a different flock with no history of MVV infection, and that were seronegative. Pathological and serological specimens were selected from 20 animals.

The specimens in Groups A and C were collected at the abattoir where the sheep were routinely slaughtered, as part of the normal management programme of the flocks (for purposes of culling or meat production).

Table 3.1: Experimental groups and number of animals sampled

POSITIVE/CONTROL GROUPS	MACROSCOPICAL PATHOLOGY	NUMBER OF ANIMALS
GROUP A Positive group Naturally infected	Macroscopically severely affected (seropositive)	10
	Macroscopically mildly affected (seropositive)	10
	Macroscopically normal (seropositive)	10
	Macroscopically affected (seronegative)	10
GROUP B Positive control group Naturally infected	Macroscopically affected	6
	Macroscopically normal	6
GROUP C Negative control	Macroscopically normal (seronegative)	20

3.4 Specimen collection

3.4.1 Serology

Prior to slaughter of animals of Groups A and C a serum sample was obtained from each individual animal, properly identified and kept cool on ice in coolboxes. Following collection of the serum the sample was refrigerated at 4°C until arrival at the laboratory where the serum was decanted and stored in a clean tube at -20°C. For the animals in Group B no serum samples were obtained but the available serological results were used.

3.4.2 Pathology

For animals in Groups A and C representative specimens of lung tissue were collected in 10% phosphate-buffered formalin. Specimens were collected to include affected areas of lung tissue as well as normal areas, if present. At least six specimens were collected from each animal. All lesions and their distribution, including the lobe from which they were collected, were recorded for each case. The macroscopical lesions were graded according to the following general criteria:
- Normal lungs: no visible macroscopical lesions.
- Mildly affected lungs: lungs of almost normal size and consitency with a slight reddish-brown or greyish discolouration. A few randomly distributed greyish foci 1-2 mm in diameter present in one or more lobes. Mediastinal lymph nodes slightly enlarged.
- Severely affected lungs: lungs markedly enlarged with or without rib impression marks; rubbery in consistency; failing to collapse with diffuse distinctive greyish discolouration with multifocal to confluent areas of consolidation. Numerous distinctive greyish 1-2 mm in diameter foci were scattered evenly through lung lobes. Mediastinal lymph nodes were enlarged to 2-3 times their normal size.

Not all the lungs examined necessarily exhibited all the features described; each lung was graded

on the extent of those lesions present. Histologically (see below) a more refined system of classification was used to grade normal, mildly affected, moderately affected and severely affected lungs.

For animals in Group B the available specimens were used and all information with regards to the macroscopical lesions, or any other relevant information were obtained from Prof de Martini (Department of Pathobiology, Colorado State University).

3.5 Processing of specimens

3.5.1 Histology

Formalin-fixed specimens were routinely processed, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. All histological sections were examined for the presence or absence of typical lesions for MVV infection. In those cases where lesions were present, the severity of the lesions were graded according to a modification of the system previously published by Brodie *et al.* (1992).

Lesions were classified on the basis of the following criteria:

1. The degree of severity of the interstitial pneumonia as characterized by thickening of the interstitial septa caused by the mononuclear inflammatory cellular infiltrate, hypertrophy of smooth muscle cells or fibrosis of the alveolar walls, and the presence or absence of exudate within the alveolar lumen. The degree of severity was graded as absent (0), mild (+), moderate (++) and severe (+++) with scores of 0, 1, 2 and 3, respectively, for each grading. For each animal all sections were graded and the average score calculated.
   The following criteria were used for the classification:
   - **absent** represents cases with no histological abnormalities.

- **mild** represents all cases where the lesions consisted of areas with interstitial septal thickening due to an inflammatory cellular infiltrate interspersed by areas of unaffected parenchyma.
- **moderate** represents cases reflecting diffuse interstitial septal thickening affecting almost all alveolar septa within the section, but without severe atelectasis and/or compensatory emphysema. Minimal smooth muscle hypertrophy and/or fibrosis may or may not be evident. Alveoli may or may not be filled with an inflammatory exudate.
- **severe** represents cases manifesting diffuse, interstitial septal thickening progressing to atelectasis of most alveoli and compensatory emphysema of others. Prominent smooth muscle and collagen hyperplasia were evident with or without an inflammatory exudate. Bronchiolar epithelium may be severely hyperplastic with plugging of the bronchiolar lumen by an inflammatory exudate.

2. The presence or absence of typical peribronchiolar, perivascular or interstitial lymphoid follicles as counted in 10 microscopy fields under low magnification (10x objective). The extent of change was graded as absent (0), mild (+), moderate (++) or severe (+++) with follicular counts of 0; 1-2; 3-5 and > 6, respectively, for the three categories.

Three specimens from the caudal lobe were considered to be representative and these were collected from the cranio-ventral, ventro-caudal and mid-dorsal regions of the caudal lung lobe. The average score of the severity of interstitial pneumonia and number of lymphoid follicles was calculated for each section, then the average of the three sections from each lung was calculated to provide the score for each lung. This score represents the following classes: no lesions (0-0,7); mild (0,8-1,4); moderate (1,5-2,4) and severe (> 2,4).

3.5.2 Immunoperoxidase stain

The immunoperoxidase technique as described by Brodie *et al.* (1992) was modified. For the

immunoperoxidase technique each section of each sample was mounted on a glass slide with Poly-L-lysine and deparaffinized, then blocked and incubated with the primary antibody at 4 ˚C overnight. The antibody was supplied by Prof. J.C. de Martini and is an affinity-purified monoclonal antibody 3F. Biotinylated rabbit anti-mouse IgG, strepavidin-peroxidase conjugate and a substrate chromogen solution were applied.

Sections of each individual animal were stained as follows:
- one section with the MVV monoclonal antibody (OvLV-3F).
- one section without the monoclonal antibody
- one section with an irrelevant antibody (RVF)

Sections were deparaffinized and blocked for endogenous peroxidase by immersion into 3% hydrogen peroxide in methanol for 30 minutes. Antigen retrieval was performed in citrate buffer (pH 6) in a conventional microwave oven for 10 minutes. Sections were allowed to cool for 15 minutes. They were then rinsed in distilled water followed by rinsing in PBS. Thereafter they were immersed in a 10% dilution of normal “rabbit” serum to block any background staining. Primary and irrelevant antibodies and PBS were applied to the sections and allowed to stain overnight at room temperature. Sections were then rinsed twice for 5 minutes in PBS followed by application of the biotinylated secondary RxM antibodies for 40 minutes. All sections were then rinsed in PBS and the ABC Complex Elite was applied for 30 minutes. Following rinsing with PBS, DAB substrate (30mg/100ml PBS, deactivated with 4 drops of a 3% hydrogen peroxide solution in PBS) was put on for 8 minutes. The final steps were rinsing of sections in distilled water, counterstaining with Mayer’s haematoxylin, dehydration and mounting.

The number of infected cells with viral antigen present were counted in ten microscopy fields (20X objective) for each stained slide; the average number of infected cells staining positive were calculated for each case.

3.5.3 Serology

The ELISA test was carried out on each serum sample according to the prescribed procedures (Boshoff *et al.,* 1997). The purified and recombinant GST-TM-P25 fusion proteins were diluted to 1:200 in 0.1 M sodium bicarbonate buffer at pH 9,6 and then passively adsorbed onto the surface of 96 - well microtiter plates (Greiner, Germany). Plates were incubated overnight at room temperature and then washed three times with a buffer consisting of 50mM Tris-HCl, 150mM NaCl at pH 8.0 with 0.05% (v/v) Tween 20. Plates were dried under vacuum and stored at 4°C until used. Gluthathione S-transfersae (GST) extracted from *Escherichia coli* which was transformed with pGEX-2T was used as control antigen.

