MEAT & LIVESTOCK
AUSTRALIA

On farm

Field evaluation of OJD control using Gudair

Project number OJD.009
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Abstract

A 5-year field trial was undertaken to determine the efficacy of a killed *M. a. paratuberculosis* vaccine, Gudair™, for the control of OJD in Australian merinos. The vaccine stimulated cell-mediated and humoral immune responses. Gudair™ reduced mortalities due to OJD by 90% and delayed faecal shedding for the first year post-vaccination (pv). Thereafter, the prevalence of shedders among vaccinates was reduced by 90%. The numbers of *M. a. paratuberculosis* excreted by the vaccinated groups were also reduced by at least 90% at most sampling times. However, high levels of excretion by vaccinates occurred on some occasions, and although only 7 of 600 vaccinates died from OJD, all had multibacillary disease. Thus there remains a risk that some vaccinated sheep could transfer the disease. Small reductions in liveweight gain were found in vaccinated lambs in the first year pv, but there was little effect on condition score or wool production. Vaccine injection site lesions were detected in almost 50% of sheep 2 months pv, and these persisted for at least 4 years in 20-25% of vaccinates. Data from this trial enabled the registration of Gudair™ in Australia in 2002 and underpins the pivotal role of vaccination in the current management of OJD.

Executive summary

Background and introduction:

Paratuberculosis of sheep (OJD) is known to cause significant mortality of adult sheep, particularly in self-replacing Merino flocks that have been infected for an extended period. In areas where the flock prevalence of the disease is high, eradication by destocking is restricted because of the risks of reinfection from neighbours and from local environmental sources. Producers needed management tools to deal with OJD. Vaccination against OJD has been used in several overseas countries, with encouraging results, however there were few scientifically controlled reports demonstrating its efficacy.

CSL Animal Health imported a killed *M. a. paratuberculosis* vaccine, Gudair™ (CZ Veterinaria, Porrino, Spain) into Australia. As part of the National Ovine Johne's Disease Program (NOJDP), a major field trial was undertaken from 1999 until 2004 to determine the efficacy of Gudair™ for the control of OJD in Australian merinos run under Australian pastoral conditions.

Objectives of the trial were:

1. To determine if vaccination with Gudair™ Vaccine could reduce the incidence of mortality.
2. To determine if vaccination caused any reduction in bacterial shedding of *M. a. paratuberculosis.*
3. To investigate the cellular and humoral immune response following vaccination, and to attempt to correlate this with any reduction in OJD mortality or in faecal *M. a. paratuberculosis* shedding.
4. To assess the field safety of Gudair™ in vaccinated flocks.
5. To determine changes in productivity parameters associated with either vaccination or subclinical disease.

Methodology:

On each of three farms in New South Wales experiencing significant OJD losses (5 to 15% per annum), 200 Merino lambs (age 1-4 months) were vaccinated with Gudair™, and 200 lambs were sham vaccinated with saline. Animal assessments and sample collections were conducted twice yearly until 4 or 5 years of age. We examined the impact of vaccination on mortality rate, faecal shedding of *M. a paratuberculosis* (by pooled and individual faecal culture), lamb growth, condition score and wool productivity, vaccine injection site lesions and cellular (BOVIGAM™) and humoral (PARACHEK™) immunity. Experimental sheep that were culled for normal flock management reasons, died or showed clinical signs of OJD, were assessed at necropsy or slaughter.

Results:

Clinical disease and mortality - By project end there had been only seven confirmed OJD mortalities in vaccinates compared to 80 from the controls (a significant reduction of about 90%), and the mortalities in the vaccinated sheep were delayed by at least 12 months. Vaccination reduced mortalities over the productive life of the sheep and did not simply delay the onset of mortalities. All the vaccinates, and 88% of the controls that died of OJD had multibacillary disease. Sheep with multibacillary lesions can excrete enormous numbers of organisms and the "breakdown" of a single vaccinated animal can have a disproportionate effect on transmission of the infection and the persistence of disease in the flock.

Subclinical disease – Among both the hogget culls, and the sheep that went to slaughter at trial end, there was a significant reduction in the proportion of vaccinates with subclinical OJD

compared to controls. The magnitude of this reduction was, however, less than that for clinical disease – from 49% in controls to 17% in vaccinates for the hoggets, and from 28 to 15% respectively at final slaughter.

M. a. paratuberculosis excretion - The results from farms P1 and P3 clearly demonstrated a delay and reduction in faecal excretion of *M. a. paratuberculosis*, with maintenance of the protection for the economic life of the sheep. On these farms, there was no detectable excretion of *M. a. paratuberculosis* by the vaccinated groups until 18 months pv (about 21 months of age), compared to 6 or 8 months pv (9 -11 months of age) in the controls. The reduction in prevalence of excreters averaged about 90%. On P2, the property with the highest prevalence of infection, excretion of *M. a. paratuberculosis* by vaccinates was also delayed, but only by about 4 months. At 12-36 months pv, reduction in prevalence in the vaccinates was also about 90%. At 42 and 48 mths pv there was little difference in prevalence between the vaccinated and control groups, but the control group was much smaller due to the large number of OJD mortalities. The peak prevalence of excreters over the life of the sheep was much lower and delayed in the vaccinated group. Across all farms, the numbers of *M. a. paratuberculosis* excreted by the vaccinated groups were reduced by at least 90% at most sampling times. However, the actual level of excretion was sometimes very much higher than that suggested by prevalence data alone, a reflection of the large contribution that a single (or a few) multibacillary infected sheep may make.

Immunological responses - The vaccine stimulated both cell-mediated and humoral immune responses in a high proportion of vaccinated lambs which declined over time, accompanied by a significant increase in the proportion of unvaccinated animals with positive immune reactions, presumably reflecting an increasing prevalence of OJD in this group. Among vaccinated sheep, positive IFN-γ response was negatively associated with subsequent shedding of *M. a. paratuberculosis* and with subsequent infection status. No significant association with OJD-mortality was found, but there were very few vaccinated sheep that died of OJD. Positive ELISA response in vaccinates was negatively associated with shedding, with infection and with OJD-mortality. Thus, although humoral responses are not considered to be protective, it appears that pv ELISA response may be a suitable marker for “vaccination take”. The presence of maternal antibodies was negatively correlated with pv CMI responsiveness, but no significant effect of maternal antibody on later infection status or OJD-mortality was found.

Injection site lesions - Vaccine injection site lesions were detected in almost 50% of sheep 2 months pv, and these persisted for at least 4 years in 20-25% of vaccinates. The presence or absence of a pv lesion was not associated with subsequent infection or excretion of *M. a. paratuberculosis,* so was not a useful marker for vaccination take. The presence of lesions in a proportion of the flock, however, may be a useful indication that the flock has been vaccinated, for up to 3 years after vaccination. Post vaccinal lesions did not result in losses or downgrading of carcasses on the slaughter floor.

Production measurements - These were conducted on surviving sheep at each sampling time. Thus the findings for vaccinates vs controls do not include prior losses due to OJD mortality, and the findings with regard to infection status are for subclinically infected sheep. A small, but consistent and statistically significant reduction in the weight gains of vaccinates over the first 12 months pv was seen; there were not consistent differences among adults. Vaccinates cut slightly more wool than controls at the 2003 shearing, but there were no differences at other shearings, nor in fibre diameter at any shearing. With regard to OJD infection status, subclinically infected sheep were lighter and had lower condition scores than uninfected sheep between 18 and 42 months pv. No differences in weight or condition score due to infection status were detected in growing sheep, nor in the fleece parameters at any time. Reproductive parameters were not studied.

Conclusions and recommendations:

In flocks with a high prevalence of OJD, vaccination with Gudair™ stimulated specific immune responses and significantly reduced and delayed both OJD-related mortality and the excretion of *M. a. paratuberculosis.* Protection was maintained for the economic life of the sheep. Ignoring OJD-related mortality, there were minimal effects of either vaccination or subclinical OJD on production parameters.

Data from this trial enabled the registration of Gudair™ in Australia in 2002, and the vaccine now plays a pivotal role in the management of OJD in Australia.

However, high levels of excretion by vaccinates on some occasions, and multibacillary disease in the few vaccinates that died from OJD, indicate risk that some vaccinated sheep could transfer the disease.

This trial examined sheep in only one generation in only high prevalence flocks, and unvaccinated control sheep were present. This was necessary to facilitate measurement of the effects of vaccination. In the real world, whole cohorts of lambs are vaccinated over successive years in flocks of low, medium or high prevalence, and benefits of vaccination may accrue over many years. To assure the best use of vaccination in control programs, it is essential that on-going investigations into the efficacy of vaccination under these more typical farm conditions are continued.

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1 Background and industry context

Paratuberculosis of sheep (OJD) is known to cause significant mortality of adult sheep in infected flocks (Eppleston et al. 2000), particularly in self-replacing Merino flocks that have been infected for an extended period. The central and southern tablelands of NSW are areas of high prevalence, and OJD was a cause of significant economic loss on some farms. Additional indirect losses associated with regulation of the disease, were a problem on infected farms (eg studs) which supplied live sheep to other producers. In areas where the flock prevalence of the disease was known to be high, eradication by destocking was restricted because of the risks of reinfection from neighbours and from local environmental sources. Producers needed management tools to deal with OJD.

Vaccination against OJD has been used in several overseas countries, reportedly with encouraging results (Garcia Marin JF et al. 1999; Fridriksdottir et al. 2000), however there are few scientifically controlled reports demonstrating its effectiveness. CSL Animal Health imported an inactivated JD vaccine, Gudair™ (CZ Veterinaria, Porrino, Spain) into Australia. The vaccine was evaluated as part of the National Ovine Johne's Disease Program (NOJDP). This research into vaccine efficacy under Australian conditions was conducted with in-kind support from CSL, who used the data in a registration package submitted to the National Registration Authority.

Before NSW farmers with infected sheep flocks were allowed to use the vaccine to alleviate losses caused by OJD, it was necessary to have a vaccine efficacy trial in place. Notwithstanding this requirement, the introduction and widespread adoption of an OJD vaccine has proven to be one of the most useful methods of controlling this disease in areas with a high prevalence and incidence of OJD.

At the beginning of this project it was considered that long term usage of vaccination may allow easier eradication of disease following a reduction of the incidence of OJD to negligible levels. The extent of OJD infection was probably underestimated at that time, and at least for the foreseeable future, eradication of OJD on a national scale is not an option.

2 Project objectives

The original objectives (1 – 5) when the trial was commenced in 1999 were:

1) The principal objective was to establish if the use of Gudair™ vaccine can reduce the incidence of mortality due to OJD under Australian conditions.

2) A secondary objective was to determine if faecal shedding of *M. paratuberculosis* in infected flocks was delayed or reduced following vaccine usage.

3) A tertiary objective was to determine changes in productivity parameters associated with either vaccination or subclinical disease.

