Investigations of adult sheep vaccinated with Gudair® for protection against ovine Johne's disease

Mark Hazelton

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Department of Animal Science, The University of Sydney.
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Declaration

I hereby certify that I, Mark Hazelton, have completed this work individually and that, in so doing, I did not plagiarise the work of another person or have someone else complete it on my behalf, either in whole or in part.

Summary

This observational study closely examined the gross and histopathological lesions associated with ovine Johne's disease in sheep vaccinated as adults with Gudair®. Two cohorts of Gudair® vaccinated sheep were used from two separate properties both with high OJD mortality rates (>5%). All sheep had been vaccinated outside the recommended 1 to 4 months of age, mostly as adults. Vaccination injection site lesions were prevalent (>39%) in both cohorts of sheep at least 6 months post vaccination. At necropsy, lymphatic cording was found to be a good indicator of OJD and highly correlated with the formation of a multibacillary lesion. This study indicates the possibility for misdiagnosis of OJD based on gross pathology alone and emphasises the necessity for histological confirmation. Histopathology from the two cohorts of sheep reports a high proportion of multibacillary lesions in one group and a predominance of paucibacillary lesions in the other. This suggest the use of the Gudair vaccine could effect the type of histological lesion developed in OJD infected sheep vaccinated as adults, however further research is necessary to identify additional property factors which could be involved.

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Literature Review

1.1 Introduction

Ovine Johne's disease (OJD) has become a disease of increasing significance for the Australian sheep industry, especially within the last 10 years. Rising infection levels have created an epidemic and attempts to control and possibly eradicate the disease have developed widespread debate among producers and authorities regarding effective management of OJD. Much research is underway to further understand the disease and consequently develop more effective control methods.

Ovine Johne's disease is characterised by a chronic granulomatous enteritis and regional lymphadenitis in sheep caused by *Mycobacterium avium* subspecies *paratuberculosis* (*Mptb)* (Clarke and Little, 1996). Infection usually occurs soon after birth and is followed by a long incubation period. Clinical signs are commonly not evident until at least 18 months of age and affected sheep will experience progressive weight loss over a period of weeks to months and eventually die. The slow nature of the disease allows carrier animals to spread the disease among the flock for possibly many years before it is detected (Sergeant, 2001). This means the disease can be well established in a flock before clinical signs are present. Therefore if OJD is suspected in a flock it is important to implement preventative measures and continually test for the presence of *Mptb*.

Johne and Frothingham first described paratuberculosis as "a peculiar case of tuberculosis" in a cow during 1895. Further studies by Bang (1906) confirmed that it was not tuberculosis and consequently named it Johne's disease. Johne's disease has since been found to infect a wide range of both domestic and wild animal species including cattle, sheep, goats, deer, rabbits, hares, mice and various species of birds, carnivores and macropods (Rolfe, 2003). Within this array of host species exists strain variation of *Mptb* and the particular strain of interest associated with OJD in Australia is the "S" strain (Whittington *et al*., 2000a).

OJD is commonly found in varying degrees in most sheep producing countries, including New Zealand, United Kingdom, Spain, Iceland and India. In Australia, the first diagnosis of OJD occurred in 1980 on a property in the central tablelands region of New South Wales (Seamen *et al*., 1981). The exact origin of the OJD infection in Australia is not known although it is suspected that the New South Wales cases originated from

imported New Zealand sheep during the 1970s (Denholm *et al*., 1997). Since its initial discovery, OJD has spread to other areas of New South Wales and cases have also been identified in Victoria, Tasmania, and South Australia. The most likely cause for spread of the disease is via movement of infected stock or by local spread between neighbouring flocks (Sergeant, 2001).

A 6 year, $40 million National Ovine Johne's Disease Control and Evaluation Program (NOJDP) was implemented in early 1999 to further understand and control the disease (Sergeant, 2001). The Australian distribution of OJD is illustrated according to nominated zones (Figure 1). OJD is endemic in the Management Area, present at a manageable level in the control zone, occurs sporadically in the protected zone and is undetected in the free zone.

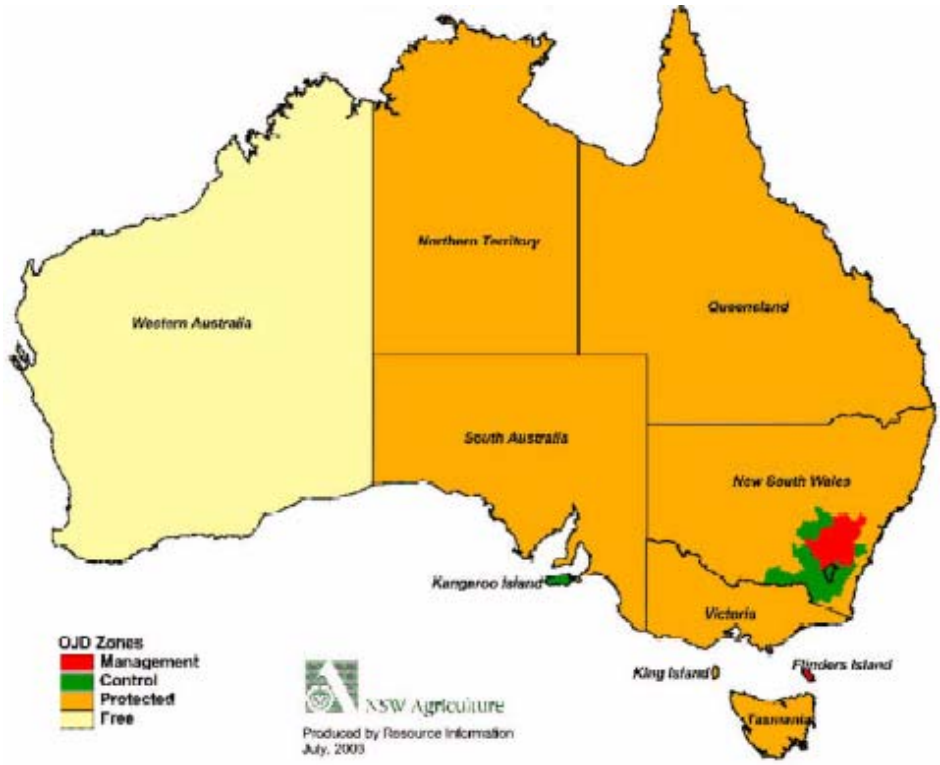

Figure 1. Current OJD Zones in Australia as of July 15, 2003 (NSW Agriculture, 2003).

At 31 December, 2000, there were 543 known infected flocks in Australia out of a potential total of between 2000 and 3700 infected flocks (Sergeant and Baldock, 2002). This estimate of flock-prevalence is broad due to some uncertainty about the sensitivity

of flock-screening tests for OJD. More than 80% of these infected flocks represent a small geographic area of central and southern New South Wales. More recent data as at March, 2003 (Table 1) presents a total of 1309 known infected sheep flocks across Australia with no infected flocks reported in Western Australia or Queensland.

State	Mar-03	Jun-02	Jun-01
NSW	1123	840	584
ACT	NA	1	2
VIC	80	45	21
TAS	41	30	20
SA	65	15	19
Total	1309	931	646

Table 1. Number of known infected sheep flocks in Australia (Langwill, 2003).

Within the last decade there has been an alarming increase in the number of infected flocks in New South Wales (Figure 2). As a part of the NOJDP, numerous research projects have commenced to estimate the full impact of OJD and better understand the disease so appropriate control measures can be implemented.

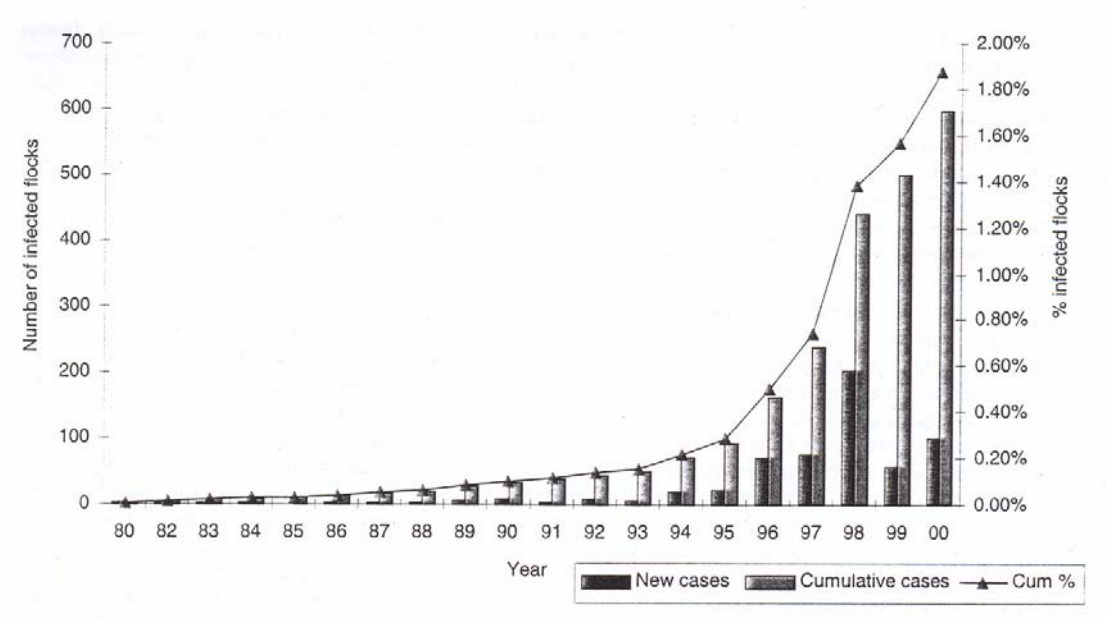

Figure 2. Annual detections of ovine Johne's disease in New South Wales to December 2002 (Sergeant, 2001).

1.2 Aetiology

Mycobacterium avium subspecies *paratuberculosis* (*Mptb*) is an acid-fast, weakly gram-positive, rod shaped bacillus of 0.5-1.5µm length (Clarke, 1997). Its complex mycobacterial cell wall is rich in lipids and is relatively impermeable which provides the acid fast property. *Mptb* differs from other members of the *Mycobacterium avium* complex as it is an obligate pathogen of animals and it does not exist in the environment in a replicative form (Whittington and Sergeant, 2001). *Mptb* is resistant to drying, acid conditions and certain disinfectants and can persist in water, soil and faeces for many months (Chiodini *et al*., 1984), however, the survival of the species is entirely dependent upon colonisation and dissemination of animals (Whittington and Sergeant, 2001).