Prior to use plates were blocked for 1 hour at 37°C using 200 µl of skimmed milk solution at 5% (w/v) in washing buffer. One hundred microliter (100 µl) of test sera, diluted to 1:100 in 5% (w/v) skimmed milk in washing buffer was added and then incubated at 37 ˚C for 1 hour. Following washing, protein G-peroxidsae conjugate (Zymed, CA, USA) diluted to 1:5000 in 5% (w/v) skimmed milk in washing buffer (100µl) was added and incubated for 1 hour at 37˚C. Plates were washed and 50µl of substrate (10mg O-phenylenediamine and 8 mg urea-hydrogenperoxide in 10ml 0,1 M sodium citrate buffer at pH 4,5) was added per well and incubated in the dark at room temperature for 5 minutes. One hundred microliter (100 µl) of 0,5 M sulphuric acid was added per well to stop the reaction. An automated ELISA plate reader (Titerk Multiskan microplate reader, Flow Laboratories, McLean, Va) was used to determine the adsorbance of each well at two wavelengths (492 and 690nm). All sera were tested in duplicate and the mean absorbance was determined for each specimen.

3.6 Statistical analysis

Data were analysed by logistic regression methods (McConway, *et al.,* 1999) using Genstat 5

(release 3). All results are presented in the form of Analysis of Deviance tables with results summarized as the relative proportions and their standard errors. Statistical analysis was performed by Mr. H. Dicks, Division of Statistics and Biometry, School of Mathematics, Statistics & Information Technology, University of Natal, Pietermaritzburg.

4. RESULTS

Positive immunoperoxidase (IMP) staining was characterized by the presence in macrophages of either golden-brown, intracytoplasmic granules or a more diffuse, golden-brown discolouration of the cytoplasm, with or without granules (Figure 4.1). In several cases giant cells were seen exhibiting similar positive staining patterns (Figure 4.2). In cases which had extensive areas of consolidation and a secondary purulent bronchopneumonia, the number of positively staining cells appeared to be more compared to the number of infected cells in other sections of the same lung. The number of giant cells were usually increased in these areas. In some cases large numbers of macrophages, admixed with neutrophils, were present in the bronchiolar lumens to form clusters of these cells (Figure 4.3). In some bronchi cells in the exudate were necrotic making distinction between the cytoplasm of individual macrophages difficult. In these areas moderate amounts of free-lying, golden-brown, granular pigment were detected (Figure 4.4). Non-specific staining was evident in some sections and usually as numerous, varying-sized, light-brown, intra-cytoplasmic globular structures in macrophages. Non-specific staining lacked the granular appearance or diffuse cytoplasmic staining as in positive reactions (Figure 4.5). In some sections bronchi, alveoli and blood vessels were filled with moderate amounts of homogenous, light-brown material (Figure 4.6) presumed to be oedema fluid. Large, black birefringent pigment in the cytoplasm of macrophages, usually surrounding bronchi, were presumed to be inhaled dust or carbon-like particles (anthracosis) (Figure 4.7).

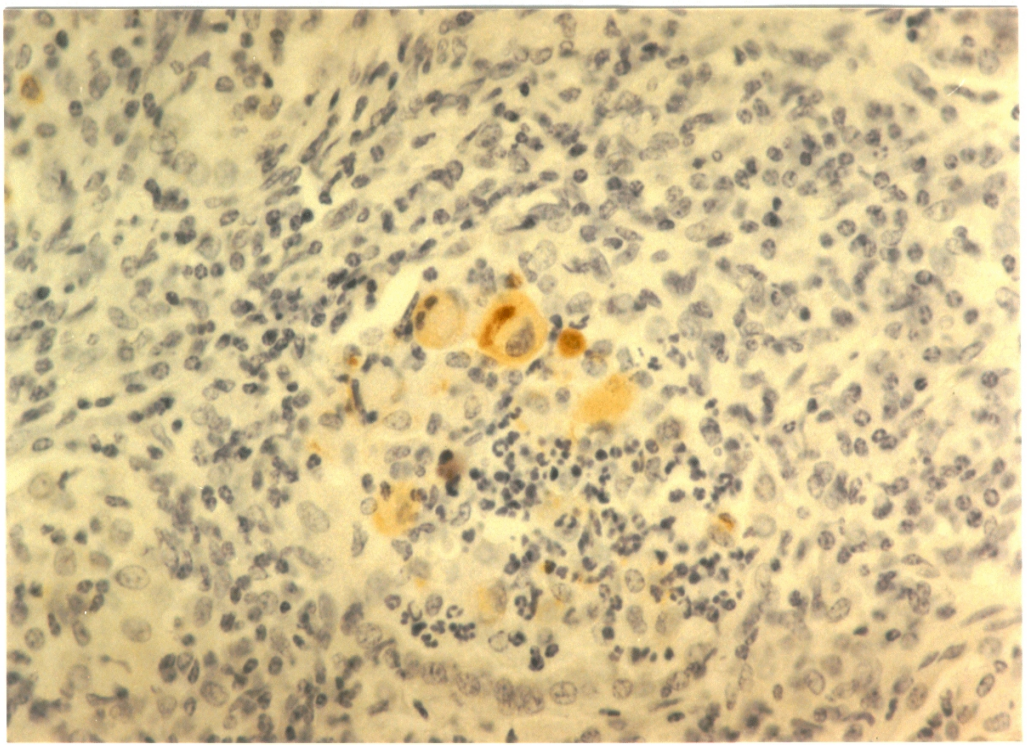

Figure 4.1: Diffuse golden brown staining of cytoplasm with distinct golden brown intracytoplasmic granules in macrophages (400X).

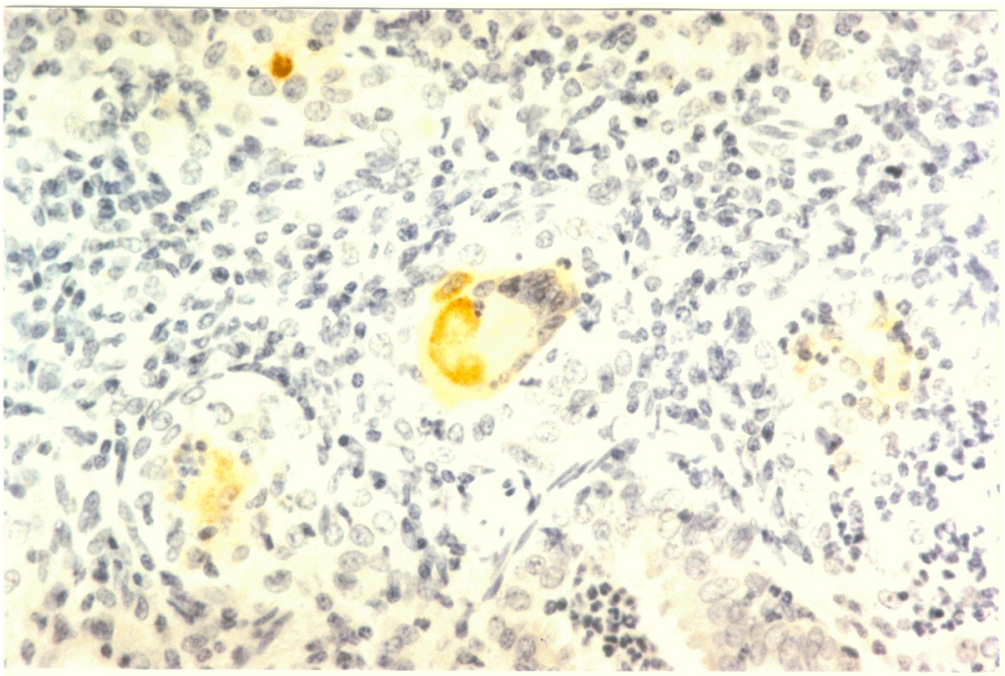

Figure 4.2: Multinucleated giant cells with diffuse golden brown discolouration of cytoplasm and golden brown intracytoplasmic granular staining of viral antigen (400X).