4) In addressing these objectives it will also be important to gather information on:

   I. Injection site and draining lymph node reactions

   II. Any occupational health and safety issues

   III. Factors associated with failure of vaccine to protect sheep

5) Monitoring of the extended use of the vaccine to ensure that vaccine efficacy in trials

reflects results of wider field usage.

There has been considerable refinement of the project objectives over time, with experience and wider use of the vaccine. For example:

An additional objective, "To investigate the cellular and humoral immune response following vaccination with Gudair™ vaccine, and to correlate this with any reduction in OJD mortality or in faecal *M.paratuberculosis* shedding" was added in a 2001 review to facilitate the registration of Gudair™ for use in Australia.

Compulsory data collection for Objective 5 was not possible once permits for vaccine use were no longer required, so that this objective was dropped from the project as reviewed in 2002. However, the investigators considered that some monitoring of the extended use of the vaccine, particularly for injection site lesions and OH&S issues, could produce valuable information at minimal cost, and so some data was collected.

The modified objectives which were designed to provide enhanced registration data, and which have guided the last 4 years of this project are listed below, in order of importance:

- The principal objective is to determine if vaccination with Gudair™ Vaccine can reduce the incidence of mortality in merino sheep due to OJD under Australian conditions.
- To assess, quantitatively via faecal culture, any reduction in bacterial shedding of *M. paratuberculosis* that may occur in vaccinated sheep in comparison with non-vaccinated controls.
- To investigate the cellular and humoral immune response following vaccination with Gudair™ Vaccine, and to attempt to correlate this with any reduction in OJD mortality or in faecal *M.paratuberculosis* shedding.
- To assess the field safety of Gudair™ Vaccine in vaccinated flocks.
- To determine changes in productivity parameters associated with either vaccination or subclinical disease.

Comprehensive annual reviews of this project were undertaken in 2001 (deferred to May 2002), 2002 and 2003. These involved research staff from NSW Agriculture, Sydney University, and representatives from MLA and CSL. In these revised objectives, the collection of immunological data was considered a priority over field safety and productivity measurements. Planned studies on reproduction were omitted due to the difficulty of collection and peripheral importance to the project. The planned studies on immunological factors associated with failure of the vaccine to protect sheep were incorporated into the broader immunological studies.

3 Methods

3.1 Trial Design

Property selection - Three properties in the Central Tablelands of NSW were selected as trial sites for intensive study. These were Property 1 (owner T Dobel), Property 2 (owner J Rosewarne) and Property 3 (owner T Toole), referred to below as P1, P2 and P3 respectively. For convenience and economy, these were within about 100 to 150 km of Orange Agricultural Institute and Rural Lands Protection Board in Bathurst. Owners were paid a co-operator's fee, and subsidised for vaccine cost for non-trial sheep on their farms. Trial sheep, however, remained the property of the farm owners, and apart from the specific requirements of this trial, were subjected to the usual farm management practices, along with the rest of the sheep on each farm.

All 3 properties lambed at least 800 ewes annually, sufficient to allow adequate selection of trial lambs. All had been running a self-replacing fine wool Merino flock for several decades and the level of management was considered to be adequate to exclude other major causes of mortality. These properties all had estimated losses due to OJD of over 5% in susceptible age groups (2, 3 and 4 year olds). On P1, seroprevalence among 100 systematically selected sheep was 7% (ELISA and AGID), and on P2 was 9% (ELISA). These results were considered consistent with the estimated level of annual mortality.

To increase the likelihood of significant bacillary challenge to the trial sheep, they were depastured in paddocks previously grazed by older sheep in which clinical cases were occurring. In addition, suspected clinical cases of OJD from the remainder of the flock were regularly put in with the trial sheep.

Lamb selection - On each property, 400 predominantly female lambs were selected to enter the trial (200 vaccinated and 200 unvaccinated controls), and double ear tagged for individual identification. This number was chosen to allow for normal farm culling and deaths unrelated to OJD, so that about 150 head of female replacement sheep in each of the treatment and control groups would remain into the third year of their life (total of 300 two year-old trial sheep per property). All selected lambs on P1 and P2 were female, but 142 wether lambs had to be included in P3 to make up the numbers. Any additional lambs on each farm not required for the trial were vaccinated. The vaccinated and control lambs in each flock were grazed together for the whole of the trial.

Lamb treatments – Each property was visited once for lamb selection and vaccination when lambs were 1-3 months old. On P1 lambs were 2-3 months old, on P2 they were 1-2 months old, and on P3 they were 1-3 months of age. Lambs in the vaccinated groups were dosed with Gudair™, while the control lambs were sham vaccinated with saline. Vaccine and saline was administered by subcutaneous injection high on the lamb's neck behind the ear using a 6mm needle, in accordance with the recommendations for the use of Gudair™. Following vaccination all lambs were given a fly strike preventative treatment over the head and neck to prevent problems from flystrike associated with discharging sinuses which may develop following vaccination.

Sampling schedule - Each trial property was visited and sheep were sampled, at the time of vaccination, approximately 2 and 6 months post vaccination (pv), then six monthly thereafter.

On P1, spring 1999 born lambs were vaccinated in December 1999, but no samples were taken for immunological testing until twelve months post vaccination. On properties 2 and 3, autumn 2000 born lambs were treated in June 2000, and immunological testing commenced at this

time. The sampling schedule on each property is given in Table 1.

Table 1. Scheduled sampling visits on each of the 3 trial properties

Nominal visit (months pv)	Date	Activity					
		Blood sample ELISA , IFN-	Blood sample AGID	Pooled faecal culture	Individual faecal culture	Liveweight, condition score	Assess vaccination lesions
		Property 1					
0	14/12/1999			•		•	
2	10/02/2000					•	•
6	13/06/2300			•		•	•
9	20/09/2000			•		•	•
12	5/12/2000	•		•	•	•	•
15	20/03/2001					•	
18	13/06/2001	•[a]		•	•	•	•
24	11/12/2001	•		•	•	•	•
30	11/06/2002	•		•	•	•	•
36	26/11/2002		•	•	•	•	•
42	11/06/2003	•		•	•[b]	•	•
48	9/12/2003			•			
54	19/05/2004	•	•	•	•	•	•
		Property 2					
0	7/06/2000	•				•	
2	9/08/2000	•		•		•	•
8	13/02/2001	•		•	•	•	•
12	6/06/2001	•[a]		•	•	•	•
18	13/11/2001	•		•	•	•	•
21	13/03/2002					•	
24	25/06/2002	•		•	•	•	•
30	3/12/2002		•	•	•	•	•
36	24/06/2003	•		•	•[b]	•	•
42	3/12/2003			•			•
48	23/06/2004	•	•	•	•	•	•
		Property 3					
0	27/06/2000	•				•	
2	6/09/2000	•		•		•	•
8	27/02/2001	•		•	•	•	•
12	20/06/2001	•		•	•	•	•
18	30/10/2001	•		•	•	•	•
21	27/03/2002					•	
24	16/07/2002	•		•	•	•	•
30	6/11/2002		•	•	•	•	•
36	5/08/2003	•		•	•[b]	•	•
42	8/12/2003			•		•	•
43	27/01/2004	•	•		•		

[a] IFN- results for these samplings were invalid

[b] Samples for IFC collected and stored, but not cultured due to insufficient resources

In addition to the intensive trial described above, Gudair™ was used under permit (NRA permit no. 3257) on approximately 70 OJD infected properties in the Residual zone of NSW in a so-called extensive appraisal. Qualitative and semi-quantitative efficacy and safety data were initially collected from these properties as part of the permit conditions. When wider use of

Gudair™ was available in 2002, formal data collection was abandoned. However, PFC data on seven properties were collected from 2 year-old sheep in the first and third years of vaccination, to provide a preliminary assessment of the effect of vaccination over several generations in a real world situation.

Note that there was a degree of "evolution" of the methodology in this trial, as the objectives varied with the needs of industry over the five-year course of the trial. Initially, a relatively limited trial to assess vaccine efficacy in the Australian situation in terms of reductions of mortalities and group faecal excretion of *M. a. paratuberculosis,* was planned, and collection of data on production traits was also emphasized. Immunological assessments were then included to facilitate the registration of Gudair™ vaccine, and with this increased attention to individual animal response, individual excretion data, and final infection status could be assessed to greatly expand the conclusions which might be drawn from the study.

3.2 Necropsy examinations

During the course of the trial, experimental sheep that were culled for normal flock management reasons, died or showed clinical signs of OJD, were sampled for assessment of paratuberculosis.

Clinically affected sheep - Post mortem examinations were conducted on any ill or moribund trial sheep during property visits. Initially, property owners were offered incentives to collect samples from moribund trial sheep (i.e. from those possibly dying from OJD) between scheduled visits, but the number and quality of such samples was very poor and this was abandoned. Subsequently, owners would contact Jeff Eppleston whenever moribund sheep were observed, and he would visit if possible and conduct necropsies. Tissue samples were collected from these sheep for histopathological examination only.

Hogget culls - On each farm, some trial sheep were culled for normal flock management reasons ("hogget cull"), and processed through the abattoir. On P1, 20 sheep (10 vaccinates and 10 controls) were culled in June 2001, 18 months pv. On P2, 30 sheep (15 vaccinates and 15 controls), and on P3, 28 sheep (10 vaccinates and 18 controls) were culled in November 2002, 29 months pv. These sheep were examined on the line for gross lesions, and tissue samples were collected from all slaughtered sheep for histopathological examination only.

Abattoir sampling at trial end - Final sampling was planned for approximately 100 sheep from each flock, to assess subclinical infection levels. This number was chosen to allow sufficient sheep with an accurate "final status" for analysis, and also was a practical number, in that trial sheep were not owned by the project, and the producer needed to be both willing and able to consign sufficient sheep to slaughter. These sheep were examined on the line for gross lesions, and tissue samples were collected for histopathological examination and for culture from all slaughtered sheep.

Sheep were slaughtered from P1 on 25/06/04 at 54 months pv.

From P2, sheep were not slaughtered until 12/10/04, 52 months pv and thus 4 months after the final faecal and immunological samples were collected. This delay was a result of the sheep's remaining the property of the cooperating farmer, the need to wean their autumn-born lambs, and to get the sheep in sufficient condition for economic return.

On P3, approximately 100 sheep (all wethers) were slaughtered on 14/11/2003, 42 months pv, and 4 days after their final sampling for immunological tests. This was an emergency measure because the property was being broken up and sold, and it was feared that we may lose access to the remainder of the trial sheep on this farm. Fortunately, this was not the case, and the

remainder were sampled in January 2004, 43 months pv, and were then sent to slaughter. These were examined on the line for gross lesions, but tissue samples for histopathological examination and culture were collected only from sheep with gross lesions.