Mptb is shed in the faeces of infected animals and transmitted to susceptible individuals either from contaminated teats during suckling, or from contaminated pasture, water or feed (Whittington and Sergeant, 2001). Shedding is particularly high in clinical cases, up to 5 x 10^{12} *Mptb* per day, and the long incubation period of the disease allows for shedding for up to 18 months before clinical signs are evident (Chiodini *et al*., 1984). As few as 10^2 organisms given orally have been shown to cause microscopic lesions in lambs in a study performed by Nisbet *et al*. (1962), although larger dose rates between 10^6 and10^9 CFU have been shown to be required in other studies to develop clinical cases of OJD (Brotherston *et al*., 1961 and Gilmour, 1965). This indicates that a relatively small amount of contaminated faeces is required to infect a large number of susceptible animals. Younger sheep, namely lambs, are most susceptible, especially those of a pre-weaning age (Abbot *et al*., 2003).

There are distinct strains of *Mptb* and strong links have been found between Australian sheep being infected with the sheep(S) strain and other species being infected with the cattle(C) strain. Therefore Johne's disease in sheep and cattle can be considered separate (Whittington *et al*., 2000a).

1.3 Pathogenesis

1.3.1 Clinical

The key signs of OJD are chronic weight loss with intermittent diarrhoea (Clarke, 1997). Weight loss in ruminants is presumed to be a direct result of protein malabsorption and loss due to the increased cellular infiltrate and oedema that occur in the intestine (Allen *et al*., 1974a). Compensatory mechanisms of increased protein synthesis by the liver are eventually exhausted and clinical signs of wasting are consequently expressed (Allen *et al*., 1974b). Sheep with clinical signs of OJD will have a low body condition score and often appear weak (Figure 3). Several implications can present from the disease including mortality, loss in feed efficiency and milk yield, decreased fertility, reduced slaughter weight at culling and shedding of wool. Diarrhoea has been found to be associated with OJD but is not evident in all clinical cases. Faeces can be hard pellets, soft and pasty or fluid-like (Carrigan and Seaman, 1990 and Clarke and Little, 1996). Due to the lengthy incubation period of the disease, clinical signs are usually expressed in older sheep.

The typical clinical sign of wasting or ill thrift is common to many other diseases and conditions. Some of these include the presence of worm parasites, malnutrition, many mineral and vitamin deficiencies, ingestion of plant toxins and carcinoma of the small intestine (Hungerford, 1990).

Figure 3. Sheep suspected of being infected with OJD and displaying typical clinical signs of wasting.

1.3.2 Gross pathology

At post mortem, most carcasses are emaciated and display diffuse thickening of the terminal ileum wall with the mucosal surfaces often presenting a granular appearance. (Clarke and Little, 1996 and Clarke, 1997). The intestinal mucosa regularly displays prominent traverse ridges and crevices and the serosal surface is usually oedematous with cording of the serosal lymphatic ducts (Carrigan and Seaman, 1990, Clarke and Little, 1996 and Clarke, 1997). Mesenteric and particularly ileocaecal lymph nodes are frequently enlarged. Gross lesions are most evident at the terminal ileum adjacent to the ileocaecal junction and less apparent both proximally and distally (Carrigan and Seaman, 1990 and Clarke and Little, 1996).

The potential for misdiagnosis of OJD on the presence of gross pathology alone has been identified, with the likelihood of experiencing "false positives" (Bush *et al*., 2003). This observation presented from a 12 month study (MLA.OJD.023) designed to estimate the biological and economic impact of OJD on 12 infected flocks with reportedly high mortality rates (exceeding 5%) due to OJD in southern New South Wales. Preliminary results illustrated 18.5% of cases with gross and microscopic lesions of thickening of the bowel without histopathological verification of OJD. This thickening was attributed mostly to serosal oedema, suggested to be due to hypoproteinemia as a result of the drought conditions experienced during 2002. This study reinforces the point that although properties may have a history of OJD losses, other diseases and conditions are also likely to produce similar clinical signs and gross lesions.

Hypoproteinemia is a condition commonly associated with gross signs of oedema (Radostits *et al*., 2000). It results from excessive loss of plasma proteins from the blood into the gastrointestinal tract due to an osmotic imbalance. Although one of the most common causes of chronic protein-losing gastroenteropathy (hypoproteinemia) in ruminants is Johne's disease (Smith, 2002), other causes are possible. Parasite infestations and malnutrition with diets low in protein can cause hypoproteinemia (Radostits *et al*., 2000). As severe drought conditions often produce diets low in protein, hypoproteinemia can result and consequently display gross thickening of the bowel, probably explaining some of the misdiagnosed cases at gross pathological examination (Bush *et al*., 2003).

As there are a number of NOJDP studies involving gross pathology of OJD further research is necessary to determine the accuracy of the presence of gross lesions of bowel thickening for the diagnosis of OJD.

1.3.3 Histopathology

Many studies have discovered two distinct histological lesions of advanced ovine paratuberculosis. These include the multibacillary and paucibacillary types and they differ in type of cellular infiltrate, degree of mycobacterial colonisation and immune response (Clarke and Little, 1996). A majority of cases have a lesion of the multibacillary form where the lesion is fully developed with an extensive, diffuse epithelioid macrophage infiltrate within the intestinal mucosa and submucosa (Clarke and Little, 1996, Perez *et al*., 1996, Perez *et al*., 1997, Perez *et al*., 1999 and Sigurdardottir *et al*., 1999). These multibacillary lesions have *Mptb* present in large numbers within the epithelioid macrophages (Appendix 1). Sheep with paucibacillary lesions have predominantly a lymphocytic infiltrate within the intestine and macrophages present in small granulomata accompanied by giant cells. *Mptb* are either few or undetectable in the paucibacillary lesion (Clarke and Little, 1996 and Clarke, 1997) (Appendix 1).

These descriptions of the multibacillary lesion are common with ovine pathology paratuberculosis type I of Stamp and Watt (1954), group I of Rajya and Singh (1961), type I of Reddy *et al*. (1984), most cases described by Carrigan and Seaman (1990), type 3b of Perez *et al*. (1996) and the multibacillary animals studied by Clarke and Little, (1996). The paucibacillary lesion is similarly described by Stamp and Watt (1954) (Type II), Rajah and Singh (1961) (Group II), Carrigan and Seaman (1990) (the minority of cases), Perez *et al*. (1996) (Type 3c) and in the paucibacillary animals explained by Clarke and Little, (1996).

The Perez *et al*. (1996) classification system is used by many studies to describe histological lesions of OJD (Appendix 2). Type 1 lesions displayed small granulomata containing aggregates of foamy macrophages in the interfollicular and basal areas of the ileal Peyer's patches. Usually these lesions show no sign of *Mptb*. Type 2 lesions have more prominent lesions in the Peyer's patches and the granulomata have extended into the adjacent mucosa with *Mptb* often present. Type 3 lesions are divided into 3 separate

categories. 3A lesions exhibit multifocal and large granulomata in the lamina propria, submucosa, serosa and draining lymph nodes. Villi are distended, the mucosa is thickened and *Mptb* are obvious. 3B type lesions display a mosaic-like sheet of macrophages and a few giant cells spread diffusely through the lamina propria and submucosa causing villous fusion and noticeable thickening of the gut. *Mptb* are abundant in the macrophages (multibacillary) and less so in the lymphoid tissues of the intestine and draining nodes. Lesions of the 3C type also show signs of a diffuse granulomatous enteritis, but display a consistent lymphocytic infiltrate within the mucosa with small, defined granulomata and giant cells scattered throughout. *Mptb* are undetectable or very sparse in these cases (paucibacillary).

The type of immune response of the host has also been found to be associated with the presence or absence of *Mptb* (Clarke and Little, 1996, Clarke, 1997 and Whittington and Sergeant, 2001). The multibacillary lesion is commonly associated with high concentrations of antibody and a weak cell-mediated immune response whilst the paucibacillary lesion reflects a high degree of cell-mediated immunity and low antibody concentrations. The stages of development and pathogenic mechanisms involved with these two types of histological lesions are not clearly understood (Clarke and Little, 1996, Clarke, 1997).

1.4 Diagnosis of OJD

A number of tests are available for the detection of OJD with several advantages and disadvantages. There are three main screening tests used in Australia (Sergeant, 2003). These include the pooled faecal culture (PFC), serology (mainly the agar-gel immuno-diffusion (AGID) test) and abattoir surveillance.

Pooled faecal culture (PFC) involves pooling faecal samples from a large number of sheep into one single sample for culture. Commonly seven pools of fifty sheep are used to give a high degree of accuracy. PFC has been shown to be a highly sensitive and specific flock test for detection of OJD-infected flocks (Whittington *et al*., 2000b) and it demonstrates a consistently higher flock-sensitivity than serology (Sergeant, 2002). The cost and time it takes to receive a final result (>3 months) are distinct disadvantages of PFC (Sergeant, 2003).

The sensitivity of the main serology test, AGID, is variant depending upon the stage of the disease and the selected sample of animals to be assessed. Sensitivity is very poor in sheep early in the course of infection and highest in sheep late in the course of infection. However, specificity is regularly greater than 99.9% with the AGID test in Australian studies (Sergeant, 2003). Due to its poor sensitivity, the use of serology is limited to assurance testing or for identification of seropositive animals for further confirmatory testing. The enzyme linked immunosorbent assay (ELISA) is a similar serology test to AGID and is reported to have similar sensitivity, however the AGID test remains more specific (Hope *et al*., 2000).

Abattoir surveillance requires the visual and manual observation of the intestinal tract after an animal's slaughter (Sergeant, 2003). The observation of gross lesions suggestive of OJD is confirmed by histopathological examination of tissue samples. Infected flocks are identified via trace-back from infected lines of sheep. Abattoir surveillance is an effective tool for estimating the regional prevalence and distribution of OJD in Australia. With the use of highly educated inspectors and animals of an appropriate age (>2 years) it could potentially be used to confirm the absence of OJD on a property (Bradley and Cannon, 2003), although there are concerns regarding its sensitivity in low prevalence flocks and regions.

The Bradley and Cannon (2003) study aimed to estimate the ability of 3 abattoir inspectors to truly diagnose OJD infected sheep using visual and manual examination of sheep viscera of 1200 sheep from OJD infected farms. They found the ability of the inspectors to detect gross lesions varied from 53% to 87%. Of the three inspectors, the best two inspectors (74% and 87% sensitivity) had a high level of agreement between their diagnoses whilst the third inspector had not undergone formal OJD inspector training. These results reflect a sensitivity level of about 70 - 75% for the ability of abattoir inspection to detect histologically positive animals and outline the need for well educated inspectors. Meanwhile, for the purposes of a negative assurance scheme, a sensitivity level of 50% is proposed to account for the lower level of heavily infected animals in low prevalence flocks (Bradley and Cannon, 2003). The examination of sheep viscera for gross signs of OJD by abattoir surveillance is a useful tool in supplementing other diagnostic testing procedures, however its application for provision of evidence of freedom from OJD is more controversial.

The effectiveness of the gamma interferon test (IFN-γ) is still being evaluated. This test has potential for use in the early detection of infection as it targets cell mediated immunity rather than humoral immunity. However, it is unlikely to become a widely used diagnostic tool because the test requires technical procedures to be performed within hours of sampling and the cost is relatively high (Sergeant, 2003).