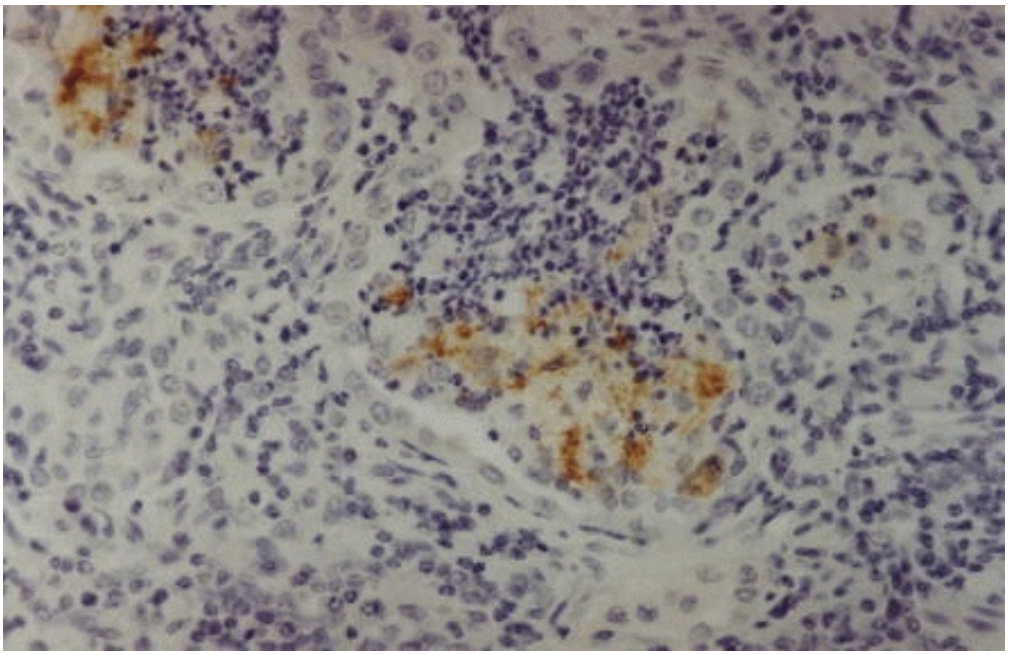

Figure 4.3: Large numbers of antigen-positive staining macrophages, admixed with neutrophils, in alveoli and bronchioli (400X).

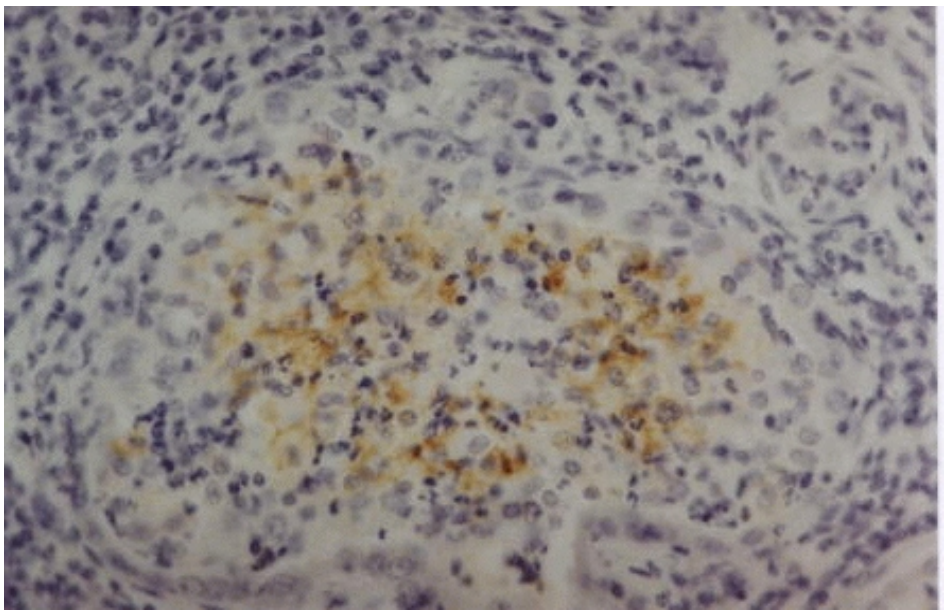

Figure 4.4: Conglomerate of antigen-positive staining cells, without clear distinction between cytoplasm of individual cells (400X).

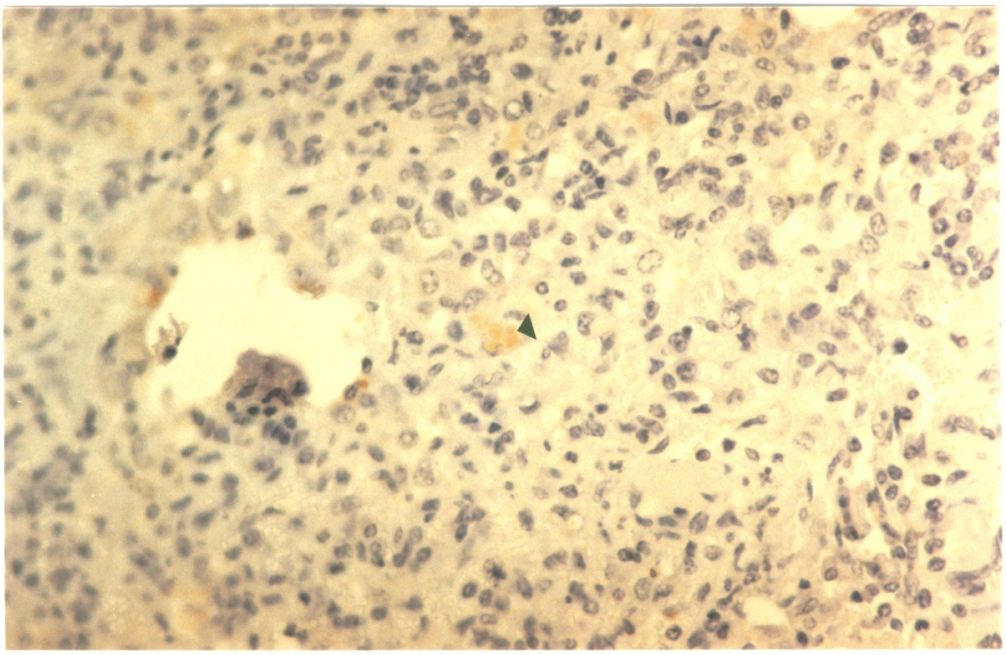

Figure 4.5: Non-specific staining seen as pale yellowish-brown intracytoplasmic globular structures (arrow head) without diffuse deep golden brown discolouration or granules (400X).

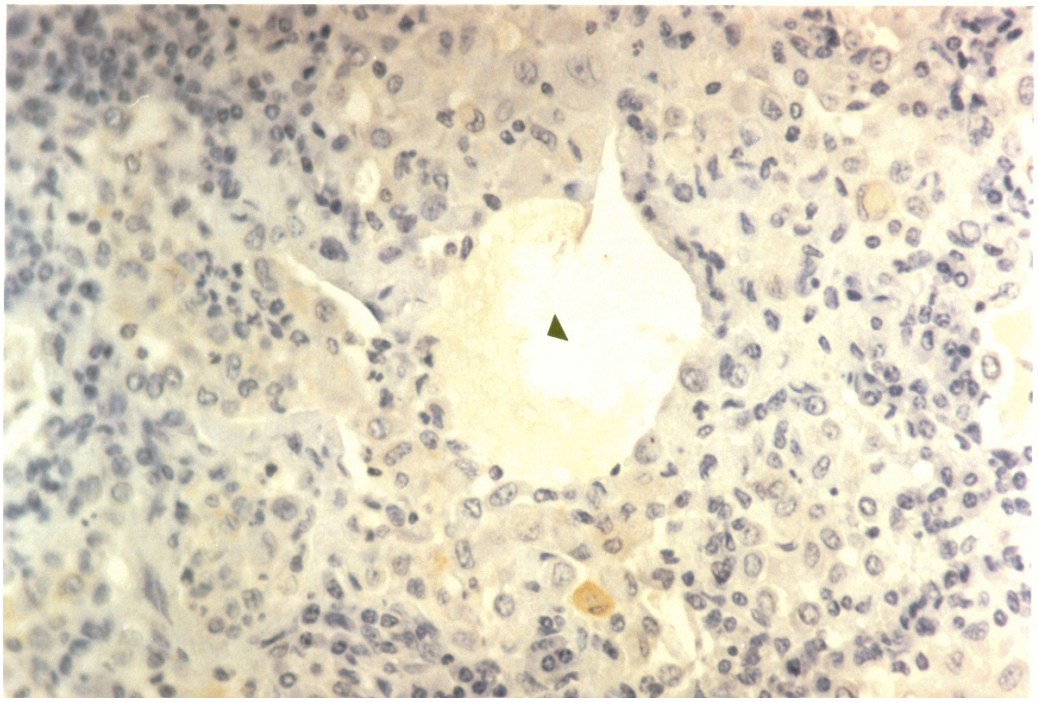

Figure 4.6: Homogenous, light brown material in alveoli and bronchi (arrow head), presumed to be oedema fluid (400X).