Histopathological examination – Samples were collected into 10% neutral buffered formalin from the ileocaecal valve (ICV), 2 sites in terminal ileum (TI) and 3 mesenteric lymph nodes (MLN) from each sheep. Fixed tissues were processed routinely for histopathology, then stained with hematoxylin and eosin (H&E) and the Ziehl-Neelsen (ZN) method. Paratuberculous lesions were graded, based on the classification of Perez (Perez et al. 1996), as focal (grades 1 or 2), multifocal (3a), severe diffuse paucibacillary (3c) or severe diffuse multibacillary (3b). The minimum criterion for a positive result was the finding of at least two clumps of macrophages with typical epithelioid morphology in a usual predilection site, with or without the presence of acid-fast bacilli.

Culture from tissues - Tissue samples for culture were held at 4^{o}C for less than 24 hours, or frozen at -80^{o}C for up to 6 months, then processed for radiometric culture. A single pooled culture from the ileocaecal valve (ICV), 2 sites in terminal ileum (TI) and 3 mesenteric lymph nodes (MLN) from each sheep was done using previously described techniques including a centrifugation step (Whittington et al. 1998; Reddacliff et al. 2003b; Reddacliff and Whittington, 2003). Confirmation of growth was by PCR and REA analysis (Whittington et al. 1998; Cousins et al. 1999).

3.3 Faecal sampling and culture

Samples for pooled faecal culture (PFC) were collected at each scheduled visit. Initially pools of 40 sheep were used. This was reduced over time to 20 sheep per pool and finally to 10 sheep per pool, to provide greater discrimination between group excretion levels, and to facilitate the identification of individual culture-positive sheep from positive pools. These samples were held at 4^{o}C, and processed within 72 hours of collection.

Samples for individual faecal culture (IFC) were collected at each scheduled visit after January 2001. In samplings up to and including June 2002, gloves were changed only between pools. From Dec 2002 gloves were changed for each sheep. These samples were held at -80^{o}C for up to 12 months until processed for culture. Samples from all positive pools were cultured. Samples from randomly selected negative pools from the 8, 12 and 18 month pv samplings on P2 and P3, and the 12, 18 and 24 month samplings on P1 were also cultured (40 samples per group).

Samples were processed for culture by routine methods (Whittington et al. 2000) using radiometric culture in modified BACTEC medium (Whittington et al. 1998) with confirmation by PCR and REA analysis (Whittington et al. 1998; Cousins et al. 1999).

Numbers of *M. a. paratuberculosis* organisms in each culture-positive sample were estimated based on the days taken for the cumulative growth index in Bactec culture to reach 1000 (dcgi1000) (Reddacliff et al. 2003a), and allowing for the effect of decontamination procedures (Reddacliff et al. 2003b). The approximate numbers of organisms excreted by each group of sheep per day were then calculated, adding the amounts contributed by each infected pool, allowing for the pooling rate, daily faecal excretion per sheep (estimated at 1 kg) and total number of pools per group.

The prevalence of individual sheep excreting *M. a. paratuberculosis* was estimated from the PFC results using the pooled prevalence calculator (Appendix 4). Method 2, with exact binomial confidence limits was used. This method assumes 100% sensitivity and specificity for PFC. This method was chosen, acknowledging that sensitivity is not 100%, thus the prevalences derived

are underestimates of the true prevalence. However, this method gives meaningful results across the spectrum of PFC results, allowing meaningful comparison between the vaccinated and control groups on each farm (ESG Sergeant, personal communication). The PFC and IFC data from this project was used in the development of the pooled prevalence calculator, which can now be accessed at http://www.ausvet.com.au/pprev/. The prevalence of sheep excreting *M. a. paratuberculosis* was also calculated directly from the IFC results at the samplings where this was done. This method also assumes 100% sensitivity and specificity, and the results can be meaningfully compared to those obtained from PFC.

3.4 Serology for M. a. paratuberculosis

Humoral immune responses were measured using a commercial ELISA test (Parachek™) (Hope et al. 2000). Samples were transported overnight at 4°C to CSL in Geelong for testing. The test was performed on the plasma from blood samples stimulated with saline only in the IFN-γ test – see below). In addition, an agar gel immunodiffusion test (AGID) (Hope et al. 2000) was performed at EMAI on serum samples collected in the final round of sampling, and from the Dec 2002 sampling. Some sheep necropsied with severe clinical disease during the course of the study were also AGID-tested at RVL Orange.

3.5 IFN-γ testing

Cell mediated immune responses were measured using a gamma interferon (IFN-γ) assay (Bovigam™)(Stewart et al. 1999). Stimulation of whole blood was done at RVL Orange within 8 hours of blood collection, using 100μL of Johnin PPD, avian PPD, with PBS as negative control. In later rounds pokeweed mitogen was included, providing a positive control to determine whether samples were suitable for IFN-γ testing. Stimulated plasma was then consigned to CSL at 4°C overnight for the enzyme immunoassay. Details on the stimulation agents are as follows:

- Nil Antigen PBS (Ca++, Mg++ free, 0.01% v/v thiomersal) from JRH Biosciences
- Avian PPD (300 μg/mL) (0.01% v/v thiomersal, Lot 2091-01301) from CSL Animal Health
- Johnin PPD (300 μg/mL) (0.01% v/v thiomersal, Lot 0404-2301:8 from CSL Animal Health, originally sourced from CVL Weybridge, UK
- Pokeweed Mitogen sourced from Sigma Aldrich (150 μg/mL, 0.01% v/v thiomersal, in PBS (Ca++, Mg++ free) from JRH Biosciences

3.6 Assessment of local reactions to vaccination – (pv lesions)

At each property visit, all experimental sheep were palpated to determine the size of pv lesions at the site of vaccination. At the first pv visit, the draining prescapular lymph nodes were examined also, but it was not possible to detect enlarged nodes. Presence/absence of lesions, diameter of the vaccination site lesions, and any discharging sinuses were recorded at each visit.

3.7 Collection of production data

Liveweight and condition score – These parameters were measured for each sheep at each visit. Liveweight at pv 0 was used as an indication of individual age at the time of vaccination, and was included as a covariate during analysis.

Wool Production - Greasy fleece weights (GFW) and fibre diameter (FD) measurements were collected on all trial properties from most sheep when shorn for the first time as adults in 2001. Further samples were collected from P1 in 2003, from P2 in 2002 and 2003, and from P3 in 2002. This data collection was of an opportunistic nature, being peripheral to the revised project objectives.

3.8 Assessment of disease/infection status of individual sheep

A sheep was classified as a **shedder** of *M. a. paratuberculosis*, if at any sampling time it had a positive IFC. For the purposes of analysis, positive results in the IFC test were considered invalid (and grouped with non-positive samples) when the sample was from a pool where gloves were not changed, and where the previously collected sample was also positive in the IFC, and yielded larger numbers of *M. a. paratuberculosis*.

A sheep was classified as **infected** with *M. a. paratuberculosis* if it was a shedder, or if at necropsy it had histological lesions consistent with *M. a. paratuberculosis* infection or was positive by culture of tissues.

Sheep which developed clinical signs suggestive of OJD, and that were shown to have severe OJD at subsequent necropsy, were classified as having severe **clinical signs/died of OJD**.

3.9 Statistical methods

The Chi square test (EpiInfo Statcalc), or Fisher exact test (if an expected cell value in the Chi square test was less than 5) was used to test the significance of the following on each farm, and stratified analysis was used to test for significant differences across all three properties, or across vaccinates and controls, where appropriate:

Reduction in the proportion of faecal pools positive in the vaccinates compared to the controls at each sampling time

Reduction in mortality due to OJD in the vaccinates compared to the controls over the whole trial

Focal/multifocal/paucibacillary vs multibacillary pathology in the vaccinates compared to the controls among sheep with severe clinical OJD, among sheep slaughtered that had lesions at trial end, and among hogget culls that had lesions

Subclinical OJD in the vaccinates compared to the controls among sheep slaughtered at the abattoir at trial end, and among the hogget culls

Association of vaccination status with subsequent IFN-γ, ELISA and AGID responses

Association of pv IFN-γ response among vaccinated sheep with subsequent shedding of *M. a. paratuberculosis*, with infection status and with OJD-mortality

Association of pv ELISA response among vaccinated sheep with subsequent shedding of *M. a. paratuberculosis*, with infection status with OJD-mortality

Association of maternal antibodies with subsequent pv immune responses among vaccinated sheep

Association of maternal antibodies with subsequent shedding of *M. a. paratuberculosis*,

with infection status and with OJD-mortality, among vaccinates and among controls

Association of presence of a vaccine-site-lesion among vaccinated sheep with subsequent shedding of *M. a. paratuberculosis*, with infection status with OJD-mortality

The agreement between markers of "vaccine take" (presence a post-vaccinal lesion, IFN-γ response, ELISA response) was assessed using Kappa coefficient that is approximately normally distributed when the number of observations is large. When Kappa coefficient approaches one, that indicates complete agreement. When the coefficient is zero or negative, that indicates there no agreement. The detailed calculation can be found in Siegel and Castellan, 1988(Siegel and Castellan, 1988).

McNemar's Chi-square test for paired observations (Motulsky, 1995) was used to compare the numbers of infected animals at final slaughter that were detected by culture or by histopathology. The binomial test for two proportions (Minitab statistical software) was used to determine whether the use of both tests in parallel was superior to either test used alone.

General linear models (Minitab statistical software) were used to compare production data across the three properties. The model included farm, vaccination status, infection status, and farm/vaccination and infection/vaccination interactions. Weight at the time of vaccination was included as a covariate in the analysis of liveweight up to 12 months of age, to remove any effect of age of lamb.

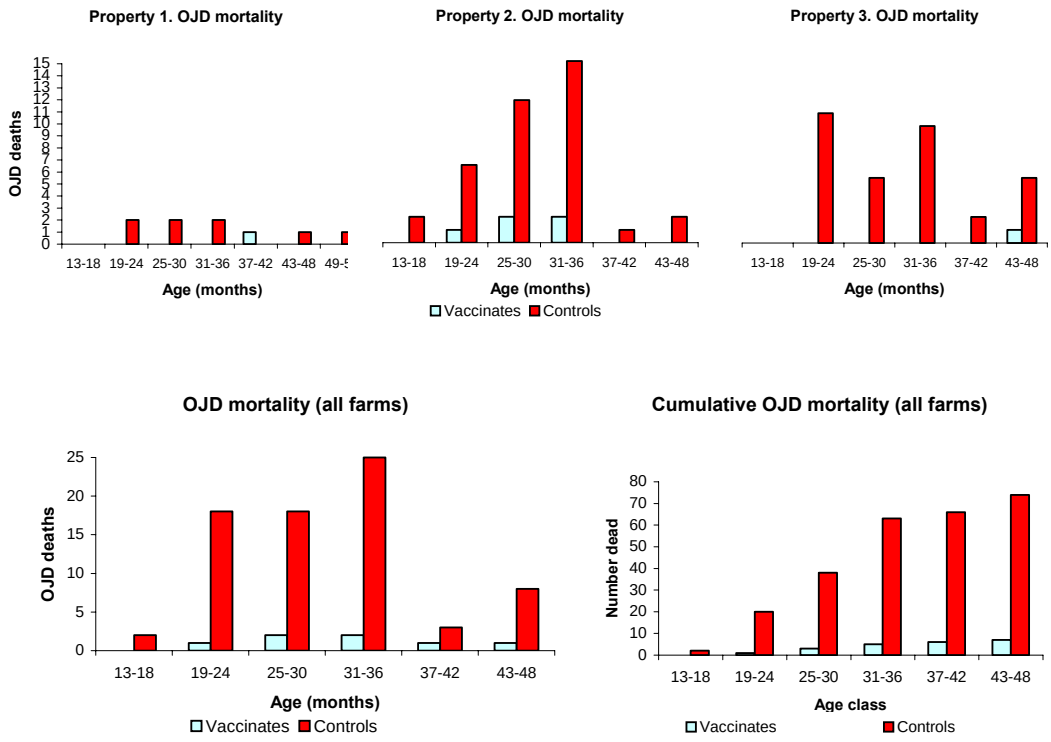
4 Results and discussion

Summary data tables and figures, and statistical analyses are given below in this report. There were considerable differences between the three farms, which became more apparent towards the end of the trial. Results are thus shown and analysed separately for each property. Where appropriate, analysis across all three farms is given. Comprehensive result details, including all individual animal raw data, are held separately at EMAI and RLPB Bathurst in an Access database.