While there are many different diagnostic tests available, histopathology remains the gold standard and is the most common test that provides a definitive diagnosis. A disadvantage of histopathology is the necessity to sacrifice the animal for collection of tissue samples unless intestinal biopsy is preferred. The culture of biopsies of intestinal tissue and associated lymph nodes has been shown to be a sensitive diagnostic tool in cattle (Pemberton, 1979, Benedictus and Haagsma, 1986 and Sockett *et al*., 1992) but has only been used experimentally in sheep. Initial results in sheep indicate that fixed biopsy tissues of the terminal ileum and associated mesenteric lymph nodes are of good quality for histological assessment of OJD (McConnel *et al*., 2003). Intestinal biopsies would have greater use as an experimental tool to further understand the pathogenesis of the disease in the live animal.

1.5 Measures of control

Due to evidence of regional distribution of OJD gathered during the mid-1990s, there has been industry pressure to develop state or national programs to control or eradicate the disease, with the hope of reducing its spread throughout Australia. Initial plans were made by the New South Wales government to control the immediate spread of OJD via movement restrictions of known or suspected infected sheep, followed by an eradication program. Victoria also implemented an eradication program and soon all States had introduced movement restrictions for sheep from known or suspected infected flocks (Sergeant, 2001).

A proposal for a national eradication program for OJD was discussed in 1997 and uncertainty as to whether the disease could be eradicated was identified in The Hussey-Morris Report (Hussey and Morris, 1998). Based on recommendations from the Hussey-Morris Report, the deeds of agreement to fund and implement a 6 year, $40 million National Ovine Johne's Disease Control and Evaluation Program (NOJDP) were signed by national and state governments in early 1999. The main aims of the program were to control the spread of OJD while further research is undertaken to determine the feasibility and cost-effectiveness of eradication, including determining the distribution and prevalence of the disease (Anonymous, 2000). The Property Disease Eradication Plan (PDEP) stipulates a minimum 15 month interval between destocking all infected flocks and restocking the property (Taylor *et al*., 2003). A more recent and continuing study evaluating the efficacy of a destocking-restocking strategy to eradiate OJD from infected Australian properties has already demonstrated a 46% failure rate. The failure of this eradication process is thought to be largely due to the inability to identify OJD free replacement stock (Taylor *et al*., 2003).

As discussed in reference to Figure 1, regional zoning of OJD across Australia was implemented in July 1999 to reduce the spread of OJD. In more detail, the four zones include: i) Residual zones – OJD is endemic but some level of control is in place, ii) Control zones – OJD may be present at a manageable level and is actively controlled, iii) Protected Zones – OJD occurs only sporadically and eradication measures are enforced and iv) Free zones – there are no known or suspected infected flocks (Sergeant, 2001). Strict movement conditions of sheep were then enforced depending upon the

classification of the zone, the flock of origin and the flock of destination. The idea was to reduce the movement of sheep from high prevalence flock areas to low prevalence or OJD-free flock areas.

It has been evident that individual property quarantines and movement restrictions have not been effective as first thought in controlling the lateral spread of *Mptb* in a high prevalence environment. It was identified that flocks with undetected infection presented a similar trading risk to known infected flocks. Thus in 2003, the NSW Residual Zone was reclassified as a Management Area (Figure 4) and individual property quarantines and trade restrictions were removed and replaced by a Ministerial Quarantine for the whole area. Trade was restricted to low risk animals (either approved vaccinates or sheep from assured flocks) and this move was welcomed by producers in the Management Area as it removed many of the disincentives for diagnosis and placed the responsibility for risk management back on the individual producer (Links *et al*., 2003).

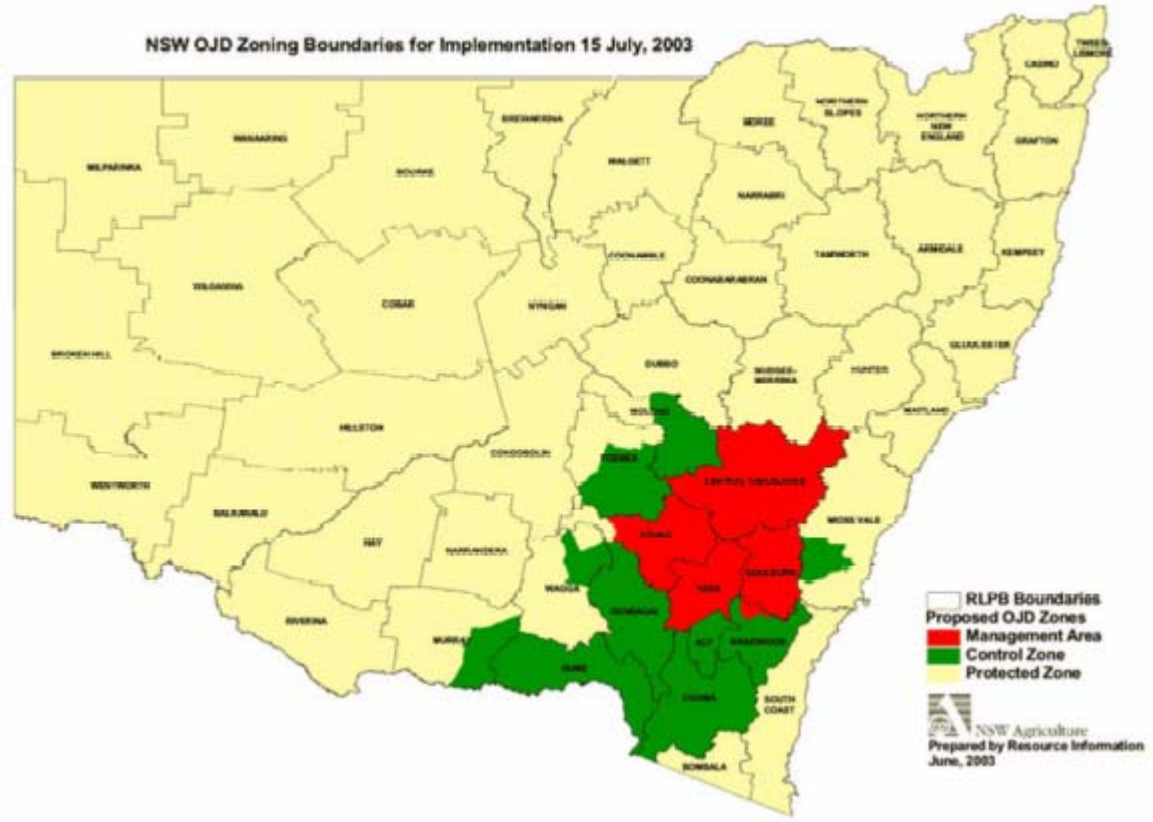

Figure 4. Current NSW OJD Zones with the Residual Zone reclassified as the Management Area (NSW Agriculture, 2003).

The recent registration of the Gudair® vaccine has provided another tool likely to be effective in the control of ovine Johne's disease. Several studies are currently underway to evaluate the vaccine and monitor its use and efficacy within Australian sheep flocks. One such study, MLA.OJD.009, involves a comparison of sheep vaccinated as lambs with non-vaccinated sheep and observes the effects of the Gudair® vaccine on immunogenicity, *Mptb* excretion, mortality, vaccination site lesion persistence and animal productivity (Eppleston *et al*., 2003). Another project, MLA.OJD.015, is a longitudinal study monitoring the changes in mortality rates following whole flock vaccination with Gudair® of a flock with a high OJD mortality rate (McGregor *et al*., 2003). A third study, MLA.OJD.033, has recently commenced looking at changes in within-flock prevalence of *Mptb* shedding following vaccination with Gudair® in high and low prevalence flocks (Windsor *et al*., 2003).

1.6 The Gudair® vaccine

The Gudair® vaccine contains a suspension of the microorganism, *Mptb*, strain 316F, inactivated by heat and adjuvanted with mineral oil in a multiple emulsion (CSL Animal Health Australia, 2002). The study by Eppleston *et al*. (2003) supports the claim that one dose (1mL) of the vaccine injected subcutaneously high on the neck behind the ear induces good immunological memory and is capable of providing immunity for the life of the sheep irrespective of whether IFN-γ responses can be detected in blood.

The vaccine was developed and manufactured in Spain (CZ Veterinaria, S.A.). The first use of the vaccine in Australia was in December 1999 for experimental purposes in the major "intensive" trial (MLA.OJD.009) to determine the effectiveness of the vaccine in Australian sheep flocks (Eppleston *et al*., 2001). Preliminary results led to the registration of the vaccine for use in Australia in June 2002. Prior to this, approval to use the vaccine in New South Wales was via a permit issued by the National Registration Authority. This permit involved certain conditions and was limited to approximately 50 flocks in New South Wales, all of which had significant mortality rates due to OJD (at or exceeding 5%) (Windsor *et al*., 2003).

In New South Wales, registration of the vaccine has provided access for flocks in the Control Zone and widened access in the Protected Zone to include: i) all infected flocks with an approved Property Disease Management Program (PDMP), ii) all monitored at-risk flocks, iii) all Market Assurance Program (MAP) flocks, and iv) all flocks trading restocking sheep to the Control Zone or the Management Area (Links *et al*., 2003).

Vaccination with Gudair® has demonstrated that it will: i) stimulate the cellular and humoral immune systems in a high proportion of vaccinates, ii) delay the onset and reduce the incidence of mortalities from OJD in vaccinates, iii) delay the onset and reduce the prevalence of shedding of *Mptb* in vaccinates, and iv) stimulate the development of lesions at or in proximity to the site of vaccination in a significant proportion of vaccinates (Eppleston *et al*., 2003). It is important to note that although the vaccine is capable of reducing the rate and delaying the onset of OJD-attributable deaths, it is not 100% effective against OJD as Eppleston *et al*. (2003) has already noted six deaths in vaccinates beginning at twenty three months of age. Whether eradication of

OJD is possible via the use of the Gudair® vaccine is questionable after reports from Iceland. Since 1966, vaccination has been compulsory in Iceland in OJD endemic areas and in 2001, the infection reappeared on two properties that had been declared paratuberculosis free, in an area where vaccination had ceased 3 years earlier (Rolfe, 2003).

Stimulated cellular and humoral immunity in vaccinates has been demonstrated by elevated IFN-γ reactions and enzyme linked immunosorbent assay (ELISA) antibody levels (Eppleston *et al*., 2003). The persistence of these parameters in the circulation is variable, however, IFN-γ reactors and ELISA antibody levels remain consistently higher in vaccinates compared to controls even at 24 months post vaccination.

Since the introduction of the Gudair® vaccine into Australia, several research projects have been designed to better understand the vaccine's role in controlling OJD within Australian sheep flocks. With a combination of the slow process of the disease and the short period for which the vaccine has been available in Australia for experimental purposes, the results are limited. Research to date is encouraging and preliminary results have answered many questions about the vaccine but there still remain many uncertainties and this research has revealed new ideas to explore.