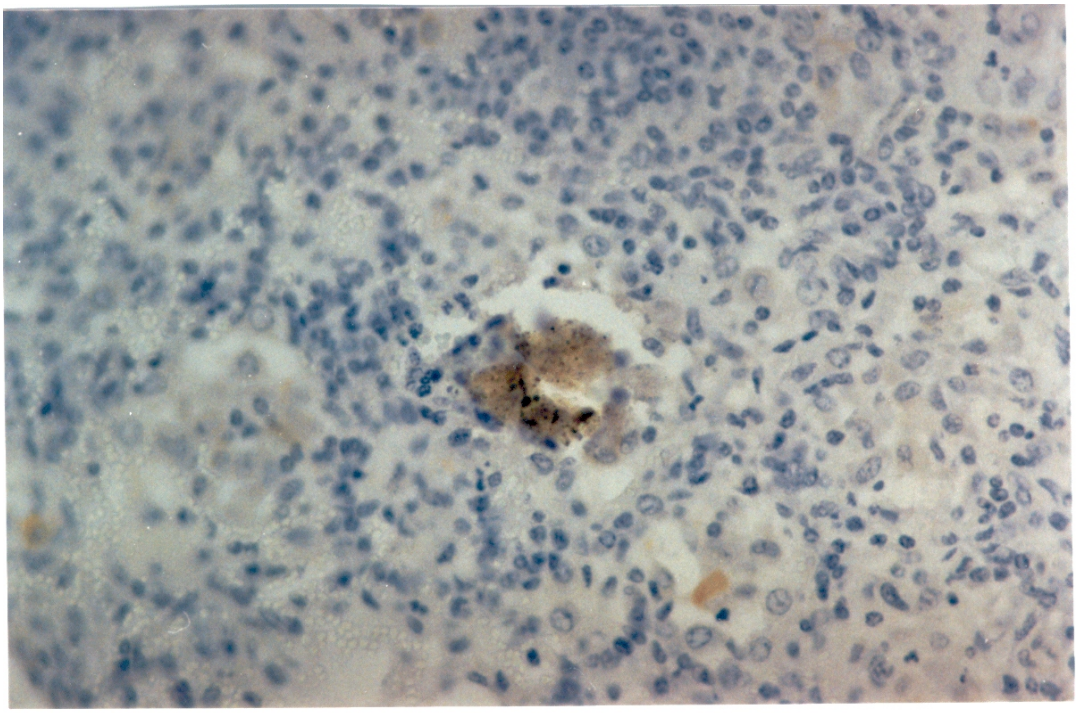

Figure 4.7: Large, black, granular staining material presumed to be dust or carbon like particles (400X).

The results of the histopathological examination, histological grading, immunoperoxidase staining and serology are recorded below for the different groups of animals. The results are summarized in Table 4.1 (page 26).

4.1 Naturally infected sheep (Group A)

Macroscopically severely affected lungs (seropositive animals, n = 10)

Ten out of ten (100%) of the animals had pulmonary lesions histologically consistent with Maedi and of these 7/10 (70%) and 3/10 (30%) of the lesions were, respectively, graded as moderate (++) and severe (+++). Lung tissues of 8/10 (80%) of animals were positive for the presence of MVV viral protein with the immunoperoxidase test. Five out of 10 (50%); 1/ 10 (10%); 1/10 (10%) and 1/10 (10%), respectively, exhibited positive staining in 6/6; 5/6; 4/6 and 1/6 sections. Two animals (20%) exhibited no staining at all. The average counts of positively infected cells per positively staining section varied from 3,6 to 25,5 per animal in this group with an average of 12,4. No lung sections stained positive in those stained with either the RVF antibody or sections stained without the monoclonal antibody. Ten out of ten (100%) of these animals were seropositive with the ELISA.

Macroscopically mildly affected lungs (seropositive animals, n = 10)

Eight out ten (80%) of the animals had pulmonary lesions histologically consistent with Maedi and of these 4/10 (40%) , 4/10 (40%) and 2/10 (20%) were graded, respectively, as moderate (++), mild (+) or normal (0) . In six out of 10 animals MVV viral protein could be demonstrated in lung sections. Two out of ten (20%), 2/10 (20%), 2/10 (20%) and 4/10 (40%), respectively, exhibited positive staining in 5/6, 3/6, 2/6 and 0/6 lung sections . The average counts of positive staining cells per section varied from 1,6 to 5,8 with an average of 3,5 in this group. No lung sections

stained positive with the RVF antibody or in the sections stained without the monoclonal antibody. Ten out of ten (100%) animals tested seropositive with the ELISA test.

Macroscopically normal lungs (seropositive animals, n = 10)

None of ten animals (100%) had histological lesions consistent with Maedi in any of the sections. One out of ten (10%) and 9/10 (90%) were graded, respectively, as having a non-specific interstitial pneumonia (+) without significant lymphoid follicles and normal (0). MVV viral protein could be demonstrated in lung sections in 3/10 (30%) animals. One out of ten (10%) and 2/10 (20%) of animals exhibited positive staining for MVV viral antigen in 2/6 and 1/6 sections, respectively. The average counts of positive staining cells in the positive staining sections varied from from one to four with an average of two. No positive staining reactions were seen when sections were stained with the RVF antibody or in sections stained with solutions in which the monoclonal antibody was deleted. Ten out of ten animals (100%) tested seropositive with the ELISA.

Macroscopically affected lungs (seronegative animals, n = 10)

Ten out of ten (100%) of animals exhibited pulmonary lesions which were histologically consistent with Maedi. Six out of ten (60%), 2/10 (20%) and 2/10 (20%) were graded, respectively, as severe (+++) , moderate (++) and mild (+). Nine out of ten (90%) of animals exhibited positive staining reflecting the presence of viral protein and 1/10 animal exhibited no staining. In the positive animals 6/10 (60%) ; 1/10 (10%), 1/10 (10%) and 1/10 (10%), respectively, had positive staining in 6/6; 4/6; 2/6 and 1/6 sections. The average count of positive staining cells per section varied from 1 - 21,8 with an average of 8,1. No positive staining reactions were seen when sections were stained with the RVF antibody or in sections stained with solutions in which the monoclonal antibody was deleted. Ten out of ten animals (100%) tested seronegative with the ELISA.

4.2 Positive control sheep (Group B)

Macroscopically affected lungs (n=6)

Six out of six (100%) of animals had histological lesions consistent with Maedi and 2/6 (33,3%); 3/6 (50%) and 1/6 (16,6%), respectively, were graded as severe, moderate and mild. Viral protein could be demonstrated in lung sections of 4/6 (66%) of animals. One out of 6 (16%), 1/6 (16%) and 2/6 (12%) of these animals, respectively, exhibited positive staining in 3/3, 2/3, 1/3 lung sections. The count of infected cells varied from 1 to 38 with an average of 16. No positive staining reactions were seen when sections were stained with the RVF antibody or in sections stained with solutions in which the monoclonal antibody was deleted. Four out of 6 (66%) animals were seropositive with the ELISA test and the other two were seronegative.

Macroscopically normal lungs (n=6)

Six out of six (100%) animals had no histologic lesions consistent with Maedi in their lung sections and were graded as normal (0). No viral protein was demonstrated in any of the lung sections. No positive staining reactions were seen when sections were stained with the RVF antibody or in sections stained with solutions in which the monoclonal antibody was deleted. Three out of six (50%) and 3/6 (50%) tested seropositive and seronegative with ELISA, respectively.