4.1 Mortality due to OJD (severe clinical OJD)

Mortalities due to OJD are shown in Figure 1 for each farm, for each age class, across the duration of the trial. Histopathological findings for all sheep that were killed or died with severe clinical signs suggestive of OJD are presented in Table 2.

Figure 1. Mortality due to OJD on each property.



Vaccination was associated with both a delay and a reduction in OJD-related mortality. OJD mortalities in control sheep began at 23, 14 and 19 months of age on farms 1, 2 and 3 compared to 41, 23 and 43 months respectively in vaccinates. This represents a mean delay of about 17 months. The total mortality due to OJD was significantly reduced on all three farms by vaccination. On P1, 10 controls and one vaccinate ($P<0.01$) on P2, 39 controls and 5 vaccinates ($P<0.000001$) and on P3, 31 controls and one vaccinate ($P<0.000001$) were confirmed as having died of OJD. Across all three farms, the magnitude of reduction in OJD mortalities due to vaccination was about 90% (a total of 80 controls and 7 vaccinates).

Over the course of the trial there were a number of sheep which went missing (particularly on

P3), or died but were not necropsied. Numbers were approximately the same for vaccinates and controls - 21and 23, 10 and 11, and 30 and 36 respectively on P1, P2 and P3.

Table 2. Histological examination of sheep killed or dying with severe clinial signs suggestive of OJD

	Number examined	Histopathological findings						% with severe lesions[a]	% multibacillary[b]
		No lesions	Focal or multifocal lesions			Severe diffuse lesions			
			1	2	3a	3c paucibacillary	3b multibacillary		
Property 1									
Vaccinates	2	1	0	0	0	0	1	50	100
Controls	11	1	0	0	0	0	10	91	100
Property 2									
Vaccinates	5	0	0	0	0	0	5	100	100
Controls	40	1	0	0	0	5	34	98	87
Property 3									
Vaccinates	4	2	0	1	0	0	1	25	100
Controls	33	2	0	0	0	5	26	94	84

[a] This is the % of sheep which died or were killed, and severe OJD was confirmed. All such sheep had grossly visible lesions at necropsy.

[b] % of sheep that died or were killed due to OJD (ie had severe diffuse lesions) that had multibacillary lesions. Sheep with only focal or multifocal lesions were infected with OJD, but died from other causes. None of these latter sheep had gross lesions.

Of the vaccinates that died due to OJD, all had multibacillary disease. Of the controls, 70 (88%) had multibacillary disease – not significantly different from the vaccinates. Sheep with multibacillary lesions can excrete sufficient organisms to infect many thousands of susceptible animals (Whittington et al. 2000). Thus, the "breakdown" of a single vaccinated animal can have a disproportionate effect on potential transmission of the infection and the persistence of disease in the flock.

4.2 Subclinical OJD – examination of culled sheep ("hogget culls")

These sheep were examined by histopathology at abattoir slaughter. Ages ranged from 21 to 31 months. Histopathological findings are presented in Table 3. No gross lesions were recorded. On each farm, fewer vaccinated than control animals had paratuberculous lesions, but this difference was significant only on farm 2 ($P<0.01$). Across all three farms there was about a 65% reduction in the proportion of vaccinates with subclinical OJD compared to controls. 6 of 35 vaccinates (17%) had lesions, compared to 21 of 43 controls (49%), ($P<0.01$). Among sheep with lesions there was no significant difference between vaccinates or controls with respect to focal/multifocal/paucibacillary or multibacillary lesions.

Table 3. Histological examination of hogget culls for lesions of OJD

	Number examined	Histopathological findings						% with lesions[a]	% multibacillary[b]
		No lesions	Focal or multifocal lesions			Severe diffuse lesions			
			1	2	3a	3c paucibacillary	3b multibacillary		
Property 1									
Vaccinates	10	10	0	0	0	0	0	0	-
Controls	10	8	0	0	0	1	1	20	50
Property 2									
Vaccinates	15	10	1	2	0	0	2	33	40
Controls	15	2		3	3	4	3	87	24
Property 3									
Vaccinates	10	9	1	0	0	0	0	11	0
Controls	18	12	0	2	1	3	0	33	0

[a] This is the % of sheep that had subclinical OJD at slaughter (as detected by histopathology)

[b] % of sheep with lesions that had multibacillary lesions

4.3 Subclinical OJD – examination of old sheep at trial end

These sheep were examined by histopathology and by culture at abattoir slaughter. Ages ranged from 44 to 57 months. Histopathological findings are presented in Table 4 and culture results in Table 5. The pattern of subclinical disease (as determined only by histopathology) in these older sheep among vaccinates and controls was similar to that seen among the younger hogget culls, although the proportion of control sheep with lesions had dropped. On each farm, fewer vaccinated than control animals had paratuberculous lesions, but this difference was significant only on farm 3 ($P<0.001$). Across all three farms there was about a 50% reduction in the proportion of vaccinates with subclinical OJD compared to controls. 24 of 163 vaccinates (15%) had lesions, compared to 41 of 144 controls (28%), ($P<0.01$). Among sheep with lesions, there was no significant difference between vaccinates or controls with respect to focal/multifocal/paucibacillary or multibacillary lesions on Farms 1 and 3, nor across all farms. On farm 2 only, there were significantly more vaccinated sheep with multibacillary lesions (3/10 compared to 0/16 controls) ($P<0.05$).

Culture of tissues at necropsy detected eleven additional infected sheep which had no histological lesions. When culture and histopathology were used in parallel, the proportion of sheep with subclinical OJD infection was higher, but the significant difference between vaccinates and controls remained: 31 of 163 vaccinates (19%) were infected, compared to 45 of 144 controls (31%), ($P<0.05$). All sheep that had severe diffuse or multifocal histological lesions, but one, were also culture-positive, but many sheep with only focal lesions were culture-negative. Overall, using both culture and histopathology, 76 sheep were found to be subclinically infected, significantly more ($P<0.05$) than the 48 sheep that were detected by culture or the 65 sheep detected by histopathology. The observation that culture and histopathology in parallel detected more infected sheep than either test alone in older sheep was consistent with findings from previous studies (Reddacliff et al. 2004; Lambeth et al. 2004;

Reddacliff and Whittington, 2004; Reddacliff et al.). However, in the current study, histopathology alone outperformed culture alone for the detection of subclinical OJD in old sheep (P<0.01). This was at odds to previous findings, in which no significant difference between the two tests was found, and the change was not due to vaccination.

Abattoir surveillance for gross lesions of OJD detected all but one of the 21 sheep with severe diffuse lesions, but only two of the 44 sheep with focal or multifocal lesions. Two other sheep considered to have gross lesions at abattoir inspection had no histological changes consistent with OJD.

Table 4. Histological examination of abattoir-slaughtered sheep at trial end for lesions of OJD

	Number examined	Histopathological findings						% with lesions[a]	% multibacillary[b]
		No lesions	Focal or multifocal lesions			Severe diffuse lesions			
			1	2	3a	3c paucibacillary	3b multibacillary		
Property 1									
Vaccinates	48	40	3	3	1	1	0	17	0
Controls	44	35	4	2	0	2	1	21	11
Property 2									
Vaccinates	58	48	2	1	0	4	3	17	30
Controls	60	44	4	6	1	5	0	27	0
Property 3									
Vaccinates	57	51	4	1	0	1	0	11	0
Controls	40	24	12	0	0	3	1	40	6
Property 3 (slaughter of all remaining sheep, only those with gross lesions examined)									
Vaccinates	4	1			2	1		75	0
Controls	13	0	2	1	2	8	0	100	0

[a] This is the % of sheep that had subclinical OJD at slaughter which was detected by histopathology.

[b] % of sheep with lesions that had multibacillary lesions

Table 5. Examination of abattoir-slaughtered sheep at trial end for evidence of OJD by culture and histopathology

	Number examined	Total culture positive	Total histo positive	Total infected[a]	Proportion of sheep culture positive	
					No lesions	Lesions[b]
Property 1						
Vaccinates	48	5	8	10	2/40	3/8
Controls	44	9	9	11	2/35	7/9
Property 2						
Vaccinates	58	8	10	11	1/48	7/10
Controls	60	10	16	18	2/44	8/16
Property 3						
Vaccinates	57	8	6	10	4/51	4/6
Controls	40	8	16	16	0/24	8/16
Property 3 (slaughter of all remaining sheep, only those with gross lesions examined)						
Vaccinates	4	3	3	3	0/1	3/3
Controls	13	10	13	13	0	10/13

[a] Sheep that were positive by culture and/or histopathology

[b] All sheep with severe histological lesions were also culture-positive

4.4 Excretion of M. a. paratuberculosis

Group excretion data - A summary of the group excretion data obtained from PFC for vaccinates and for controls at each sampling time on each farm is shown below in Table 6.

The prevalence of excretion (expressed as proportion of positive pools) in the vaccinated sheep was less than that of the controls at every sampling time after 2 months pv on each farm, and this difference was statistically significant on a majority of occasions. Stratified analysis was done for 12, 18, 24, 30, 36 and 42 months pv across all three farms, and for 48 mths pv across farms 1 and 2. Differences in prevalence were very highly significant ($p<0.001$) at 12, 18, 24, 30 and 36 months and highly significant ($p<0.01$) at 42 months, whilst at 48 months, the difference was almost significant ($p=0.05$).

These data are easier to comprehend graphically, especially when looking for patterns over time and for differences between farms. Figures 2-4 display the data for each farm in two forms. Figures 2a, 3a and 4a show the % of pools positive – a simple measure of prevalence, mostly suitable for comparing the vaccinates and controls at a particular sampling, but mostly unsuitable to compare data between sampling times because the pool size varied. Figures 2b, 3b and 4b show the prevalences obtained from pooled prevalence calculator, and allow meaningful comparisons of prevalence over time, as well as showing the 95% confidence limits for these estimates.