A number of issues regarding the use of the Gudair® vaccine in Australian sheep flocks have been raised by producers and their advisors. For instance, there is a perception by producers that a visible, palpable tissue reaction at or in proximity to the site of vaccination is an indicator of successful vaccination (Windsor *et al*., 2003). This particular lesion is expected with the type of adjuvant used in the vaccine and is outlined in the product literature as a side effect but there is no evidence supporting the claim that the presence of a vaccination site lesion indicates protection against OJD. Vaccination site lesions do not seem to occur in all vaccinates. In one particular study, only 42% of vaccinates had experienced vaccination site lesions at 2 months post vaccination (Eppleston *et al*., 2003). This figure reached a plateau by 24 months post vaccination with 20% of vaccinates having palpable lesions and the average diameter of the lesion had not decreased with time as expected according to the product literature. A New Zealand study does report a decline in size of the vaccination site lesion over 2 years but does not comment on the proportion of vaccinates with vaccination site lesions

(Thompson *et al*., 2002). These lesions may cause further implications at the abattoir as it possible for a carcass to require additional trimming depending upon the location of the vaccination site lesion at slaughter.

Research into the use of different types of adjuvants in commercial vaccines has demonstrated a reduction in the local inflammatory response at the site of inoculation (Reyes *et al*., 2002). MONTANIDE® ISA 266 adjuvant offers the same efficiency in controlling the OJD infection as Complete Freund's Adjuvant (CFA) which is used in the Gudair® vaccine, but the vaccination site lesion is less pernicious. This is thought to be due to a better distribution of the antigen when using the MONTANIDE® ISA 266 adjuvant. Consequently the use of MONTANIDE® ISA 266 adjuvant could be considered as a possible alternative in the preparation of vaccines against OJD.

There is also little knowledge on the presence of Gudair® vaccine injection site lesions in sheep and their subsequent OJD intestinal lesion type at the gross and histological level. The effect of the vaccine on subsequent OJD lesion types either at the gross or microscopic level is not clearly understood. There is no evidence to support the considered view that vaccination of sheep may increase the likelihood that they will develop paucibacillary OJD lesions rather than a multibacillary lesion when they eventually 'breakdown' (Windsor *et al*., 2003).

It is recommended to vaccinate lambs between the ages of 1 to 4 months, although the effect of vaccination in sheep outside this recommended age range is not completely known. There is limited data on the effects of the vaccine in older aged sheep (e.g. hoggets and mature ewes and wethers), with initial findings reporting no apparent effect of the vaccine in reducing the death rate from OJD in adult sheep which were presumably exposed to the disease as lambs or weaners (McGregor *et al*., 2003). Further examination into the effects of vaccination in older sheep is warranted.

While there is much evidence supporting the effectiveness of Gudair® vaccine there are still some controversial issues concerning its use. One major drawback is that it does not provide complete protection from infection so it therefore cannot be solely used to eradicate the disease. Another concerning issue, is the safety of the vaccine. One documented case demonstrates serious reactions after self-inoculation (Windsor *et al*.,

2003). More research involving vaccinated sheep could hopefully lead to a better understanding and more effective use of the vaccine in Australian sheep flocks.

1.7 Conclusion

OJD is a disease of increasing importance within Australia. Initial attempts to control and eradicate the disease via regional zoning and movement restrictions appear to have had limited success. The recent registration of the Gudair® vaccine has provided a new approach to the control of OJD. Current research has demonstrated the ability of Gudair® to delay and reduce effects of OJD, although it has also revealed new aspects to explore. Several studies are continuing in an effort to further understand the disease process and establish an effective diagnostic test, with some albeit limited research involving vaccinated sheep. The use of some "wasted" and vaccinated adult sheep from a cooperating producer for undergraduate necropsy classes has presented an opportunity to more closely examine the effects of the vaccine. During the severe drought conditions in late 2002, several sheep displayed gross bowel thickening without histological evidence of OJD. The ability to attain more sheep from the same "hospital mob" provides an opportunity to further examine these findings and possibly determine how accurate the observation of gross thickening of the bowel is for field diagnosis of OJD in vaccinated sheep. Extra data and tissue samples collected from another project (MLA.OJD.015) also allows for a more detailed study of vaccinated sheep. Both cohorts of sheep provide an opportunity to conduct an observational study on older vaccinated sheep, with the potential to offer new understanding on the vaccine's impact upon sheep vaccinated outside the nominated age range of 4-16 weeks.

Such studies could provide information to producers and advisors that will impact upon management decisions relating to vaccinated sheep and the use of the vaccine. It may also shed some light on the possibility for veterinarians and farmers to misdiagnose OJD in the field based on the presence of gross thickening of the bowel. After close observation of the gross and histological nature of intestinal lesions, results may add to the knowledge of the pathogenesis of *Mptb* in sheep vaccinated as adults.

Materials and Methods

2.1 Animals used

Sheep used in this observational study were sourced from 2 separate properties. Fifty eight sheep (flock 1) were supplied from a property located on the southern tablelands of New South Wales near Goulburn. This property is 1929 Ha in size and had a total sheep number of 10 575 as of January 1, 2003. It was diagnosed as an OJD infected property in 1998 and estimated mortality rates due to OJD in March 2002 were about 8%, placing it in the high prevalence category (>5% mortality rate). One neighbouring property is known to be infected with OJD, however the status of the other 7 neighbours is unknown. The impact of drought conditions was noted in October 2002. Since then, the producer has experienced an increase in mortalities suspected to be due to OJD and he has also noted malnutrition as a problem. The 1998 drop appeared to be the most affected generation, especially the wethers. Cell grazing was the preferred grazing system but this was altered to a set stocking regime due to the lack of feed at the time of drought. This was done to utilise all available pasture and additional feed and water was supplied. All sheep were fed either triticale or barley depending upon its availability. Sheep used for data collection belonged to the same "hospital mob" described in the MLA.OJD.023 project (Bush *et al*., 2003). They were all of an adult age (3+ years old) and were culled from their mobs following the manager's decision that they displayed obvious signs of wasting.

As the mortality rate due to OJD was greater than 5% the producer was able to apply for use of the Gudair® vaccine. Lambs and one year old sheep were initially vaccinated at the end of 2001 and older sheep (2+ year olds) were vaccinated using excess vaccine in August/September 2002. As sheep used in this observational study were all of an adult age (3+ years old), this means the sheep will have been vaccinated for between 6 and 9 months at the time of euthanasia and necropsy.

The second property from which sheep were sampled in this study (flock 2) is a Merino enterprise also located on the southern tablelands of New South Wales. This cohort of sheep was sourced from the same property which is involved with the MLA.OJD.015 project (McGregor *et al*., 2003). Paratuberculosis was diagnosed in the flock in 1996 when annual sheep losses from all causes were estimated to be between 3-5%. Back then the flock grazed 1420 Ha and comprised of about 10 000 adult sheep.

Since the diagnosis of OJD and at the time these sheep were used for data collection, the enterprise has reduced in size to approximately 1000 Ha and 9000 adult sheep. An estimation of the OJD mortality rate from farmer inventory records was 25% in early 1999. After a detailed investigation of mortalities in this flock by the MLA.OJD.015 project it revealed an OJD attributable mortality risk to be 16.3% in September 1999.

In 1997, a cell grazing system was introduced involving management of the flock in mobs of up to 5000 sheep, in paddocks of about 10 Ha, with a change of paddocks every 2 to 3 days. Weaner sheep are run separately from adult sheep until they are about 15 months of age. Shearing occurs in August-September and lambing in September-October. Ewes are set-stocked during lambing. In the year leading up to the slaughter of the sheep used in this trial (September, 2001), significant management changes were implemented to combat the increasingly severe drought conditions. Sheep were sold on several occasions during the year and the remaining sheep were grazed at increased stocking rates to facilitate hand-feeding, provision of water and to enable close monitoring of feed intake and changes in body condition. Supplementary feed included chopped straw, palm kernels, commercial mineral mix and the best available protein supplement.

Seventy eight sheep in flock 2 were slaughtered for data collection on 17th September, 2001. Sheep slaughtered ranged from 2 to 7 years of age and were vaccinated with the Gudair® vaccine according to the dates outlined in Table 2.

Sheep vaccinated	Date of Gudair® vaccination	Months since vaccination at time of slaughter
1999 drop - 2100 lambs	17/05/2000	16
2460 ewes in lamb and all of an adult age	12/08/2000	13
3500 wethers all of an adult age	19/08/2000	13

Table 2. Gudair® vaccination dates of sheep from flock 2.

Sheep from both flocks were selected from the producer's "hospital mob" where sheep had very poor body condition scores and were therefore of no commercial value. The sheep were considered unfit for transport on welfare grounds.

2.2 Data and tissue collection

Collection from flock 1 began on the 24th March 2003 and was completed on the 2nd June, 2003. At the initial examination, 8 sheep were transported to Elizabeth Macarthur Agriculture Institute (EMAI) for a final year vet necropsy class. Three other subsequent classes were held at 3-weekly intervals throughout the rest of the semester. A similar necropsy class was also held for final year agricultural science students from which data and tissue samples were also collected. A Gross Pathology Course held by The University of Sydney Post Graduate Foundation at EMAI also provided an opportunity to sample a further 20 sheep from flock 1. In total, 58 sheep were used from flock 1 for data and tissue collection.

Sheep were restrained and gross data was recorded from these sheep including age, sex, condition score, confirmation of vaccination (3 hole ear-punch) and presence of soft faeces or diarrhoea. The size and location of any vaccination injection site lesions was noted. Euthanasia of the sheep was performed with a dose of barbiturate (Lethobarb®, Virbac, Peakhurst, Australia) injected intra-venously into the jugular vein. Sheep were then weighed and placed in left lateral recumbency to facilitate examination of the abdominal contents with the rumen in a ventral position. The animal was skinned and the thoracic and abdominal contents were exposed. The terminal ileum was observed for any gross signs of thickening and if present, it was graded on a scale of 1(slight) to 5(severe). A grading of 0 means the appearance of the terminal ileum was normal. If the ileocaecal lymph nodes appeared swollen their size was recorded and any signs of lymphatic cording were graded on a scale of 1(slight) to 5 (severe). Any other abnormalities were also noted.

For histopathology, the ileocaecal junction, one ileocaecal lymph node and a cross-section of the terminal ileum within 10cm of the ileocaecal junction were taken from each sheep and placed in 10% buffered formalin.