4.3 Negative control sheep (Group C)

Macroscopically normal lungs (seronegative animals, n = 20)

Twenty out of twenty animals had no histological lesions consistent with Maedi, all lungs appearing normal macroscopically. Seventeen out of twenty were graded as normal (0) and 3/20 as having mild interstitial pneumonia. All six lung sections of each of the animals (100%) stained negative for the presence of viral antigen with the MVV and, RVF antibodies and sections stained without monoclonal antibody. All twenty animals (100%) were negative with the ELISA.

TABLE 4.1: Results of histopathology, immunoperoxidase and serology

ANIMAL INDENTIFICATION		HISTOPATHOLOGY	IMMUNOPEROXIDASE			SERO-
GROUP	NUMBER		OVF-LV	RVF	NONE	LOGY
A	1	2	1/6	0/6	0/6	P
A	2	3	6/6	0/6	0/6	P
A	3	3	6/6	0/6	0/6	P
A	4	2	0/6	0/6	0/6	P
A	5	2	0/6	0/6	0/6	P
A	6	2	6/6	0/6	0/6	P
A	7	3	6/6	0/6	0/6	P
A	8	2	4/6	0/6	0/6	P
A	9	2	6/6	0/6	0/6	P
A	10	2	5/6	0/6	0/6	P
A	11	2	5/6	0/6	0/6	P
A	12	2	3/6	0/6	0/6	P
A	13	1	5/6	0/6	0/6	P
A	14	1	0/6	0/6	0/6	P
A	15	2	3/6	0/6	0/6	P
A	16	1	2/6	0/6	0/6	P
A	17	0	0/6	0/6	0/6	P
A	18	2	0/6	0/6	0/6	P
A	19	0	0/6	0/6	0/6	P
A	20	1	2/6	0/6	0/6	P
A	21	0	1/6	0/6	0/6	P
A	22	0	0/6	0/6	0/6	P
A	23	0	0/6	0/6	0/6	P
A	24	0	0/6	0/6	0/6	P
A	25	0	0/6	0/6	0/6	P
A	26	0	0/6	0/6	0/6	P
A	27	0	1/6	0/6	0/6	P
A	28	0	0/6	0/6	0/6	P
A	29	0	0/6	0/6	0/6	P
A	30	1	2/6	0/6	0/6	P
A	31	3	6/6	0/6	0/6	N
A	32	3	6/6	0/6	0/6	N
A	33	2	6/6	0/6	0/6	N
A	34	3	6/6	0/6	0/6	N
A	35	1	0/6	0/6	0/6	N
A	36	1	1/6	0/6	0/6	N
A	37	2	2/6	0/6	0/6	N
A	38	3	4/6	0/6	0/6	N
A	39	3	6/6	0/6	0/6	N
A	40	3	6/6	0/6	0/6	N
B	41	3	0/3	0/3	0/3	P
B	42	2	3/3	0/3	0/3	P
B	43	2	2/3	0/3	0/3	N
B	44	2	1/3	0/3	0/3	N
B	45	3	0/3	0/3	0/3	P
B	46	1	1/3	0/3	0/3	P
B	47	0	0/3	0/3	0/3	P
B	48	0	0/3	0/3	0/3	N
B	49	0	0/3	0/3	0/3	N
B	50	0	0/3	0/3	0/3	N
B	51	0	0/3	0/3	0/3	P
B	52	0	0/3	0/3	0/3	P
C	53	0	0/6	0/6	0/6	N
C	54	0	0/6	0/6	0/6	N
C	55	0	0/6	0/6	0/6	N
C	56	0	0/6	0/6	0/6	N
C	57	0	0/6	0/6	0/6	N
C	58	0	0/6	0/6	0/6	N
C	59	1	0/6	0/6	0/6	N
C	60	0	0/6	0/6	0/6	N
C	61	0	0/6	0/6	0/6	N
C	62	0	0/6	0/6	0/6	N
C	63	0	0/6	0/6	0/6	N
C	64	1	0/6	0/6	0/6	N
C	65	0	0/6	0/6	0/6	N
C	66	0	0/6	0/6	0/6	N
C	67	0	0/6	0/6	0/6	N
C	68	0	0/6	0/6	0/6	N
C	69	0	0/6	0/6	0/6	N
C	70	1	0/6	0/6	0/6	N
C	71	0	0/6	0/6	0/6	N
C	72	0	0/6	0/6	0/6	N

4.4 Relationship between histologic grading and IMP results

Following grouping of animals into the different categories (severe, moderate, mild and normal) according to the histologic grading system, the numbers of animals that could/could not be diagnosed as infected by means of IMP staining, was compared according to the different histologic categories. Results were compared after examining all sections from animals in groups A, B and C and results are set out below in Table 4.2. For Groups A & B it was found that in the majority of animals, 82%, 81% , 75% an 12%, respectively, which were graded as severe (+++), moderate (++), mild (+) and normal, MVV viral antigen could be demonstrated. The percentages were higher for the severe and moderate categories compared to the mild category of lungs. In the negative group 15% of animals had a histologic grading of mild (+) but no viral antigen was demonstrated in any lung sections.

Table 4.2: Numbers of animals positive/negative per histologic grading category

	GRADING	NUMBER OF ANIMALS POSITIVE	NUMBER OF ANIMALS NEGATIVE	TOTAL NUMBER OF ANIMALS
POSITIVE GROUPS A & B	+++	9 (82%)	2 (18%)	11
	++	13 (81%)	3 (19%)	16
	+	6 (75%)	2 (25%)	8
	0	2 (12%)	15 (88%)	17
NEGATIVE CONTROL GROUP C	+++	0	0	0
	++	0	0	0
	+	0	3 (15%)	3
	0	0	17 (85%)	17

As the sample size was uneven between Groups A and B (6 *vs* 3 sections) the same comparison was made again but only three randomly selected sections were examined from each animal in Groups A and C and the findings compared with the three sections examined of each animal in Group B. Results are summarized in Table 4.3: It was found that the findings remained the same for all animals in the positive groups of animals graded as severe (+++) and all animals in the negative group graded as mild (+) and normal (0). However, in the animals from the positive groups graded as moderate the numbers of animals diagnosed as positive dropped from 13 (81%) to 12 (75%). The number of animals which could be positively diagnosed stayed the same for the group graded as mild (+). In the group graded as normal (0) the number of animals that could be diagnosed as infected dropped to zero.

Table 4.3: Numbers of animals positive/negative per histologic grading category

GROUPS OF ANIMALS	GRADING	NUMBER OF ANIMALS POSITIVE	NUMBER OF ANIMALS NEGATIVE	TOTAL NUMBER OF ANIMALS
POSITIVE GROUPS A & B	+++	9 (82%)	2 (18%)	11
	++	12 (75%)	4 (25%)	16
	+	6 (75%)	2 (25%)	8
	0	0	17 (100%)	17
NEGATIVE GROUP C	+++	0	0	0
	++	0	0	0
	+	0	3 (15%)	3
	0	0	17 (85%)	17

4.5 Relationship between histologic grading and number of sections staining positive per animal

Following a similar approach of grouping animals according to the different histological grading categories, the number of sections per animal, that exhibited positively staining cells were compared, between the different histological categories. For this correlation only the results from animals in Group A were compared as six sections were examined from each animal in this group. In Group B only three sections were available per animal and therefore this group was not included. Results are summarized below in Table 4.4. It was found that in those animals categorised as severe (+++) almost all (89%) exhibited positive staining for MVV antigen, in all six sections, and only11% in four out of six sections. In the moderate (++) category the number of animals staining positive in 6/6; 5/6; 4/6 ;3/6; 2/6; 1/6 and 0/6 were more evenly distributed with only 23 % of animals exhibiting positive staining in all six sections. In the mild (+) category the majority of animals (43%) exhibited positive staining in 2/6 sections only.