Table 6. Summary of pooled faecal culture results

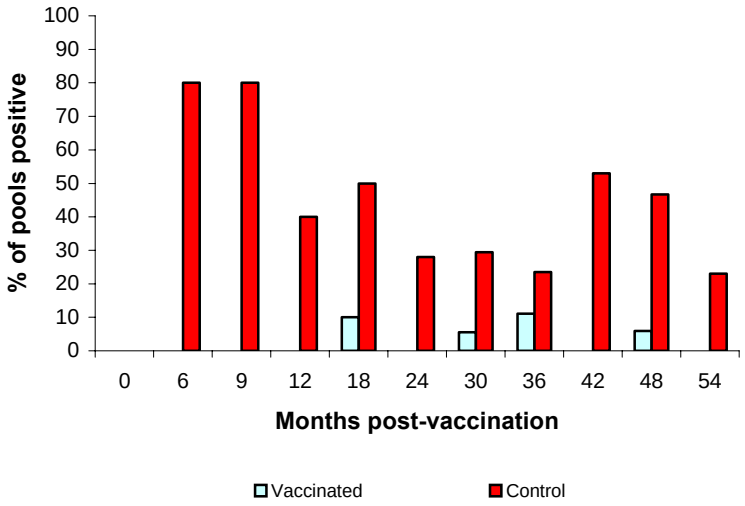
Date sampled	Months post-vacc[a]	Vaccinates			Controls			% reduction of prevalence in vaccinates[b]
		Proportion of pools positive	Pool size	dcgi1000 of +ve pools	Proportion of pools positive	Pool size	dcgi1000 of +ve pools	
Property 1								
Dec-99	0	0/5	40	-	0/5	40	-	-
Jun-00	6*	0/5	40	-	4/5	40	35,41,33,40	100
Sep-00	9*	0/5	40	-	4/5	40	47,34,38,36	100
Feb-01	12*	0/10	20	-	4/10	20	38,40,37,38	100
Jun-01	18	1/10	20	29	5/10	20	30,40,33,36,24	84
Nov-01	24	0/9	20	-	5/18	10	20,32,29,25,30	100
Jun-02	30	1/18	10	29	5/17	10	35,30,23,30,33	83
Dec-02	36	2/18	10	31,26	4/17	10	27,41,39,18	56
Jun-03	42**	0/18	10	-	9/17	10	31,25,40,28,31,33,29,59,28	100
Dec-03	48**	1/17	10	41	7/15	10	39,29,32,46,30,44,20	90
Jun-04	54	0/16	10	-	3/13	10	23,37,45	100
								91
Property 2								
Aug-00	2	0/5	40	-	0/5	40	-	-
Feb-01	8*	0/10	20	-	5/10	20	40,38,34,43,30	100
Jun-01	12**	1/10	20	44	8/10	20	29,40,32,39,29,45,38,47	93
Nov-01	18	2/10	20	34,33	9/20	10	39,44,32,33,25,28,26,17,29	81
Jun-02	24**	6/19	10	30,35,25,33,32,44	18/19	10	20,18,23,25,26,25,26,21,21,17,26,25,29,21,28,21,23,14	85
Dec-02	30**	4/17	10	32,35,29,24	12/15	10	21,27,21,22,24,27,34,77,30,34,27,26	82
Jun-03	36*	4/17	10	34,20,28,27	9/14	10	43,23,32,18,39,31,30,34,19	73
Dec-03	42	8/17	10	33,20,39,34,47,47,39,45	7/13	10	25,32,35,31,31,47,21	17
Jun-04	48	2/16	10	38,34	2/12	10	34,34	28
								70
Property 3								
Sep-00	2	0/5	40	-	0/5	40	-	
Mar-01	8	0/10	20	-	2/10	20	43,43	100
Jun-01	12**	0/10	20	-	10/10	20	35,42,26,42,35,30,29,35,36,35	100
Nov-01	18**	3/20	10	40,24,29	16/20	10	28,27,27,25,27,27,25,25,24,24,35,26,20,20,24,17	89
Jul-02	24**	2/19	10	36,39	12/17	10	40,33,24,25,36,26,27,33,25,37,34,24	90
Dec-02	30**	4/19	10	32,32,32,30	14/17	10	26,22,48,28,33,32,34,29,27,26,33,42,34,26	86
Jun-03	36**	3/17	10	38,24,25	10/13	10	25,25,36,49,24,30,29,27,27,31	86
Dec-03	42	3/11	10	41,47,46	4/8	10	32,20,34,27	53
								86

[a] Significant reductions in proportion of positive pools in the vaccinated group (Fisher exact test) are shown * ($p<0.05$) or ** ($p<0.01$)

[b] Based on estimated prevalences using the pooled prevalence calculator.

Figure 2. Faecal excretion of *M. a. paratuberculosis* on Property 1

a. Percentage of positive faecal pools. Pools contained faeces from 40 sheep (0,6,9 mths), 20 sheep (12,18 mths; 24 mths vaccinates), or 10 sheep (24 mths controls; 30,36,42,48,54 mths).



b. Prevalence of sheep excreting *M paratuberculosis* in faeces. This was calculated from the results of the pooled cultures, assuming 100% sensitivity and specificity using method 2 from the Pooled Prevalence Calculator. Error bars show the 95% binomial confidence interval.

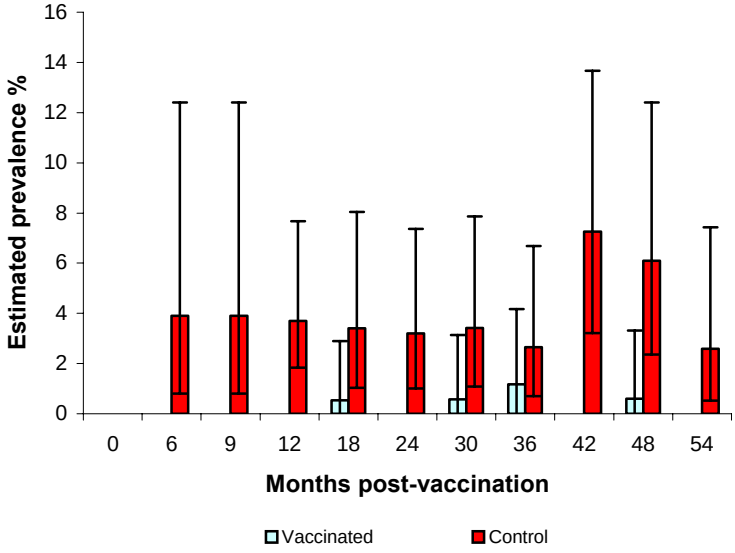
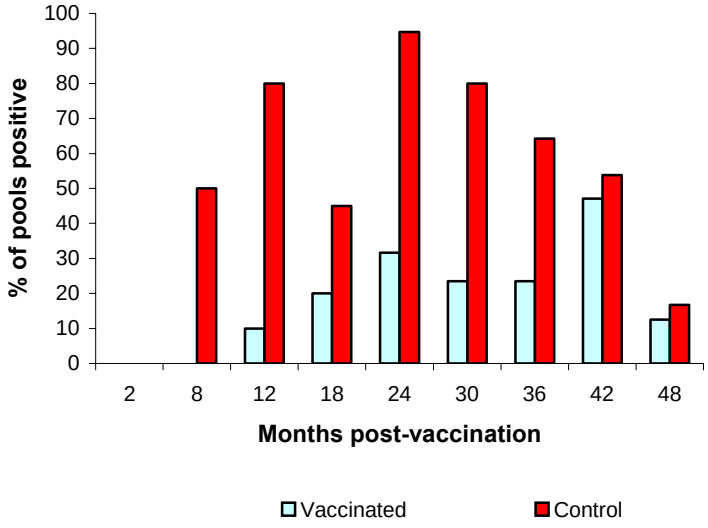


Figure 3. Faecal excretion of *M. a. paratuberculosis* on Property 2

a. Percentage of positive faecal pools. Pools contained faeces from 40 sheep (0,2 mths), 20 sheep (8,12 mths; 18 mths vaccinates), or 10 sheep (18 mths controls; 24,30,36,42,48 mths).



b. Prevalence of sheep excreting *M paratuberculosis* in faeces. This was calculated from the results of the pooled cultures, assuming 100% sensitivity and specificity using method 2 from the Pooled Prevalence Calculator. Error bars show the 95% binomial confidence interval.

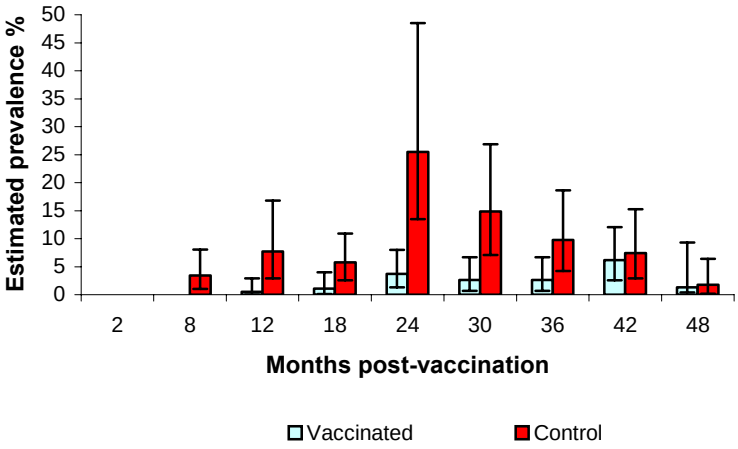
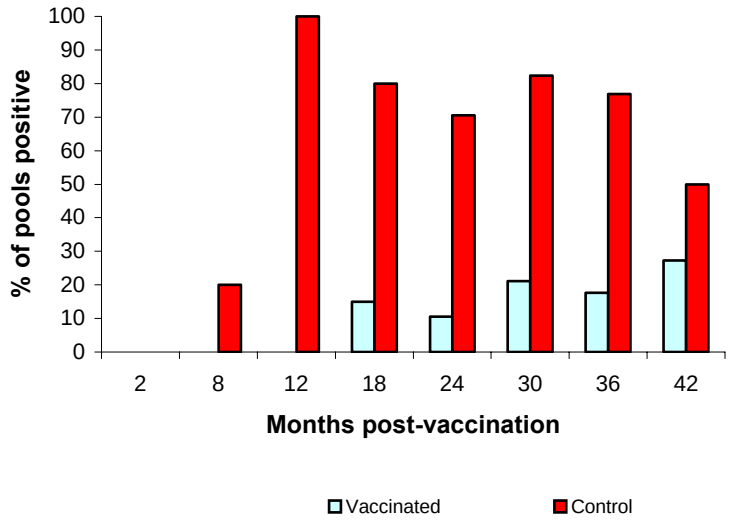
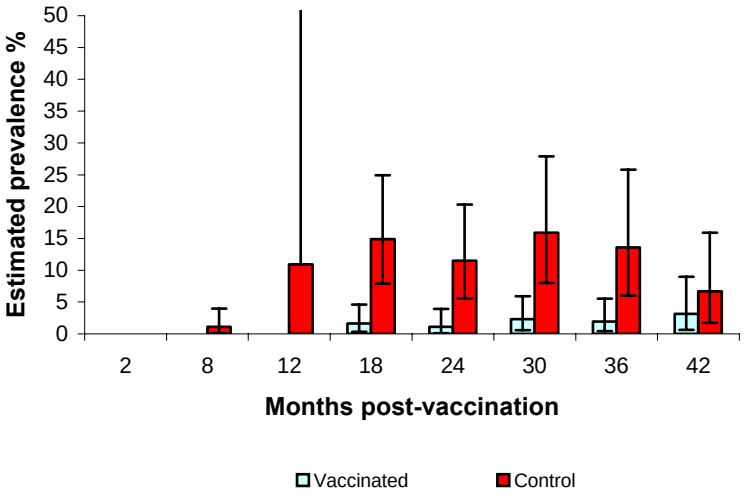