Sheep from flock 2 had data and tissues collected at Wollondilly abattoir on 17th September, 2001. Data recorded included ear-tag colour/age, size and texture of vaccination injection site lesion if present and gross signs of OJD type intestinal lesions were graded on a scale of 0 (nil) to 5 (severe). A sheep with 0 gross signs displayed intestines as found in the normal state and lymph nodes of normal size while sheep with a grading of 1 showed slight serosal oedema and reduced mesenteric fat. A grading of 2 reflected an increase in serosal thickening of the terminal ileum and a change in size and/or texture of the ileocaecal lymph node. Other gradings from 3 to 5 were based on the degree of reduction of mesenteric fat in proportion to the increase in serosal thickening of the terminal ileum plus lymph node pathology. Any sheep displaying gross signs of lymphatic cording along with severe serosal and lymph node changes were allocated a grading of 5. For further histological examination, samples were taken of the ileocaecal junction, one ileocaecal lymph node and a cross-section of the terminal ileum within 10cm of the ileocaecal junction. These tissues were preserved in 10% buffered formalin. This data was collected by Helen McGregor, Peter Windsor and Craig Kristo.

2.3 Slide preparation

Cross-sections of the terminal ileum, ileocaecal junction and ileocaecal lymph node were selected from the sampled tissues and preserved in 10% buffered formalin. Cassettes containing the selected tissues were sent into the Veterinary Pathology Diagnostic Services at The University of Sydney for further processing. This involved the use of a Miles Tek Vacuum Infiltration Processor (VIP) which processes the tissues through the following solutions according to a 12 hour schedule; 70% ethanol (2 changes, 30 minutes each), 95% ethanol (2 changes, 45 minutes each), 100% ethanol (3 changes, 1 hour each), 100% Xylol (2 changes, 1 hour each) and Paraplast Paraffin wax (3 changes, 1 ¼ hours each). This procedure removes water and fat from within the tissue and these are replaced with paraffin wax.

The tissues are then embedded in Paraplast Paraffin wax (melting point 56°C). After setting, the tissue blocks formed are sectioned at 5µm and these sections are floated on a water bath set at 45°C containing reverse osmosis water and 10mL of non

inactivated horse serum for adhesion. The sections are then mounted on glass microscope slides and allowed to dry overnight at 56°C.

Two replica slides of each section were made so one could be stained using Whitlock's Haematoxylin (progressive) and Alcoholic Eosin (Appendix 3a) while the other is stained according to the Ziehl-Neelsen method (Appendix 3b).

2.4 Histopathology

The slides were then viewed under the microscope and any OJD lesions were classified according to the Perez *et al*. (1996) classification system (Appendix 2). If a slide did not present any OJD lesions then it was classed as having no significant lesion (NSL). The severity of all the histological OJD lesions was graded on a scale of 1 to 3. A grading of 1 showed a mild lesion and a grading of 2 represented a moderate lesion while a grading of 3 demonstrated a severe lesion.

2.5 Data analysis

Percentages were calculated for all the data. Any percentages of significance were supported using a statistical computer program, Minitab. Contingency tables and chi-square tests were used to compare differences observed among the histological types of the two cohorts of sheep. A number of analyses of variance (ANOVA) were performed to determine if any significant correlations were apparent.

Results

3.1 Flock 1

3.1.1 Clinical Examination

All 58 Gudair® vaccinated sheep were 3+ years old and this particular cohort of sheep included 15 ewes and 43 wethers. All sheep were confirmed to be vaccinated by the producer. Only 2 sheep had a body condition score of 3 while the rest had a body condition score of 2.5 or less. Thirty eight sheep had their weights recorded with a mean weight of 28.7kg and a range of 23.3 – 39.7kg. Of the OJD infected sheep (diagnosed histologically) that had their faces examined (40), 53% had soft faeces or diarrhoea.

3.1.2 Gross Lesions

Vaccination injection site lesions were found on 45% (26) of the sheep (Figure 5). The average size of the lesions was 2.1cm in diameter and ranged from 1 to 5cm. These lesions were commonly found 5 to 10cm below the ear on the same side of the neck as the site of injection. Enlarged prescapular lymph nodes were palpated in 29% (17) of the sheep.

Many of the sheep displayed classic gross signs of OJD including a thickened terminal ileum, enlarged ileocaecal lymph node and lymphatic cording (Figures 6 & 7). No sign of bowel thickening was evident in 25% (12) of the OJD infected sheep and 4 out of the 10 sheep diagnosed as OJD negative displayed mild signs of bowel thickening. Lymphatic cording was present in 44% (21) of the OJD infected sheep and all sheep displaying varying degrees of lymphatic cording were diagnosed histologically as having OJD.

3.1.3 Histopathology

Of the 58 sheep, 83% (48) were diagnosed histologically as being infected with OJD and this included 12 (25%) ewes and 36 (75%) wethers. Twice as many mulitbacillary (3B) lesions were noted compared to paucibacillary (3C) lesions when classified according to the Perez *et al* (1996) classification system (Figure 8). Over 70% of the OJD infected sheep displayed a histological lesion of a severe nature. The histological lesion types of the sheep were found to be significantly different ($p<0.05$)

Figure 5. An example of a vaccination injection site lesion.

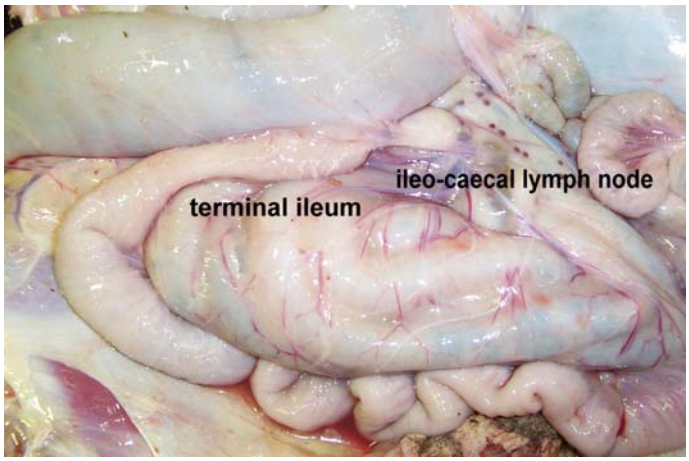

Figure 6. Gross pathology of a severe case of OJD.

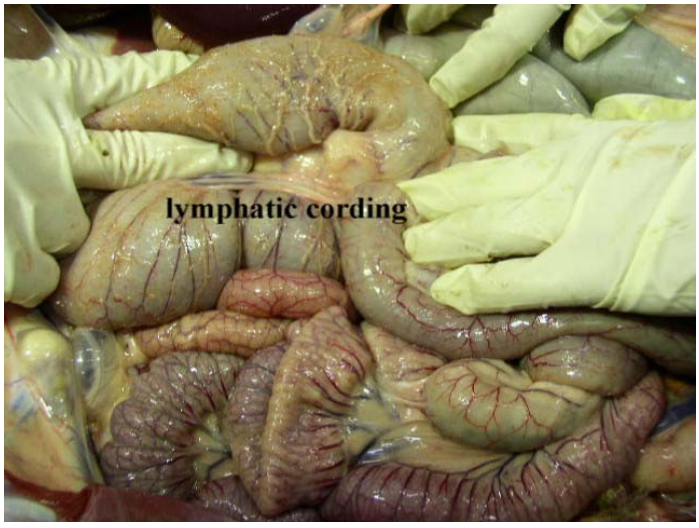

Figure 7. Severe case of lymphatic cording.

among sheep displaying lymphatic cording and sheep not displaying lymphatic cording (Figure 9). A multibacillary lesion was observed in 85% of sheep displaying lymphatic cording.

3.1.4 Alternative diagnoses

Table 3 outlines other conditions which were diagnosed in the sheep from flock 1.

Alternative diagnosis	No. of OJD positive sheep	No. of OJD negative sheep
serous atrophy of the fat reserves	1	-
serosal oedema of the bowel mesentery	2	-
cysticercosis	1	-
pizzle rot	1	1
peritonitis	1	-
endoparasitism (Trichostrongylus axei) of the abomasum	1	-
pneumonia presumed to be due to caseous lymphadenitis (CLA)	-	1
CLA subcutaneous lesion	1	-
blowfly strike	1	1
adenocarcinoma	-	1

Table 3. Alternative diagnoses in sheep from flock 1.

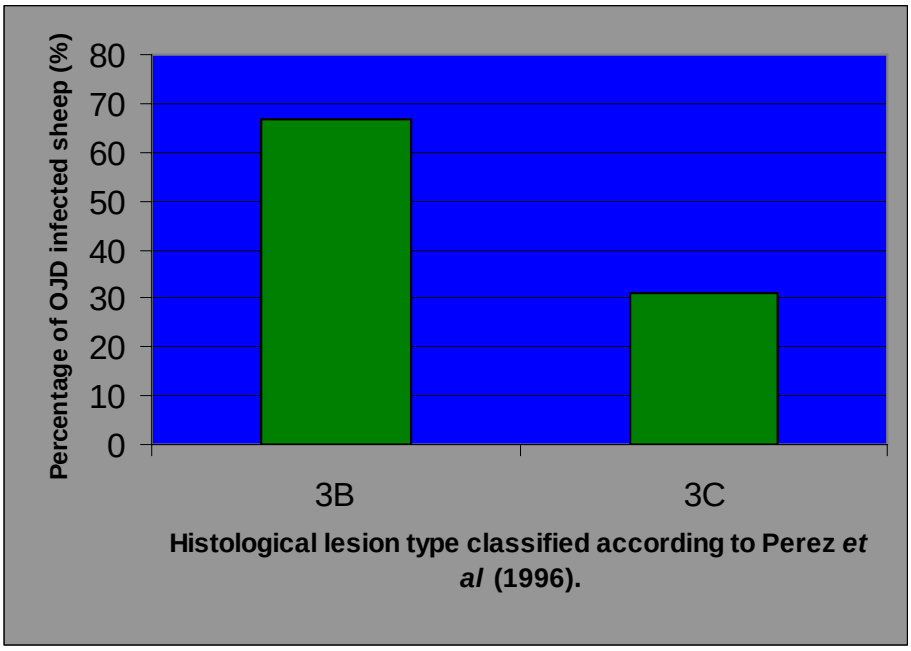

Figure 8. Classification of histological OJD lesions in flock 1.