Table 4.4: Correlation between histologic grading and number of sections staining positive in Group A

HISTOLOGIC GRADING	NUMBERS OF ANIMALS GROUPED ACCORDING TO NUMBER OF SECTIONS WITH POSITIVE STAINING PER ANIMAL IN GROUP A							TOTAL
	6/6	**5/6**	**4/6**	**3/6**	**2/6**	**1/6**	**0/6**	
+++	8(89%)	0	1(11%)	0	0	0	0	9
++	3(23%)	2(15%)	1(8%)	2(15%)	1(8%)	1 (8%)	3(23%)	13
+	0	1(14%)	0	0	3(43%)	1(14%)	2(28%)	7
0	0	0	0	0	0	2(18%)	9 (88%)	11
TOTAL	11	3	2	2	4	4	14	40

4.6 Relationship between number of sections examined per animal and sensitivity of IMP

In order to evaluate the influence that the number of sections examined per animal may have on the sensitivity of the IMP the findings of Group A was analysed. Only Group A (n = 40) was used as six sections were available per animal in this group. In Group B only three sections were available from each animal which made comparison, of this kind, between these two groups difficult. From all animal exhibiting positive staining in Group A three sections were selected from each, in a random manner, and the number of animals which could then be detected as MVV infected determined. In a similar random selection process this was repeated to select one section from each animal, and the number of animals that could be diagnosed as MVV infected determined. This selection process did not take into account the sampling site and sections of different sites are therefore compared. Results are set out below in Table 4.5.

It was found that in the nine animals graded as severe (+++) all (100%) could be diagnosed as infected regardless of the number of sections examined. However, in the group of 13 animals graded as moderate (++) the percentages of animals that could be diagnosed as infected decreased, respectively, from 77% to 70% to 46% when six, three and one section was examined. Similarly the percentages dropped from 71% to 71% to 14%, respectively, when six, three and one section was examined in the group of 7 animals graded as mild (+). In the group graded as normal no animals could be diagnosed as infected when three and one sections were examined per animal. Statistical analysis was performed on two of these groups (i.e sample size six and sample size one). For the sample size of six the findings of all the South African flocks including negative Group C were taken into account.

Table 4.5: Influence of number of sections examined on sensitivity of IMP

HISTOLOGIC GRADING	NUMBER OF ANIMALS POSITIVE PER NUMBER OF SECTIONS EXAMINED PER ANIMAL			TOTAL NUMBER OF ANIMALS IN HISTOLOGIC GROUP
	6 SECTIONS	3 SECTIONS	1 SECTION	
+++	9 (100%)	9 (100%)	9 (100%)	9
++	10 (77%)	9 (70%)	6 (46%)	13
+	5 (71%)	5 (71%)	1 (14%)	7
0	2 (18%)	0	0	11
TOTAL NUMBER OF ANIMALS POSITIVE	26 (65%)	23 (58%)	16 (40%)	

4.7 Relationship between the histologic grading and the average number of positive staining cells

The average count of positive staining cells were determined, as set out under materials and methods, for each animal which exhibited positive staining for viral antigen in both Groups A and B. Animals were then grouped according to their different histologic grades and the average cell count then calculated for each histologic grading group. It was found that the average count of positive staining cells decreased, respectively, from 12, 7, 8 to 1 for lungs graded as severe (+++), moderate (++), mild (+) and normal (0). Results are summarized in Table 4.6 below:

Table 4.6: Comparison between the histologic grading and the average number of positive staining cells

HISTOLOGIC GRADE	NO OF ANIMALS	AVERAGE COUNT OF POSITIVE STAINING CELLS
+++	11	12
++	16	7
+	8	8
0	17	1

4.8 Relationship between sampling site and positive staining

Specimens were collected from six different standardised sites from each animal in Group A. Positive and negative staining results were recorded for each site for the 40 animals of Group A. The total number of positive staining sections per site were compared to establish the possible influence of sampling site on results. For this comparison only the findings of Group A, not including Group B, were used for similar reasons as already explained above. The sampling sites were as follows:

1 - cranial lobe

2 - cranial lobe

3 - cranial lobe

4 - cranial border region of caudal lobe

5 - ventral border region of caudal lobe

6 - dorsocaudal region of caudal lobe

It was found that the sites which gave the highest number of positive results were the ventral borders and dorsocaudal regions of the caudal lobe with 50% of sections exhibiting positive staining. The percentages for the other sampling sites varied: 35%,42,5%, 42,%% and 47,5%.

Comparative results are summarized in Table 4.7 below:

Table 4.7: Correlation between sampling site and positive IMP staining

	SAMPLING SITE					
	1	2	3	4	5	6
NO OF ANIMALS	40	40	40	40	40	40
NO OF ANIMALS POSITIVE	14	17	17	19	20	20
PERCENTAGE OF ANIMALS POSITIVE	35%	42,5%	42,5%	47,5%	50%	50%

4.9 Satistical analyses of relationship between the number of sections examined, histologic grade and IMP results

The statistical analysis were performed on the following subsets of data , namely :

A- All sections of all animals (i.e. RSA Groups A & C and the USA Group B) with total number of 72 animals and 396 lung sections - tables 4.8(a) and 4.8(b).

B- Three lung specimens per animal (i.e. random specimens selected from animals in Group A and plus the three sections of animals in Group B) with total number of 52 animals and 156 lung sections - tables 4.9(a) and 4.9 (b).

C- Six lung sections per animal (Groups A & C only) with total number of 60 animals and 360 lung sections - tables 4.10(a) and 4.10(b).

D- One random sample per animal from Group A only with total number of 40 animals and 40 lung sections - tables 4.11(a) and 4.11 (b).

From the analysis of deviance tables below (Tables 4.8, 4.9, 4.10, 4.11) highly significant differences between the proportions of IMP positives associated with the four different histologic grade categories were obtained, the chi-square statistic indicating $P<0.001$ in all cases. The estimated proportions of IMP positives for each histologic grade together with their standard errors are summarized in Table 4.12. As is to be expected the standard errors for each proportion increases with decreases in sample size. The more severe the histologic grade the higher the prediction for all four subsets of data. The difference in prediction between the severe, moderate, mild and normal categories differed within all the different subsets and in decreasing order per respective category. The proportions of IMP positives based on sample size six and three compared (subset B), compared well with the analysis based on the sections obtained from all 72 animals (subset A). However, this cannot be said when comparing data from the single lung specimens (subset D) illustrating the prediction in the moderate, mild and moderate categories to be the lowest of all these four data subsets.

Table 4.8(a) : Logistic regression analysis for subset A (all data from animals in Groups A, B and C. *n* = 72 animals, 396 lung sections)

	d.f.	**deviance**	**Mean deviance**	**deviance ratio**	**approx chi pr**
Regression	3	222	73.991	73.99	< 0.001
Residual	68	141.6	2.083		
Total	71	363.6	5.121		

Ratios are based on dispersion parameter with value 1.

Table 4.8(b): Predicted proportions from logistic regression model for subset A (all data from animals in Groups A, B ,C. *n* = 72 animals, 396 lung sections)

Grade	Prediction	Standard error
Normal	0.01075	0.00754
Mild	0.20635	0.05099
Moderate	0.54023	0.05343
Severe	0.86667	0.04388

Table 4.9(a) : Logistic regression analysis for subset B (three sections of animals in Groups A and B. *n* = 52 animals, 156 lung sections)

	d.f.	deviance	Mean deviance	deviance ratio	approx chi pr
Regression	3	74.69	24.895	24.9	<.001
Residual	48	77.74	1.620		
Total	51	152.42	2.989		

Ratios are based on dispersion parameter with value 1.

Table 4.9(b): Predicted proportions from logistic regression model for subset B (three sections of animals in Groups A and B. *n* = 52 animals, 156 lung sections)

Grade	Prediction	Standard error
Normal	0.000039	0.000528
Mild	0.333333	0.096225
Moderate	0.5	0.072169
Severe	0.787879	0.071165

Table 4.10(a) : Logistic regression analysis for subset C (six sections of animals in Groups A and c. *n* = 60 animals, 360 lung sections)

	d.f.	deviance	mean deviance	deviance ratio	approx chi pr
Regression	3	231.3	77.111	77.11	< .001
Residual	56	106.5	1.901		
Total	59	337.8	5.725		

Ratios are based on dispersion parameter with value 1.