Figure 4. Faecal excretion of *M. a. paratuberculosis* on Property 3

a. Percentage of positive faecal pools. Pools contained faeces from 40 sheep (0,2 mths), 20 sheep (8,12 mths; 18 mths vaccinates), or 10 sheep (18 mths controls; 24,30,36,42,48 mths).



b. Prevalence of sheep excreting *M paratuberculosis* in faeces. This was calculated from the results of the pooled cultures, assuming 100% sensitivity and specificity using method 2 from the Pooled Prevalence Calculator. Error bars show the 95% binomial confidence interval.



Although all farms in this trial were selected based on having severe OJD, an overview of the results reveals differences in the prevalence of OJD between the three properties. P1 had the lowest prevalence of sheep excreting *M. a. paratuberculosis*. Prevalence in the unvaccinated

controls was fairly stable, around 2-4% from 6 to 54 months pv, with a slight increase to about 7% at 42 and 48 months. Note, however, the very wide confidence limits for these low prevalences. On P3, the prevalence of excreters was higher, but also fairly stable over time, ranging between 5 and 15% from 12 to 42 mths pv, and without a distinct peak. P2, on the other hand, showed a distinct peak in prevalence, reaching 26% in control sheep at 24 mths pv. By 48 mths, the prevalence in these control sheep had dropped to less than 2%, presumably because many of the infected sheep had died.

The absence or reduction of faecal shedding in the vaccinated groups compared to the controls at each sampling time on farms P1 and P3 was very clear. On these farms, there was no detectable excretion of *M. a. paratuberculosis* by the vaccinated groups until 18 months pv (about 21 months of age), whereas excretion, was detected in the controls as early as 6 or 8 months pv (9 -11 months of age). The reduction in prevalence of excreters averaged about 90%, and was maintained throughout the trial - to 54 months pv (almost 5 years of age) on P1, and to 42 months pv (almost 4 years of age) on P3. The results from these two farms thus clearly demonstrate a delay and reduction in faecal excretion of *M. a. paratuberculosis*, with maintenance of the protection for the economic life of the sheep.

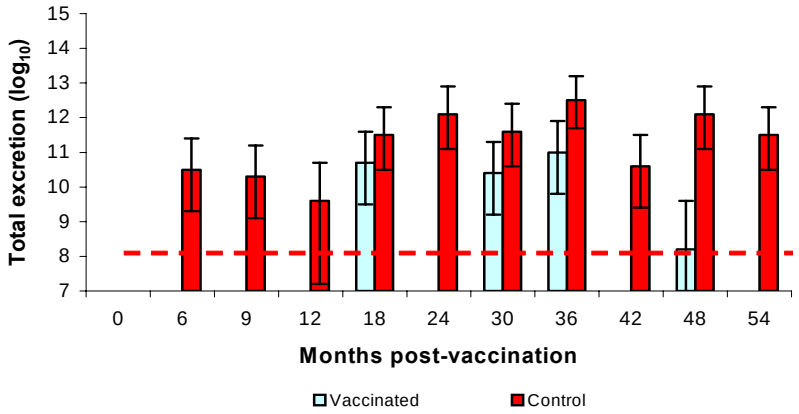
The results for P2 – this was the property with the highest prevalence of infection – differed, especially as the sheep aged. A smaller delay in the excretion of *M. a. paratuberculosis* was apparent, with first detectable excretion by vaccinates when they were 15 months old (12 months pv), compared to 11 months of age (8 mths pv) for the controls. Comparative results at samplings from 12-36 months pv were similar to the other two farms, with significantly lower prevalence of excretion in the vaccinated groups. The mean reduction in prevalence in the vaccinates was also similar (86%) up to 36 mths pv. However, at the final 2 samplings at 42 and 48 mths pv, there was little difference in prevalence between the vaccinated and control groups, bringing the mean reduction in prevalence over the trial for P2 down to 70%. Note, however, that the control group was by this time much smaller than the vaccinated group due to the large number of OJD mortalities which had occurred among the controls. Moreover, the peak prevalence for excreters over the life of the sheep was very much lower, and delayed, in the vaccinated group - 6% at 42 months pv compared to 26% at 24 months pv for the controls (a 77% reduction). In the vaccinated group, prevalence at the final sampling was lower than at 42 months, also indicating maintenance of protection.

Figure 5 shows the total amount of *M. a. paratuberculosis* excreted by each group at each sampling. This is a measure of the potential for environmental contamination with the organism, and thus the risk to subsequent generations of sheep. Note that the vertical scale is a log scale, so that a one unit reduction equates to a 90% reduction in the number of organisms reaching the pasture.

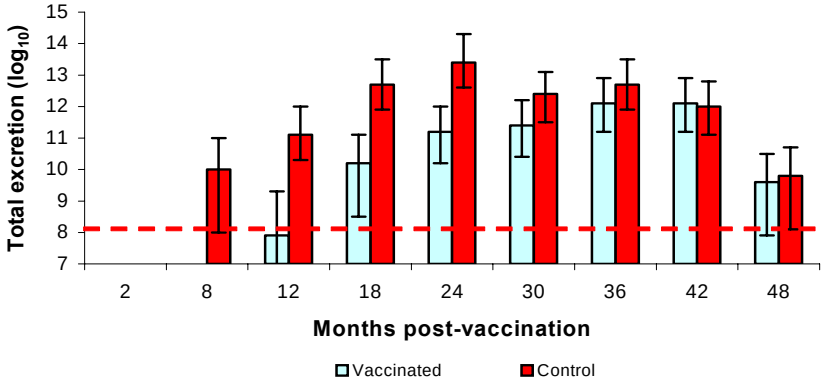
At each sampling time throughout the trial on P1, and up to 30 months pv on the other farms, the numbers of *M. a. paratuberculosis* excreted by the vaccinated group were at least one log lower than the controls. Note, however, that the actual level of excretion can be very much higher than indicated by prevalence data alone. For example, at 18 mths pv on P1 and P3, the numbers of *M. a. paratuberculosis* excreted by the vaccinates equal or exceed the numbers excreted by the control groups at any previous sampling, but the prevalence of excreters among the vaccinates was still very low. This is a reflection of the large contribution that a single (or a few) multibacillary infected sheep may make. One such sheep may excrete in excess of 10^{10} organisms per gram of faeces (Whittington et al. 2000), possibly equivalent to the excretion levels of many hundreds of sheep in the early stages of the disease or with paucibacillary infections.

Figure 5. Estimated total excretion of *M. a. paratuberculosis* by vaccinated and control groups on each farm

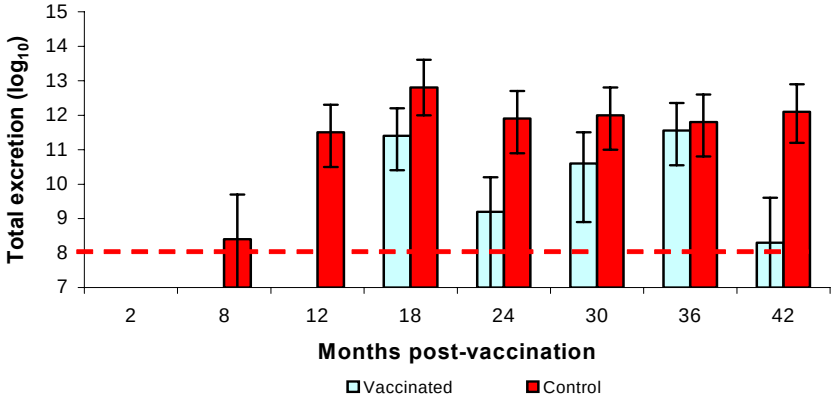
Property 1.



Property 2.



Property 3.



Indicates the maximum excretion that could go undetected because of the analytical sensitivity of the culture technique

Individual faecal culture – The IFC data from positive pools and from the final sampling of all surviving sheep were used to classify individual sheep as excreters of *M. a. paratuberculosis*. It was then possible

to calculate directly the prevalence of individual excreters, based on the assumption that all sheep in negative pools were IFC negative, and that the IFC test was 100% sensitive and specific (Figure 6). Comparison with the prevalences estimated from PFC (Figures 2b-4b) indicates that the two methods gave a similar pattern of results. Analysis of prevalence estimation for PFC and/or IFC was the subject of a separate study, and is published elsewhere. (See Appendix 4)

However, it is also clear that PFC does miss occasional sheep that are excreting *M. a. paratuberculosis*. At the final sampling, from a total of 55 negative pools (10 sheep per pool or 550 sheep), 5 sheep were shown to be positive by IFC. The dcgi1000 for these 5 sheep ranged from 46 to 63 days, all indicating that very low numbers (about one to 6 organisms) of viable *M. a. paratuberculosis* were present in the BACTEC inocula. Thus the failure of PFC to identify such samples was expected. At earlier samplings, 26 culture-negative pools of 20 sheep and 15 culture-negative pools of 10 sheep (670 sheep in total) were cultured also by IFC (Table 7). Eleven IFC samples were positive, and again the dcgi1000 for most samples indicated that few viable organisms were present, and all but two of these positive results were from control pools. However, the dcgi1000 ranged from 37 to 50. On average, for pools of 20 sheep, one might expect individual samples with less than about 20 viable organisms not to give positive results in PFC. This corresponds to dcgi1000 of more than about 42 days. Four of the eleven positive IFC samples from these early negative pools had dcgi1000 values that were less than this, but none had values that would classify them as heavy shedders. The negative results for their respective pools may be the result of sampling error, irregular distribution of organisms between faecal pellets or the error inherent in the calculations used to quantify the organisms. Overall, from 1220 sheep from pools with negative PFC results, 16 sheep were found to be positive by IFC, a false negative rate of only 1.3%.