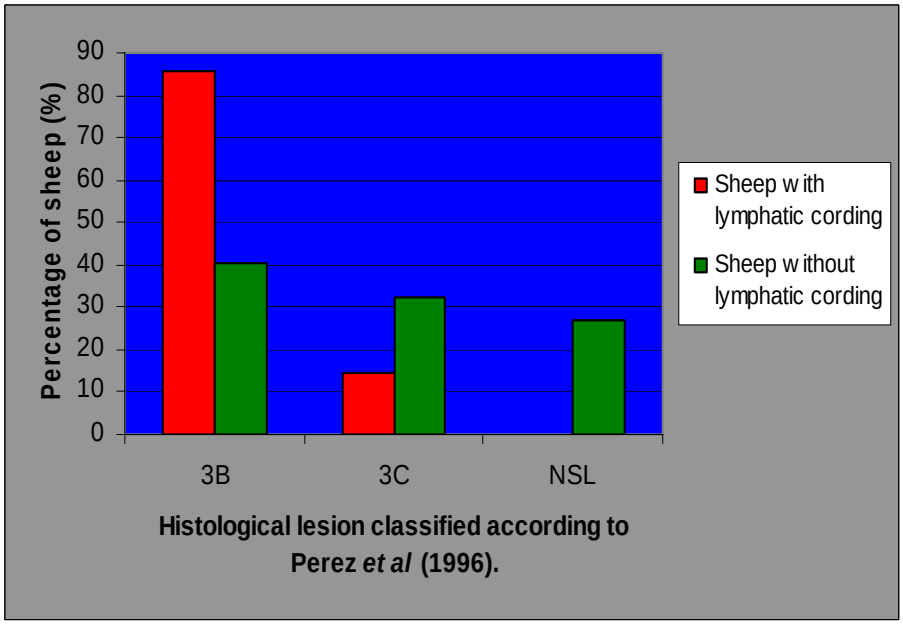

Figure 9. Histological classification of sheep with and without lymphatic cording.

3.2 Flock 2

3.2.1 Gross Lesions

At the time of slaughter the age of the sheep ranged from 2 to 7 years with over 75% being 3+ years old. Vaccination injection site lesions were present in 39% (30) of the sheep on the side of their neck. The size of these lesions averaged 2.6cm in diameter and ranged between 0.5 to 5cm. All the sheep diagnosed OJD negative (11) displayed gross signs of OJD with a grading of at least 2 with one sheep scoring a severe gross signs grading of 5.

3.2.2 Histopathology

Of the 78 sheep, 86% (67) were diagnosed histologically as being infected with OJD. Other diagnoses were not made. A significantly different proportion of histological lesion types were found amongst the OJD infected sheep from flock 2 when compared with flock 1. Over twice as many paucibacillary lesions were observed from flock 2 in comparison to the number of multibacillary lesions (Figure 10).

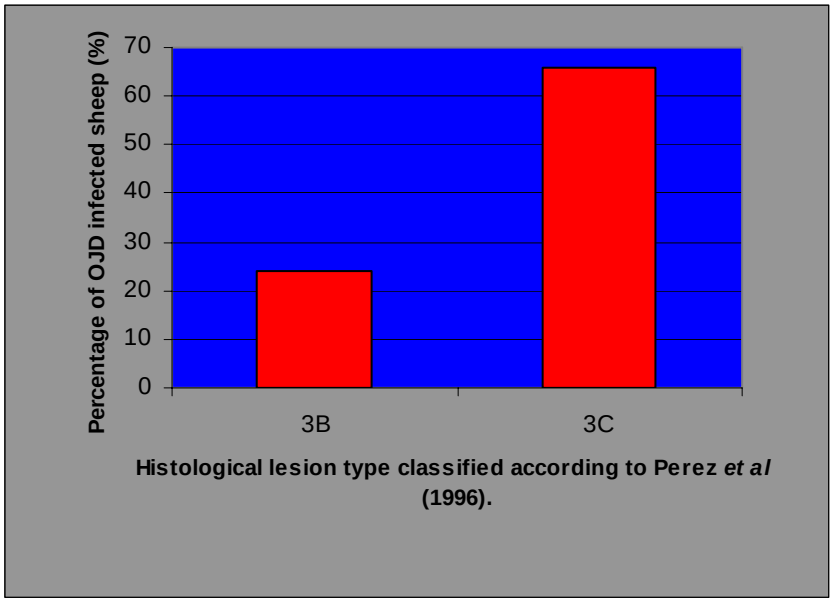

Figure 10. Classification of histological OJD lesions in flock 2.

Histological lesions of a severe nature were noted in 51% (34) of the cases and of a moderate nature in a further 28% (19) of the cases. A mild lesion was observed in 21% (14) of the OJD infected sheep.

Discussion

Ovine Johne's disease is of increasing significance in the Australian sheep industry as it continues to spread in southern and western New South Wales. There is debate between producers and authorities on approaches to control OJD with antagonism towards the zoning arrangements and quarantine policies. A new OJD control program is due to be effective from July, 2004 and it will inevitably include the deregulation of these zones. The control of OJD via the newly introduced Gudair® vaccine will become increasingly important, as will our need to understand how best to use the vaccine in order to maximise its potential.

No studies have been completed in Australia to determine how effective the vaccine is in sheep vaccinated outside the recommended age range of 1 to 4 months. The ability to source sheep from flocks already involved with adjacent studies provided an opportunity to closely examine the gross pathology and histopathology in sheep vaccinated as adults. It allowed us to add to the knowledge on diagnosis of OJD based on gross signs and to further investigate vaccination injection site lesions and their impact on gross and histological lesions.

4.1 Effectiveness of Gudair® in adult sheep

A large majority of the sheep involved in this study were vaccinated as at least 2 year olds with the exception of 11 sheep from flock 2 that were vaccinated at 8 months of age which is still outside the recommended 1 to 4 months. Diagnosis of OJD based on the presence of histological lesions presented very high levels of infection in sheep from both properties. Flock 1 demonstrated an OJD diagnosis rate of 83% while 86% of sheep from flock 2 were diagnosed with OJD. This indicates that the vaccine is not very effective at providing protection against OJD when administered to adult sheep. It is suspected that many of these sheep have already been exposed to *Mptb* as lambs prior to vaccination and they are subsequently developing lesions as they get older. Therefore producers should expect to continue to see mortalities in high prevalence flocks in the short to midterm time period post vaccination if sheep are vaccinated as adults.

4.2 Clinical assessment

Sheep used for this study were culled from their flock because of concerns that they were unproductive and even unsaleable. In combination with knowledge of high estimated mortality rates due to OJD, the producers had suspected these sheep had OJD due to their "wasted" appearance. Body condition scores and weights were only recorded for flock 1 and both these were well below average for fine wool Merinos. A typical fine wool Merino would have a body condition score of 3-4 and a body weight of 40-45kg.

Of the OJD infected sheep from flock 1 that had their faeces texture examined, 52.5% had either soft faeces or diarrhoea. This is consistent with findings reported by Carrigan and Seaman (1990) and Clarke and Little (1996). Carrigan and Seaman (1990) found 50% of a group of unvaccinated OJD infected sheep to have either soft, non-pelleted faeces or diarrhoea and similarly, Clarke and Little (1996) found 49% of their group of unvaccinated OJD infected sheep to have soft faeces or diarrhoea. This conveys to producers that the presence of soft faeces or diarrhoea can be indicative of OJD, however it is not present in all cases. It is also important to note that soft faeces and diarrhoea can be associated with other conditions as observed in 3 out of the 10 OJD negative sheep. Therefore soft faeces or diarrhoea is an unreliable clinical indicator of OJD.

4.3 Gross lesions and pathology

Vaccination injection site lesions were palpated in approximately 40% of the sheep from both flocks. This proportion is a little higher than those reported by Eppleston *et al.* (2003) for the time period of 6-16 months post vaccination, however, the lack of wool on the sheep after shearing prior to examination may have allowed for better detection of these vaccination injection site lesions. In combination with results from Eppleston *et al.* (2003) it is reasonable to suggest that producers can expect about 40% of sheep to form palpable injection site lesions post vaccination, irrespective of age of vaccination.

Further comparisons between sheep with vaccination injection site lesions and sheep without vaccination injection site lesions confirmed no significant results. There was no significant correlation between the presence of a vaccination injection site lesion

and the gross pathology or histological lesion type. This indicates that the presence of a vaccination injection site lesion is unlikely to influence the degree of bowel thickening or the formation of a particular type of histological lesion. The presence of a palpable vaccination injection site lesion does not appear to be a useful indicator that adult sheep vaccinated with Gudair® have sufficient protection against the effects of OJD to alter the clinical outcome as questioned by Windsor *et al.* (2003).

Enlarged prescapular lymph nodes were found on 29% (18) of the sheep from flock 1. Both vaccination injection site lesions and enlarged prescapular lymph nodes are required to be trimmed from the carcase in the abattoir and could add an extra cost to carcase preparation. However, feedback from processors indicates that these lesions would not present an issue as Australian carcases are routinely heavily trimmed.

While many of the OJD infected sheep displayed the classic signs of bowel thickening, an enlarged ileocaecal lymph node and lymphatic cording, 25% of the OJD positive sheep had no sign of bowel thickening. Additionally, 4 out of the 10 sheep diagnosed as OJD negative displayed mild signs of bowel thickening. This presents the possibility for misdiagnosis of OJD as discussed by Bush *et al.* (2003) based solely on gross signs of bowel thickening. Similarly, Carrigan and Seaman (1990) discuss the possibility of overlooking mild gross changes in the intestines and attributing the emaciation to another cause. This data also supports the Bradley and Cannon (2003) study that expresses the need to have educated inspectors on the abattoir floor to be able to detect OJD based on gross pathology. These findings indicates that "false positives" based on gross pathology are likely to be common and that confirmation of diagnosis via histopathology is a necessity. Bowel thickening can be caused by other conditions and is not only indicative of OJD. For example, hypoproteinaemia can result in malnourished animals which have been starving during a drought. These concepts are important for producers, veterinarians and inspectors to understand when diagnosing OJD.

As all 21 sheep from flock 1 that displayed lymphatic cording were diagnosed with OJD, lymphatic cording is considered to be a very good diagnostic indicator of OJD. This supports reports by Carrigan and Seaman (1990), Clarke and Little (1996) and Clarke (1997). Corded lymph vessels were often noted on the serosal surface of the thickened intestines by Carrigan and Seaman (1990) and lymphatic cording is regarded as

a common gross lesion in a review by Clarke (1997). A significant correlation was found between the presence of lymphatic cording and the subsequent lesion type. It was found that sheep displaying lymphatic cording had a multibacillary lesion in 85% of the cases. Therefore these sheep are likely to be large excretors of *Mptb*. Similar prominent cording of the serosal lymphatic ducts was reported in 83% of mutlibacillary animals and 71% of paucibacillary animals in a study of 45 unvaccinated OJD infected sheep by Clarke and Little (1996). In our study, 54% of the multibacillary and 20% of the paucibacillary vaccinated OJD infected sheep displayed lymphatic cording. Is this difference an influence of vaccination?

There was no significant correlation found for either of our flocks between the type of histological lesion and the degree of bowel thickening from flock 1 or the degree of gross signs from flock 2. For example, sheep with severe gross pathology were not found to have one particular type of histological lesion while sheep with mild gross pathology were not found to have a different type of histological lesion. This suggests the degree of inflammation and not the type of histological lesion influences the visual appearance of bowel thickening.

4.4 Histopathology

A significant finding was the difference between the types of histological lesions in the 2 different cohorts of sheep (Figure 11). Two thirds of the OJD infected sheep from property 1 had a histological lesion of a multibacillary type and one third had a lesion of the paucibacillary type, whereas the reverse was observed with OJD infected sheep from property 2. This raises the question, is this observational difference attributable to the use of the vaccine? For example, is it possible the vaccine is capable of having an impact upon the immune system during the developing stages of the lesion, thus forming a particular type of histological lesion in sheep vaccinated as adults?