Table 4.10(b): Predicted proportions from logistic regression model for subset C (six sections of animals in Groups A and C. *n* = 60 animals, 360 lung sections)

Grade	Prediction	Standard error
Normal	0.0119	0.00836
Mild	0.2	0.05164
Moderate	0.52564	0.05654
Severe	0.96293	0.02518

Table 4.11(a) : Logistic regression analysis for subset D (one section of animals in Group A . *n* = 40 animals, 40 lung sections)

	d.f.	deviance	mean deviance	deviance ratio	approx chi pr
Regression	3	30.15	10.0512	10.05	<.001
Residual	36	23.69	0.658		
Total	39	53.84	1.3805		

Ratios are based on dispersion parameter with value 1.

Table 4.11(b) : Predicted proportions from logistic regression analysis for subset D (one section of animals in Groups A *n* = 40 animals, 40 lung sections)

Grade	Prediction	Standard error
Normal	0.000009	0.000563
Mild	0.142857	0.13226
Moderate	0.461538	0.138264
Severe	0.999964	0.001216

Table 4.12: Estimated proportions (prediction) and standard errors of IMP positives per histologic grade

GRADE	DATA SUBSET A	DATA SUBSET B	DATA SUBSET C	DATA SUBSET D
NORMAL	0.01075 (0.00754)	0.000039 (0.000528)	0.0119 (0.00836)	0.000009 (0.000563)
MILD	0.20635 (0.05099)	0.333333 (0.096225)	0.2 (0.05164)	0.142857 (0.13226)
MODERATE	0.54023 (0.05343)	0.50000 (0.072169)	0.52564 (0.05654)	0.461538 (0.138264)
SEVERE	0.86667 (0.04388)	0.78789 (0.071165)	0.96293 (0.02518)	0.999964 (0.001216)

Estimated proportion (prediction) followed by standard errors in brackets.

DISCUSSION

The immunoperoxidase (IMP) technique had been used successfully to study certain aspects of lentiviral infection in sheep, but the sensitivity and specificity of the technique were not determined in these studies. The present study took into account the degree of severity of the lesions, both macroscopical and histological, the number of tissue sections exhibiting positive staining for viral antigen per animal; the average number of infected cells staining positive for viral antigen in these sections, and the site of sampling.

Positive staining for viral antigen in tissue sections was characterized by the presence of either golden-brown intracytoplasmic granules or a diffuse golden-brown discolouration of the cytoplasm, with or without granules, mostly in macrophages and in multinucleated giant cells in some animals. Diffuse staining of the cytoplasm is thought to be the result of viral protein production by the macrophage (Brodie, *et al.*, 1992; Lujan, *et al.*, 1994) whilst positive granular staining may also represent engulfed virus particles/proteins (Lujan, *et al.,* 1994). Multinucleated giant cells have been reported in cases with Maedi and their presence is considered to be a response to chronic inflammatory changes in the lung and are not as such specific to the lesions of Maedi (Lujan, *et al.,* 1994). Some authors associate the presence of multinucleated cells with viral strains of increased virulence (Georgsson, *et al.,* 1989) as *in vitro* replicating MVV produces multinucleated syncytia (Petursson, Georgsson & Palsson, 1990).

The IMP technique lacked sensitivity. Thirty animals (57,7%) out of the total number of 52 animals from the naturally-infected flocks (Groups A and B), could be diagnosed as infected with MVV. In group A (naturally- infected South African flock) 26/40 (65%) of animals were diagnosed as infected with MVV compared to 4/12 (33,3%) of animals in the positive

control group B. This discrepancy, with a seemingly higher sensitivity in Group A, could be influenced by three factors. Firstly there may be viral strain differences between these two groups, affecting the virulence and hence viral protein expression. Secondly the number of sections examined between these two groups were different and double the number (6 *vs* 3) were stained from animals in Group A compared to Group B. As discussed later in this section the number of sections examined may influence the sensitivity of the IMP technique significantly. Thirdly the proportion between affected and normal lungs was also different and in Group A 29/40 (72,5%) animals had histologic lesions whilst only 6/12 (50%) in Group B exhibited lesions. The absence or presence of histologic lesions may also influence the sensitivity (discussed later in this section). Animals in the negative control group (Group C), originating from a seronegative South African flock, were negative for MVV infection by means of the IMP test. None of the animals in any of the groups exhibited any positive staining cells in sections stained with the irrelevant antibody and in sections stained without antibody. All animals in Group A were either seropositive or had typical histologic lesions. Animals in Group B that exhibited no histologic lesions and were seronegative were confirmed to be infected by other means such as the PCR and AGID techniques. Animals testing seronegative, despite being infected, and in some instances with typical histopathological lesions have been reported in the literature (Petursson, 1990). A possible explanation may be suppression of the immune system over time resulting in the decline of production of antibodies as it is postulated that MVV has immunosuppressive properties. It may also be influenced by the type of serological test employed and it is well known that the various serological tests differ in their sensitivity and specificity. It is also postulated that virus strains of lower virulence produce a lower serological response compared to viral strains of higher virulence (Petursson, 1990). In this study 9/10 (90%) of the seronegative animals in Group A, that had typical histopathologic lesions, were diagnosed as infected by means of IMP stained histological sections.

It was found that the severity of lesions had an influence on the ability to confirm infection in lung sections by applying a specific IMP stain. Macroscopic assessment of the severity of lung lesions was found to be subjective and it only really allowed for distinction between unaffected, mild and severe macroscopical lesions. This subjectivity was mirrored by the

sometimes less than satisfactory correlation between the macroscopic assessment, especially the moderate category, and the histological grading of the same lung. In many cases lungs did exhibit a mild degree of non-specific interstitial pneumonia. This was seen in the positive and negative South African flocks without other typical histological lesions to indicate Maedi. Findings were usually those of mononuclear cellular infiltrates in alveolar septal walls which were diffuse or multifocal and patchy, resulting in some thickening of the alveolar walls. This probably only represent mild or the onset of maedi, or an incidental finding, or may be related to unknown immunogenic stimulation such as inhaled environmental antigens (dust) or low-grade bacterial infections (eg *Arcanobacterium pyogenes*) giving rise to these mild lesions. All animals in both Groups A and B were kept under circumstances where inhalation of dust was possible. In the flock from which the animals in Group A originated many animals exhibited *A. pyogenes* abscesses in their lungs at slaughter. Brodie *et al.* (1992) found no correlation between antibody titres to respiratory viruses and lentiviral infection. However, they observed an association between lentiviral induced pneumonia and secondary bacterial infection, particularly *Corynebacterium pseudotuberculosis*. To what extent circulating *A. pyogenes* may have influenced development of lesions remains undetermined.

For the purpose of this study animals were therefore grouped according to their histological grading and all findings then compared. Findings for Groups A and B were that in those groups with lung lesions graded as severe, 9/11 (82%) animals exhibited positive staining. Animals with moderately affected lungs exhibited positive staining in 13/16 (81%). In the group of animals which had mild lesions, 6/8 (75%) could be diagnosed as infected. In the normal appearing animals, 2/17 (12%) were diagnosed as infected. These conclusions were made combining findings of the two groups based on six and three sections examined per animal, respectively, for Groups A and B. In comparison the findings of the three randomly selected sections for each animal in Group A and the three section of the animals in Group B the findings were slightly different. The numbers of animals staining positive remained the same for the animals that were severely affected. In the moderately affected group fewer animals revealed positive staining, the number decreasing to 12/16(75%). The numbers of animals staining positive in the mildly affected group remained at 6/8 (75%). In the group appearing normal no positive staining was observed. In both the studies by Lujan *et al.* (1994) and Brodie *et al.* (1992) similar observations were made that animals with pulmonary

lesions exhibited a higher degree of positive staining.