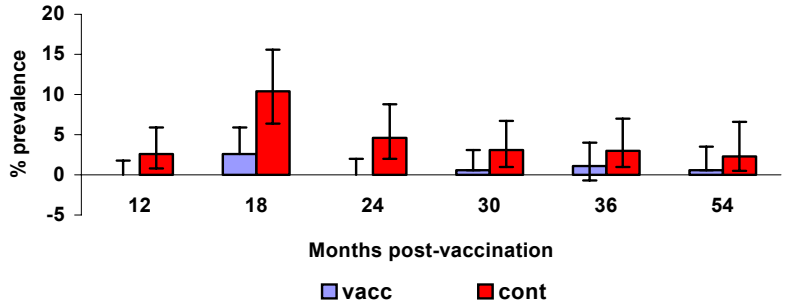
Table 7. Individual faecal culture results from selected PFC-negative pools.

Sampling time (months pv)		Property 1			Property 2			Property 3		
		n	pool size	Samples +ve (dcgi1000 range)	n	pool size	Samples +ve (dcgi1000 range)	n	pool size	Samples +ve (dcgi1000 range)
9	**vaccinates**	40	20	0	40	20	1 (44)			
	controls	40	20	2 (47-49)	40	20	1 (43)			
12	**vaccinates**	40	20	0	40	20	1 (44)	40	20	0
	controls				40	20	4 (37-50)	40	20	1 (39)
18	**vaccinates**	40	10	0	39	20	0	40	20	0
	controls	30	10	1 (40)	39	10	0	38	20	0
24	**vaccinates**							40	20	0
	controls							40	10	0

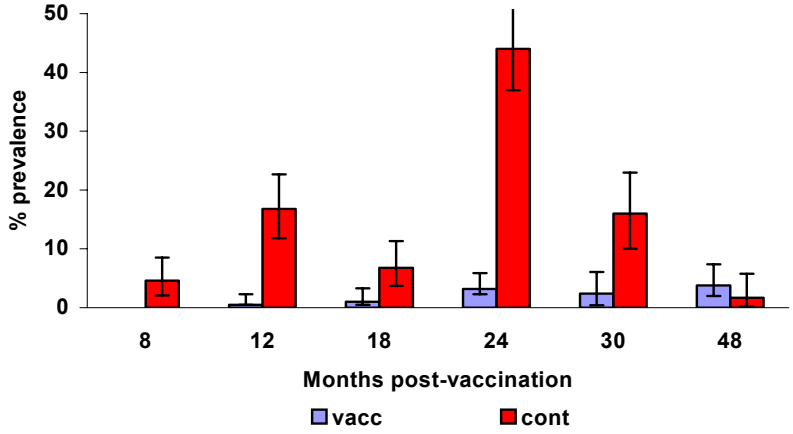
Figure 6. Prevalence of sheep excreting *M a paratuberculosis* in faeces, based on individual faecal culture.

At the final sampling on each farm, all sheep were tested by IFC. At all other samplings, sheep from positive pools were tested by IFC. Sheep from pools testing negative in PFC were classified as negative.

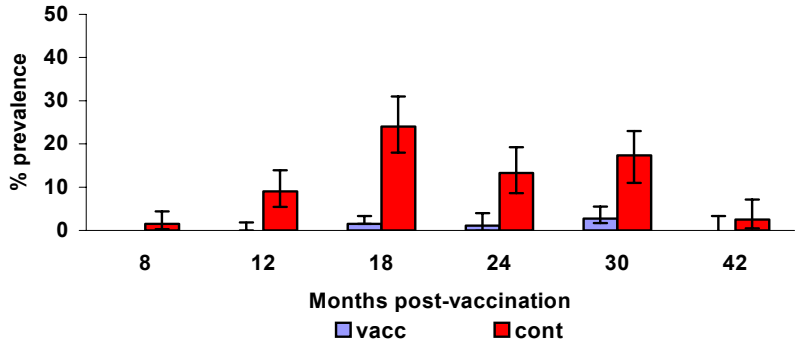
Property 1



Property 2



Property 3



4.5 Immunological responses

Table 8 presents the numbers and proportions of sheep with positive IFN-γ responses and ELISA antibody levels at each sampling time on each farm. A comparison of the percentage of sheep with positive responses in the vaccinated and control groups is illustrated graphically in Figures 7 and 8.

The stimulation of both the cell mediated and humoral immune systems by vaccination is evident from the elevated IFN-γ reactions and ELISA antibody levels in vaccinates compared to controls, and from the rapid increase in these levels among vaccinates that occurred between vaccination and 2 months pv on farms 2 and 3. The first immunological monitoring on P1 did not occur until 12 months pv.

Table 8. Proportions of trial sheep with positive immune responses.
The proportions are shown as number of sheep positive/number sampled

	Months post-vaccination	Age (months)	Gamma interferon test		ELISA	
			Controls	Vaccinates	Controls	Vaccinates
Property 1						
	0	3	ns	ns	ns	ns
	2	5	ns	ns	ns	ns
	8	11	ns	ns	ns	ns
	12	15	46/194	172/196	6/194	168/196
	18	21	5/189*	35/190*	12/191	130/191
	24	27	21/172	99/175	0/172	74/175
	30	33	13/169	97/165	4/169	74/176
	42	45	9/158	30/170	3/160	98/173
	54	57	9/129	23/156	1/130	25/157
Property 2						
	0	2	0/199	0/199	12/199	14/199
	2	4	4/198	171/199	4/198	94/199
	8	10	6/197	121/197	1/197	124/197
	12	14	0/196*	7/195*	4/197	112/195
	18	20	110/189	152/191	8/191	58/191
	24	26	86/181	102/186	11/182	78/186
	36	38	32/136	72/166	10/136	48/166
	48	50	21/121	37/158	1/121	22/158
Property 3						
	0	2	0/204	0/207	3/204	0/207
	2	4	1/204	177/207	1/204	173/207
	8	10	25/196	144/199	0/196	117/199
	12	14	21/197	94/187	3/199	125/199
	18	20	40/196	70/190	2/196	82/194
	24	26	45/172	75/180	9/174	86/183
	36	38	35/130	60/170	11/130	78/170
	42	50	13/71	22/106	4/77	56/106

ND Not sampled
* Unreliable result, stimulation by antigens failed

Cell-mediated (IFN-γ) responses (Figure 7)

In controls, positive IFN-γ responses were detected by 8-12 months pv and are likely to be due to environmental exposure to *M. a. paratuberculosis*. The proportion of vaccinates with positive IFN-γ responses was maximum at the first pv test on each farm, and then declined. These

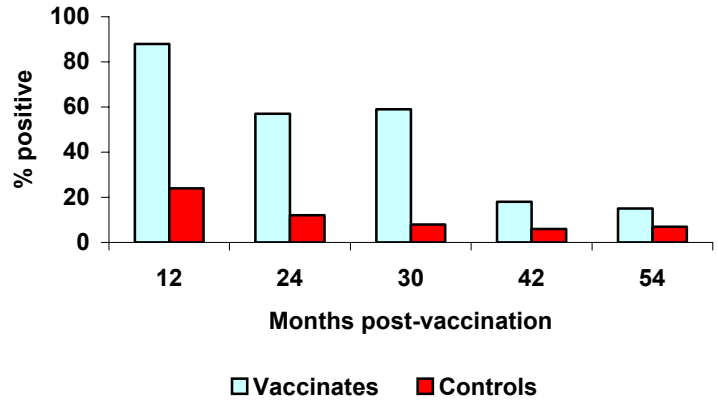
proportions remained higher than in the controls at every subsequent sampling time, although at 24 and 48 mths on P2, and 36 and 42 months on P3, differences between the two groups were not statistically significant. Property 2 appeared to have had a very high level of environmental *M. a. paratuberculosis* challenge as evident by a high mortality rate, and 58% IFN-γ reactors in controls at 18 months pv. This large amount of environmental challenge may also explain the rise in the number of IFN-γ test positives amongst vaccinates on Property 2 at 18 months pv. Environmental *M. a. paratuberculosis* challenge of vaccinated sheep could lead to a boosting of waned IFN-γ responses.

Association of pv IFN-γ status with subsequent OJD status (excretion, infection status and OJD-mortality)

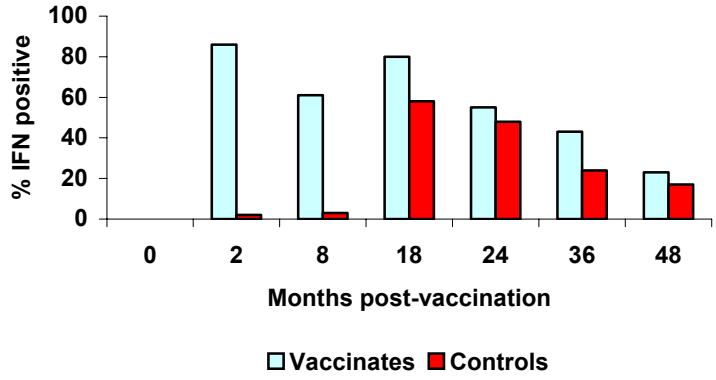
Table 9 shows the numbers of vaccinated sheep that were classified as shedders, as infected with *M. a. paratuberculosis* or as having died of OJD, according to their post-vaccination IFN-γ response. Positive IFN-γ response was negatively associated with shedding ($P<0.05$) on all farms, and negatively associated with infection ($P<0.01$) on farms 1 and 2. Stratified analysis across all farms demonstrated a negative association ($P<0.0001$) with infection. No significant association with OJD-mortality was found, but there were very few vaccinated sheep that died of OJD.

Figure 7 . Proportion of animals showing positive gamma interferon responses

Property 1.



Property 2.



Property 3.