Results from Clarke and Little (1996) find 69% (31) of a group of unvaccinated sheep to have multibacillary lesions and 31% (14) to have paucibacillary lesions which are similar proportions to those from property 1. Carrigan and Seaman (1990) experienced 88% (44) of a group of unvaccinated sheep to have multibacillary lesions and 12% (6) to have paucibacillary lesions. These observations indicate that a greater

proportion of multibacillary animals compared to paucibacillary animals is to be expected in an OJD infected population as observed in flock 1.

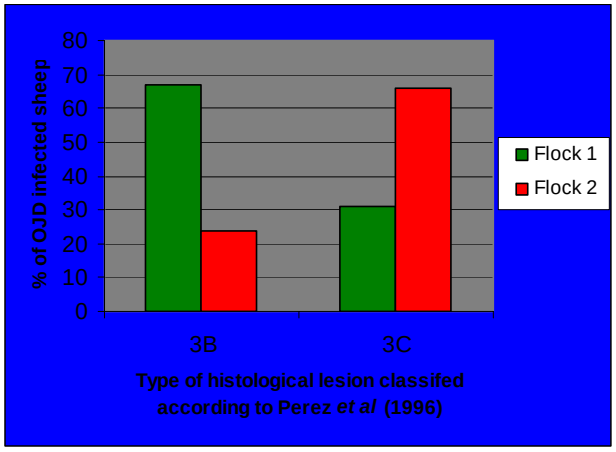

Figure 11. Comparison of types of histological lesions in OJD infected sheep from flock 1 and 2.

What possible reasons are there to explain the reversal of this multibacillary to paucibacillary ratio between property 1 and property 2. Being an observational study, there are a number of factors which need to be considered when attempting to determine why this difference has occurred between the two properties in this study.

Firstly, the genotype of the sheep could have impacted upon the results as both properties have studs and therefore have their own bloodlines and these may react differently to the vaccine. However, they are both fine wool Merino breeders and the explanation of this hypothesis would require extensive research on the genetics of immunological responses to OJD. Sheep from flocks on property 2 may well be worthy candidate flocks for such a study.

Secondly, the time of data collection could have affected the type of histological lesion. Data collected from sheep from property 2 in 2001 was at the beginning of a severe drought whereas data collected from sheep from property 1 was at the end of the drought. The drought influenced management changes to the grazing protocol of both properties. Property 1 changed from a cell grazing system to a set stocking regime thereby decreasing stocking rates while property 2 increased stocking rates by restricting grazing to a smaller area to facilitate hand feeding. This could well have intensified exposure to infection on property 2. Current drought feeding advice promotes semi-lot

feeding to reduce energy expenditure of grazing, however this may increase challenge of *Mptb* in OJD infected flocks. Further research on the benefits of lot-feeding versus the effect of increased OJD exposure in droughts appears to be of importance.

Thirdly, post-exposure vaccination in older sheep indicates the vaccine could be acting like a booster. In the case of property 2, the increased challenge from *Mptb* from a more intensive grazing protocol and a higher initial property infection status, could increase or boost the immune response and direct developing lesions in a paucibacillary direction. However, in sheep where the disease is already too far advanced and the antigenic load is too great, a sufficient immune response is not created and the lesion develops in a multibacillary direction.

Fourthly, the length of time for which the sheep have been vaccinated before the data was collected could also be a factor. Sheep from property 1 had only been vaccinated for 6-9 months prior to euthanasia whereas sheep from property 2 were vaccinated 13-16 months prior to slaughter. This may suggest that the sheep from property 2 had more time for repeated stimulation of the immune system with a greater degree of challenge from the *Mptb* and this may lead to the more likely formation of a paucibacillary lesion.

These results raise the question as to whether the vaccine is capable of being used as a tool in high prevalence flocks of adult sheep to induce the formation of a paucibacillary lesion in sheep already infected with OJD? This would therefore reduce *Mptb* excretion rates and contamination levels and consequently reduce the risk of exposure of the infection to newborn lambs or other sheep.

Nevertheless, this is only one interpretation of these observations and as indicated more research is necessary to support these hypotheses.

4.5 Limitations and Further Research

There were several limitations to this study due to the availability of resources and a restricted time period. Sheep from flock 2 were slaughtered in September, 2001. I was relying upon the records of the gross pathology of the sheep and was unable to obtain precise and accurate data as recorded for flock 1. A more detailed study on the gross pathology of the sheep from flock 2 would have been performed. However, the

histopathology from flock 2 was very useful for comparison with results from flock 1. Pooled faecal culture (PFC) would have been useful to examine *Mptb* excretion rates however the cost of this procedure at $100 per culture was considered too excessive for this study.

It would have been appropriate to compare these results with unvaccinated sheep but this was simply impractical. As sheep were sourced from commercial operations that were aiming to control and reduce OJD prevalence, producers considered it would not be effective to leave any sheep unvaccinated. Therefore unvaccinated sheep were unable to be sourced as the entire flock had already been vaccinated on each property. A major study in vaccinated lambs is being conducted on 3 properties in the Bathurst region (MLA.OJD.009), consisting of 200 lambs vaccinated with Gudair® while another 200 lambs were left unvaccinated on each property. Preliminary results from this study have recorded 67 deaths from the unvaccinated sheep and 6 deaths from the vaccinated sheep. All 6 vaccinate mortalities have had multibacillary lesions while 88% (59) of the unvaccinated sheep mortalities have had multibacillary lesions (P. Windsor, personal communication). It would be interesting to examine the pathology of the unvaccinated lambs as well as the vaccinated lambs from the MLA.009.OJD trial so a comparison can be made with the results from the vaccinated adults in this study.

Preliminary results from a current study on property 2 (MLA.015.OJD) have provided some insight into the level of multibacillary animals and paucibacillary animals pre-vaccination. The creditability of this data is limited because the sample number is low, however it still provides some information which relates to this study. Prior to vaccination, a small group of sheep was sampled from the same "hospital mob" that was used in this study and tissues were submitted for histopathology. A similar proportion of multibacillary lesions and paucibacillary lesions were found in these sheep pre-vaccination when compared to sheep vaccinated as adults in this study from property 2. Tissues were sampled in April 2000 and the histopathology classification indicates 64% (14) with paucibacillary lesions and 36% (8) with multibacillary lesions. This indicates that there may be a particular property effect that is inducing the formation of a greater number of paucibacillary lesions instead of the vaccine. A closer examination of the differences between these 2 properties may reveal certain pre-disposing risk factors

which impact upon the immune system differently and stimulate the formation of different types of histological lesions.

Conclusion

This study has provided new information on the effects of the Gudair® vaccine in sheep vaccinated as adults. It has demonstrated that within a short time frame post vaccination, the vaccine is not very effective at providing protection against OJD in adult sheep which have most likely been exposed to infection as lambs. The study has emphasised the potential for misdiagnosis of OJD based on gross signs and stresses the importance of histopathology to confirm OJD infection. Producers can expect 40% of vaccinated sheep to develop vaccination injection site lesions, however, these lesions do not provide an indication of protection against OJD. Further, this study identified differing proportions of multibacillary lesions and paucibacillary lesions from 2 different cohorts of sheep from 2 different properties. This raises the question, is the vaccine capable of inducing a paucibacillary lesion in sheep vaccinated as adults and consequently reducing excretion rates of *Mptb*? Preliminary pre-vaccination data from another study suggests that a property or genotype effect rather than the vaccine may be influencing the high proportion of paucibacillary lesions experienced on property 2. Further research will be necessary to explore the property or genotypic characteristics and/or vaccine capabilities that could encourage the formation of paucibacillary lesions in OJD infected sheep and thus minimise excretion rates of *Mptb* and limit the spread of OJD within Australia.

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Appendix 1

Multibacillary Lesions – 3B

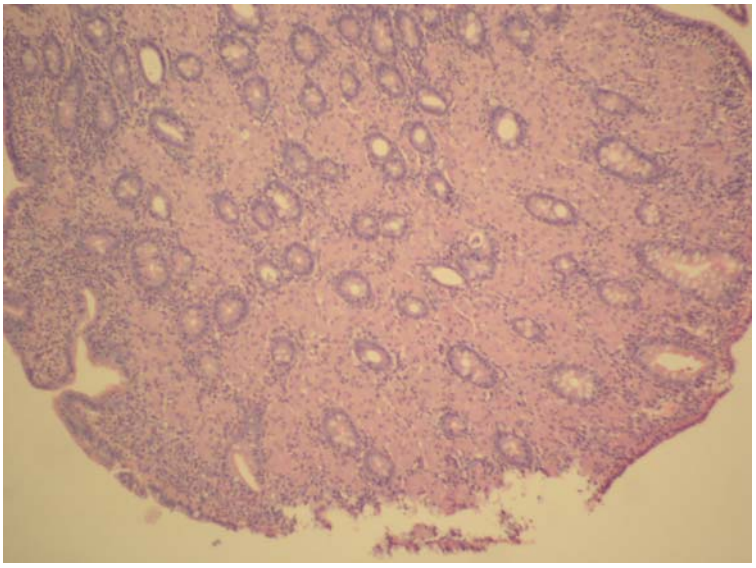

Figure 12. A Haematoxylin and Eosin stain of a section of the mucosa of the terminal ileum showing extensive infiltration between the villi of pink epitheliod macrophages in the lamina propria (Mag. x100).

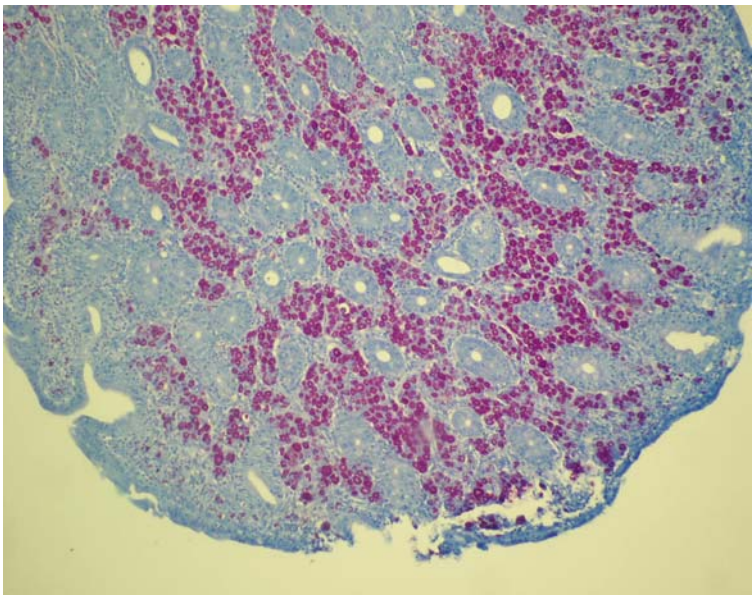

Figure 13. A Ziehl-Neelsen stain of a section of the mucosa of the terminal ileum showing the presence of thousands of rod shaped *Mptb* in the epitheliod macrophages (Mag. x100).