The severity of lesions also had an influence on the number of sections per animal that exhibited positive staining. In Group A (naturally infected South African flock) 26/40 (65%) were positive for MVV infection with IMP staining and 27%; 7,5%, 5%, 5%, 10% and 10% of these animals, respectively, exhibited positive staining in 6/6; 5/6 ; 4/6; 3/6; 2/6 and 1/6 of sections. By comparing the results in Table 4.4 it was found that in animals with severe lesions the majority (8/9 or 89%) exhibited positive staining in all six lung sections. In contrast, in all six lung sections in animals with mild lesions, not a single animal stained positively. The sensitivity drops should fewer than 6 sections be examined per animal and by randomly selecting and examining three and one sections per animal, respectively, this was demonstrated by the findings as summarized in Table 4.5. In the group with severe lesions the sensitivity remained good for that group at 100% after examining six, three and one sections, respectively. In the group of animals with moderate lesions the sensitivity changed from 77%, 70%, to 46%, respectively, for six, three and one sections examined. In the group of animals with mild lesions the sensitivity decreased from 71% to 71% to 14% when examining six, three and one sections, respectively. Overall the sensitivity decreased from 65% to 58% and 40% , respectively, for the animals that were diagnosed as MVV infected when six lung sections were examined compared to examining three and one sections.

Statistical analysis confirmed that the proportions of sections that were positive by means of IMP staining differred significantly between the four histologic categories of normal, mild, moderate and severe. The more severe the lesions the higher proportion of sections could be diagnosed as infected by means of the IMP technique. It also confirmed that the ability to detect MVV infection in sections decreases significantly with a smaller sample size.

In accordance with the findings of Brodie *et al.* (1992) and Lujan *et al.* (1994) it was observed that the average number of infected cells were higher in animals with more severe lesions compared to animals with mild or no lesions. In this study the cell count varied from 12 to 7 to

8 to 1 for animals with severe, moderate, mild and no lesions. Comparing results from the different sampling sites in animals from Group A it was found that the areas which gave the most consistent positive staining results were the latero-ventral and caudo-dorsal regions of the caudal lobes. At both these sites 50 % of animals were confirmed to be infected with MVV. Results from the other sampling sites varied from 35% to 47,5%.

In conclusion and to allow for even comparison of the sensitivity of the IMP technique to diagnose MVV infection in formalin-fixed lung tissues, the results from both Groups A and B, in this study, was 51,9% if three lung sections are examined per animal. In a study performed by Lujan *et al*. (1994) four different monoclonal antibodies were employed to study MVV in the cells obtained by broncho-alveolar lavage of infected animals and it was found that only 8 out of 20 (40%) of animals had positively staining macrophages within the bronchoalveolar fluid. It would therefore seem that formalin-fixed lung specimens are better suited for the diagnosis of MVV. However, several factors may have an influence on these findings such as viral strain differences, the specific monoclonal antibody used, the technique employed and variables in different laboratories. In the study by Lujan *et al.* (1994) they have employed monoclonal antibodies against the p15, p25 and *env* viral protein. It was found that the monoclonal antibody against the p15 protein gave the best results. The monoclonal antibody used in this study was directed at the p25 protein.

As a routine screening test of individual animals to detect MVV infection it may have very limited value in many cases. It seems clear that the chances of confirming a diagnosis of MVV infection by means of IMP will be greatly enhanced by selecting specimens from animals with severe macroscopical and histological lesions. This would allow, for example, the selection of specimens with the best possible change of confirming infection in flocks, where large numbers of animals are investigated on a flock basis, eg. abattoir surveys without available serological results. The IMP technique has limited application in infected flocks where animals exhibit no or minimal macroscopic lesions suggestive of Maedi. In this study the majority of infected animals (88%) that were histologically normal could not be diagnosed as infected by means of the IMP. For routine screening serology may the better diagnostic option in such cases.

REFERENCES

1. BOSHOFF, C.H., DUNGU, B., WILLIAMS, R., VORSTER, J., CONRADIE, J.D., VERWOERD, D.W. & YORK, D.F. 1997. Detection of maedi-visna virus antibodies using a single transmembrane-core p25 recombinant protein ELISA and a modified receiver-operating characteristic analysis to determine cut-off values. *Journal of Virological Methods,* 63: 47 -56.

2. BRODIE, S. J., MARCOM, K. A., PEARSON, L, D., ANDERSON, B.C., de la CONCHA- BERMEJILLO, A., ELLIS, J.A. & de MARTINI, J.C. 1992. Effects of virus load in the pathogenesis of lentivirus-induced lymphoid interstitial pneumonia. *The Journal of Infectious Diseases*, 166: 531-541.

3. CUTLIP, R. C. & LAIRD, G.A. 1976. Isolation and characterisation of a virus associated with progressive pneumonia(maedi) of sheep. *American Journal of Veterinary Research*, 37:1377-1382.

4. DAWSON, M. 1980. Maedi visna: a review. *The Veterinary Record*, 106: 212-216.

5. DUNGWORTH, D. L., 1993. Retrovirus infections: Ovine progressive pneumonia (Maedi). *Pathology of domestic animals.* 4th edn. Edited by Jubb, K. V. F., Kennedy, P. C. & Palmer, N. Academic Press inc. San Diego.

6. GENSTAT 5, Release 3 .1995: *Genstat committee, Statistics, department of experimental statistcs*. United Kingdom.

7. GEORGSSON, G., HOUWERS, D.J., PALSSON, P.A. & PETURSSON G., 1989. Expression of viral antigens in the central nervous system of visna-infected sheep: an immunohistochemical study on experimental visna strains of increased neurovirulence; *Acta Neuropathologica*, 77:299-306.

8. HOFF-JORGENSEN, R. 1990. Diagnostic methods. *Maedi-visna and related diseases*. Edited by Petursson, G., & Hoff Jorgenson, R. Kluwer Academic Publishers.

9. HOUWERS, D. J. 1990. Economic importance, epidemiology and control. *Maedi-visna and related diseases*. Edited by Petursson, G., & Hoff Jorgenson, R. Kluwer Academic Publishers.

10. HOUWERS, D. J., GIELKENS, A. L. J. & SCHAAKE, J. 1982. An indirect enzyme-linked immunosorbent assay (ELISA) for detection of antibodies to maedi-visna virus. *Veterinary Microbiology,* 7: 209-219.

11. LUJAN, L., BEGARA, I., COLLIE, D.D.S. & WATT, N.J. 1994. Ovine lentivirus (Maedi-visna virus) protein expression in sheep alveolar macrophages. *Veterinary Pathology*, 31: 695-703.

12. McCONWAY, K. J., JAMES, M. C. & TAYLOR, P.C. 1999. Statistical modelling using GENSTAT. Arnol publishers.

13. 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. *Clinical Immunology and Immunotherapy*, 65**:** 254 -260.

14. PALSSON, P. A. 1990. Maedi-visna. History and Clinical Description. *Maedi-visna and related diseases*. Edited by Petursson, G., & Hoff Jorgenson, R. Kluwer Academic Publishers.

15. PETURSSON, G. 1990. Maedi-visna: Etiology and immune response. *Maedi-visna and related diseases*. Edited by Petursson, G., & Hoff Jorgenson, R. Kluwer Academic Publishers.

16. PETURSSON, G., GEORGSSON, G. & PALSSON, P. A. 1990. Maedi-Visna Virus. *Virus Infections of Ruminants*, Edited by Dinter, Z. and Morein, B. Elsevier Science Publishers, Amsterdam.

17. PAYNE, A., YORK, D. F., DE VILLIERS, E. M., VERWOERD, D. W., QUERAT, G., BARBAN, V., SAUZE, N. & VIGNE, R. 1986. Isolation and identification of a South African lentivirus from jaagsiekte lungs. *Onderstepoort Journal of Veterinary Research*, 53: 55-62.

18. VERWOERD D. W., TUSTIN, R. C. & WILLIAMSON, A. L. 1994. Maedi-Visna. *Infectious diseases of livestock with special reference to Southern Africa*. First edition. Edited by Coetzer, J.A.W., Thomson, G.R. & Tustin, R.C. Oxford University Press.

19. VORSTER, J.H., DUNGU, B., MARAIS, L.C., YORK, D.F., WILLIAMS, R. & BOSHOFF, C.H. 1996. A perspective on maedi-visna in South Africa. *Journal of the*

South African Veterinary Association, 67: 2-3.