Paucibacillary Lesions – 3C

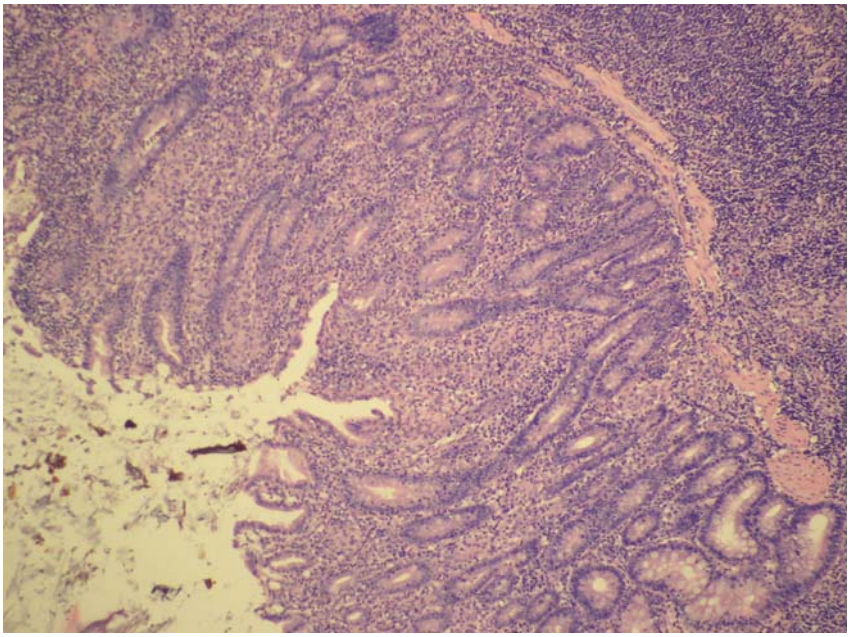

Figure 14. A Haematoxylin and Eosin stain of a section of the mucosa of the terminal ileum presenting a pleiomorphic appearance. This results from a multicellular immune response and includes macrophages as well as other cell types like lymphocytes and histiocytes (Mag. x100).

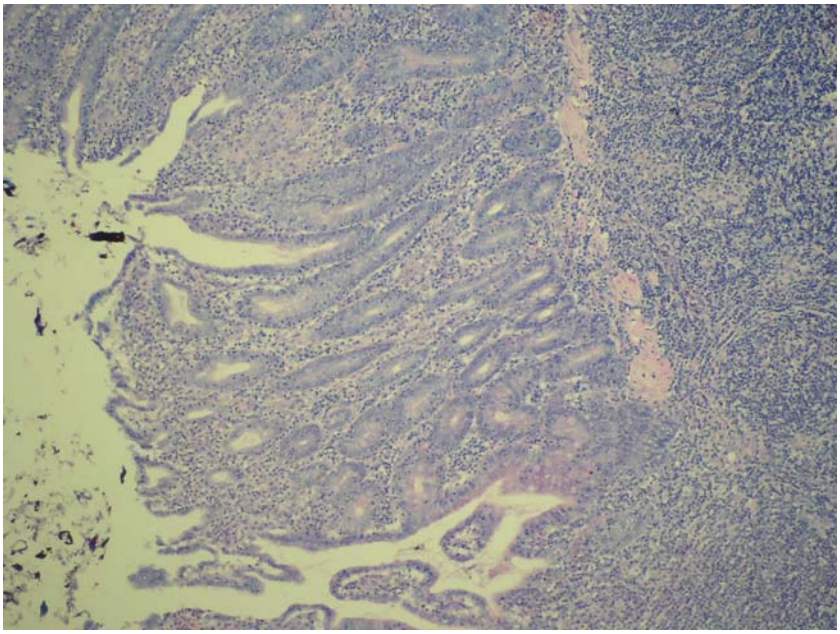

Figure 15. A Ziehl-Neelsen stain of a section of the mucosa of the terminal ileum showing the absence of the rod shaped *Mptb* in the lesion (Mag. x100).

Appendix 2

The Perez *et al*. (1996) OJD lesion classification system

Type 1 Lesions

- Location
    - only in the lymphoid tissue, never in the intestinal mucosa.
    - PPs: interfollicular spaces, in the basal zone, less often at the apex
    - MLNs: paracortex or interfollicular area, related to subcapsular or peritrabecular sinuses (MLN less often affected than PP)
- Type
    - granulomata formed by macrophage-like cells (nuclei large and clear with obvious nucleoli, abundant slightly foamy cytoplasm, lightly stained by H&E and sometimes with clear vacuoles) often with small numbers of lymphocytes and cells with elongated nuclei
    - no AFB are seen.

Type 2 Lesions

- Type
    - granulomata well delineated, round, variable in number, never enough to result in diffuse enteritis
    - AFB occasionally seen in granulomata in mucosa, but not in PPs/MLNs.
- Location
    - PPs: granulomata in a row from the most basal zone of the interfollicular area to the apex, penetrating into the lamina propria. Granulomata in the villi are always associated with granulomata in an adjacent PP
    - MLNs: similar to type 1 lesions. Less frequently seen than those in PPs and always smaller in size

Type 3 Lesions

- Granulomatous lesions affect PPs, associated mucosa and mucosa that is not associated with lymphoid tissue. There are 3 subtypes:

Subtype 3a

- Type
    - Lesions sporadic, multi-focal
    - AFB are seen in granulomata in the mucosa
- Location
    - PPs and associated mucosa:
    - lesions are similar to type 2
    - granulomata in the lamina propria are larger, extend from PPs, involve more villi, cause enlargement of villi
    - Areas of mucosa not associated with PP's:
    - granulomata are small and well delineated in lamina propria of villi and/or the basal area
    - Submucosa and serosa:
    - foci of inflammatory cells (mostly lymphocytes and macrophages) are seen around lymphatic and blood vessels
    - MLNs: granulomatous lesions

Subtype 3b (Multibacillary)

- Type
    - Diffuse granulomatous enteritis creates a mosaic formed by macrophages, epithelioid cells, a few giant cells (2-3 nuclei), small numbers of lymphocytes and other leukocytes
    - AFB in abundance, numbers in mucosa > than in lymphoid tissue.
- Location
    - PPs: granulomata in the interfollicular areas, follicles and domes, with infiltrates giving a mosaic-like appearance, among lymphoid aggregates
    - Mucosa:

- villi thickened, apices flat and wide, fused, fewer crypts due to infiltration
- in some sheep the mucosa is less thickened, epithelioid cells are seen in the villi (mostly the apex) and diffuse enteritis is due to confluence of numerous small granulomata

- Submucosa:
- lymphocyte and plasma cell infiltrates, initially perivascular, but extending to the muscular layer, with lymphatics dilated and thrombi composed of macrophages seen within

- Serosa:
- Lymph-angitis/angiectasis, perivascular lymphocyte/plasma cell aggregates

- MLN's:
- multi-focal or diffuse granulomatous lymphadenitis
- subcapsular sinuses usually contain macrophages
- serosal lesions similar to those in the gut serosa

<u>Subtype 3c (Paucibacillary)</u>

- Type
    - diffuse granulomatous enteritis, but the cell types differ from type 3b
    - AFB rarely seen, and then only in small numbers.
- Location
    - PPs:
    - lesions similar to type 3b, but with pyknotic macrophages and giant cells
    - Mucosa:
    - diffuse granulomatous enteritis, but the predominant cells are lymphocytes in the lamina propria of the villi and the basal area; macrophages are seen among the lymphocytes either scattered or in small, well defined granulomata of up to 20-25 cells
    - Submucosa:
    - frequently oedema, with variable numbers of lymphocytes and plasma cells
    - Serosa: similar to type 3b
    - MLNs:

- multifocal granulomata in the paracortical and interfollicular areas
- Langhans giant cells (some with >30 nuclei) may be present
- pyknotic macrophages and perivascular infiltrates are seen in the serosa of the LNs

NSL lesions – No significant lesion

Appendix 3a

Haematoxylin and Eosin (H&E) staining method

Solutions: Whitlock's Haematoxylin (after Mayer)
Scott's Blueing solution
Alcoholic Eosin

Method:

1. Dewax section and bring to water – 2 changes of xylol (3 minutes each) and 2 changes of 100% ethanol (2 minutes each). Place sections in Alcoholic piric acid for 15 minutes to remove the acid haematin precipitate. Wash in running tap water until the yellow colour is removed.
2. Stain in Haematoxylin for 3 minutes.
3. Wash in running tap water for 2 minutes.
4. Scott's Blueing solution for 2 minutes.
5. Wash in running tap water for 2 minutes.
6. Rinse in 70% ethanol.
7. Eosin for 28 seconds.
8. Dehydrate rapidly to 100% ethanol.
9. Clear in several changes of xylol.
10. Mount in DPX.

Results: Nuclei – blue
Background – orange/pink

Appendix 3b

Ziehl – Neelsen staining method (1882, 1883)

This staining is used to demonstrate the presence of *Mycobacteria sp.* bacillus (acid fast bacilli). *M. tuberculosis* is the tubercle bacillus and is difficult to demonstrate because of the lipid capsule surrounding it. Carbol fuchsin is forced into the tissue with heat and is then removed by acid or alcohol. The stain I removed from almost all structures except the lipid capsule which resists decolourisation. This gives rise to the name acid fast bacteria.

Solutions: 1. Carbol Fuchsin:

Basic Fuchsin (5g)
100% Ethanol (50mL)
5% Phenol (500mL)
(5g phenol in 500mL distilled water)

Dissolve the basic fuchsin in absolute alcohol then add to the phenol solution. Mix well. Filter before use.

2. Methylene blue (0.1%):

Methylene blue (0.1g)
Distilled water (100mL)

3. Acid alcohol:

Hydrochloric acid (0.5mL)
70% Ethanol (100mL)

Method:

ALWAYS USE A POSITIVE CONTROL

1. Dewax and bring sections to water – 2 changes of xylol (3 minutes each), 2 changes of 100% ethanol (2 minutes each), 2 changes of 95% ethanol (30 seconds each) and 2 changes of 70% ethanol (30 seconds each). Wash in running tap water for 2 minutes.
2. Filter carbol fuchsin onto slide.

3. Heat to steaming, leave for 2 minutes, repeat heating and leave for 10 miniutes.
4. Wash in water.
5. Differentiate in acid alcohol until sections are pale pink.
6. Wash in water.
7. Counterstain in 0.1% methylene blue for 10-15 seconds.
8. Wash in water.
9. Dehydrate, clear and mount in DPX.

Results: Acid fast bacilli – red
Nuclei – blue
Other tissue – pale blue