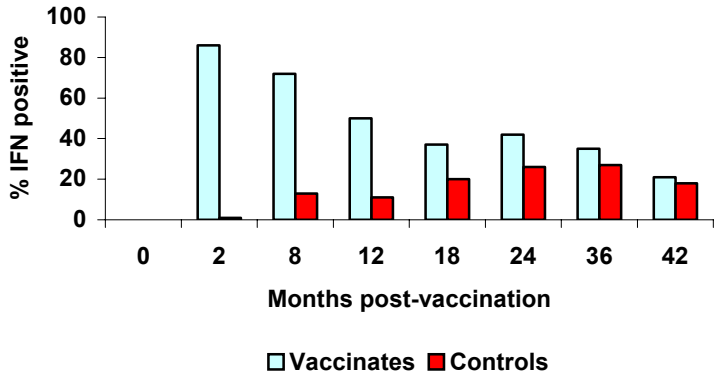


Table 9. Association of γ-IFN response[a] post-vaccination with subsequent OJD status

		Number of sheep							
		Shedding status			Infected			OJD Mortality	
	γ-IFN	Positive	Negative		Positive	Negative		Positive	Negative
Property 1									
	Positive	2	170	*	7	165	**	0	172
	Negative	3	21		5	19		1	23
Property 2									
	Positive	11	160	*	17	154	**	4	167
	Negative	6	22		9	19		1	27
Property 3									
	Positive	3	174	*	12	165		0	177
	Negative	3	22		3	22		1	24

[a] 2 months pv on P2 and P3, 12 mths pv on P1

* Significant difference (P<0.05, Fisher exact test)

** Significant difference (P<0.01, Fisher exact test)

Humoral responses

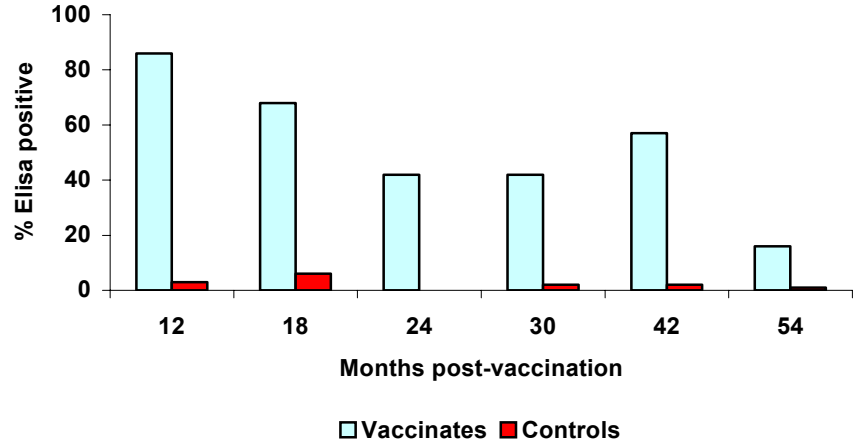
The pattern of ELISA results (Figure 8) was similar on Properties 1 & 3, with high levels of seroconversion in vaccinates, which declined slowly for about 18 months, and then levelled out. On Property 2, the most severely affected property, antibodies to *M. a. paratuberculosis* were present in 6-7% of lambs at the time of vaccination. This suggests the presence of passive maternal antibody, which may have affected the ELISA responses to vaccination. On this property fewer sheep responded initially, and by 8 months pv the percentage of reactors peaked at only 63%, in comparison with levels above 80% on the other two farms. At the 24 months pv sampling there was a large increase in the percentage of vaccinates with positive ELISA results, which may indicate an immune memory effect as vaccinates are exposed to increasing levels of infection. Among control sheep, seroconversion occurred from about 12 months and the proportion of ELISA positives was greatest at 24 months pv. These seroconversions likely reflect the development of advancing disease.

In a separate DPI-funded small trial (unpublished observations, Appendix 3), AGID was compared to ELISA for the monitoring of early post-vaccination serological response. In summary, very few sheep were AGID-positive post-vaccination. Highest rates of seroconversion in the AGID (but still only a low 10%) were seen 2-6 months pv. At other times the seropositive proportion was ≤ 4%. This low rate of seroconversions in the AGID among vaccinated sheep was unexpected and at odds with a previous report from Spain (Garcia Marin JF et al. 1999). Seropositivity rates in the ELISA ranged from 42 to 92%, consistent with the current trial. Thus, AGID was subsequently used on all sheep in the current trial at the December 2002 sampling (36 month pv on P1 and 30 month pv on P2 and P3), and at the end-of-trial sampling (Table 10). Low proportions of seropositive vaccinates were also found. There were no consistent differences in the proportion of sheep with positive AGID responses between the vaccinates and controls. In December 2002, the proportion of vaccinates with positive AGID results was significantly greater than the controls (P<0.05) on P1, whereas the reverse was true on P2 (P<0.01). At this time, less than 6-9% of vaccinates across all farms were AGID-positive, and these positive results may reflect seroconversion post-vaccination, since less than one in ten of those sheep were subsequently recorded as having died of OJD. On farms 2 and 3, 15-17% of control sheep were AGID-positive, and this probably reflects *M. a. paratuberculosis* infection, since about 50% of those sheep died of OJD. There were no differences in proportion of AGID-

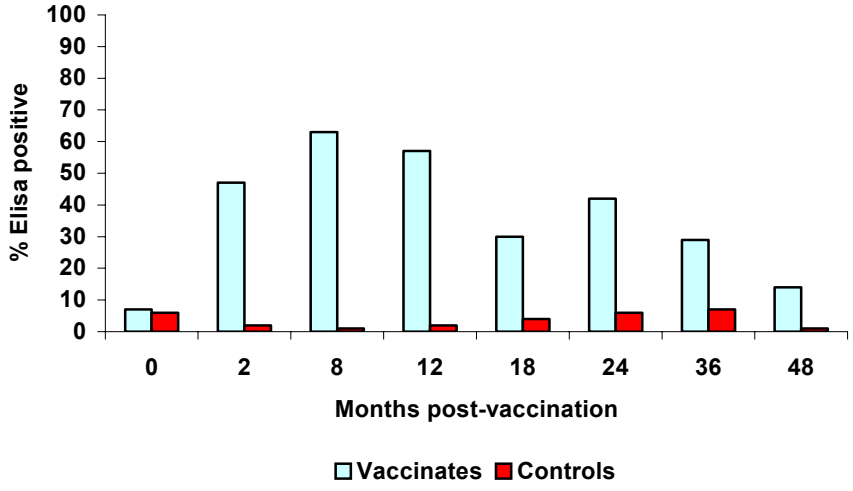
positive sheep between vaccinates and controls on any property at the final sampling. AGID tests were also performed on some sheep when euthanased with clinical OJD, and almost all of these sheep were AGID-positive at the time of death, consistent with published reports(Perez et al. 1997).

Figure 8. Proportion of animals showing ELISA responses

Property 1.



Property 2.



Property 3.

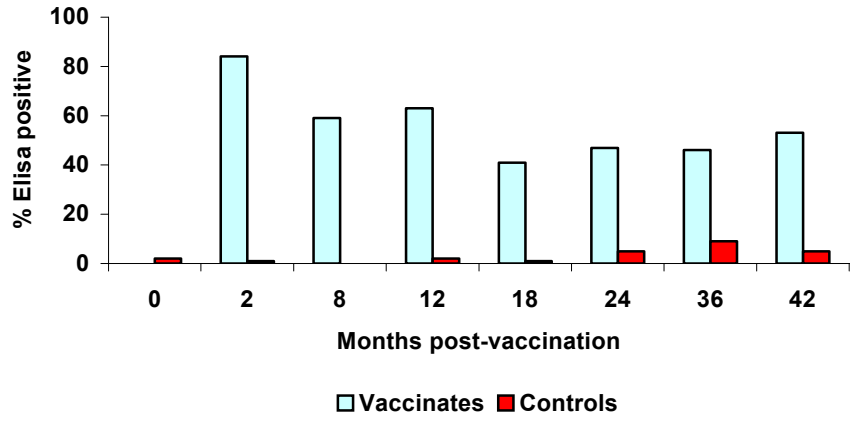


Table 10. Summary results - numbers of sheep positive or negative in the AGID test

		Time of sampling (months post-vaccination)				
		30-36[a]			42-54[b]	
		Controls	Vaccinates		Controls	Vaccinates
Property 1						
	Positive	5	15	*	5	5
	Negative	159	160		125	153
Property 2						
	Positive	21	10		6	10
	Negative	119	158		115	148
Property 3						
	Positive	21	13	**	11	9
	Negative	142	167		107	150

[a] 30 mths pv on P2 and P3, 36 mths pv on P1

[b] 42-43 mths pv on P3, 48 mths pv on P2, 54 mths pv on P1

Proportions for controls and vaccinates significantly different, * (P,0.05), ** (P,0.01)

Association of pv ELISA status with subsequent OJD status (excretion, infection status and OJD-mortality)

Table 11 shows the numbers of vaccinated sheep that were classified as shedders, as infected with *M. a. paratuberculosis* or as having died of OJD, according to their post-vaccination ELISA response. Positive ELISA response was negatively associated with shedding on P2 ($P<0.05$) and P3 ($P<0.01$), negatively associated with infection on P3 ($P<0.05$), and negatively associated with OJD-mortality on P2 ($P<0.05$). Stratified analysis across all farms demonstrated a negative association with shedding ($P<0.0001$), with infection ($P<0.01$), and with OJD-mortality ($P<0.05$).

Thus it appears that pv ELISA response is at least as good as pv IFN-γ response in predicting the subsequent OJD status of vaccinated sheep. Humoral responses are not considered in themselves to be protective (Perez et al. 1997), but in the case of vaccinated animals may be a marker for “vaccination take”.

Table 11. Association of ELISA response[a] post-vaccination with subsequent OJD status

		Number of sheep								
		Shedding status			Infected			OJD Mortality		
	γ-IFN	Positive	Negative		Positive	Negative		Positive	Negative	
Property 1										
	Positive	3	165		9	159		1	167	
	Negative	2	26		4	24		0	28	
Property 2										
	Positive	3	91	*	9	85		0	94	*
	Negative	14	91		17	88		5	100	
Property 3										
	Positive	2	171	**	9	164	*	0	173	
	Negative	4	25		6	23		1	28	

[a] 2 months pv on P2 and P3, 12 mths pv on P1

* Significant difference (P<0.05, Fisher exact test)

** Significant difference (P<0.01, Fisher exact test)

Association of maternal antibodies with the subsequent development of immune responses, and OJD status on Property 2

The effect of detectable maternal antibodies on the subsequent development of ELISA and IFN-γ responses in vaccinated sheep was assessed by comparing the post-vaccinal immunological results for lambs with and without maternal antibody (Table 12). Of the 14 vaccinated lambs that had maternal antibody, 64% were IFN-γ positive at 8 weeks pv compared to 88% of 185 vaccinates without maternal antibodies (P <0.05), indicating that the presence of detectable antibodies was negatively correlated with CMI responsiveness following treatment. A similar, but statistically non significant trend was observed with regard to ELISA results. 29% of lambs with maternal antibody were positive 2 months pv, compared to 49% of 182 lambs without maternal antibody.

To ascertain whether the association of maternal antibodies with reduced post-vaccinal CMI responses is of practical significance we examined the association of detectable maternal antibodies on the subsequent OJD status in both vaccinated and unvaccinated sheep (Table 13). Separate tests of significance were done for vaccinates and for controls, as well as stratified analysis across both groups. No significant effect of maternal antibody on later infection status or OJD-mortality was found in either group. With regard to infection status, for control sheep only, detectable maternal antibody was negatively associated with infected status (P<0.05). One would expect that lambs with maternal antibodies might be exposed to higher numbers of *M. a. paratuberculosis*, in that their mothers may be more likely to be excreting the organism, but the results from this study indicate that such sheep, whether vaccinated or not, are not at greater risk of subsequent OJD. Another possibility was that the detection of maternal antibody may simply be a reflection of the age of the lambs - younger lambs would be more likely to exhibit maternal antibodies. Indeed, lambs with maternal antibody were smaller (mean weight 11.8 kg) than those without (12.4 kg), but the difference was not significant (P=0.4, student's t test).

Table 12. Association of maternal antibodies with post-vaccination immune responses in vaccinated lambs on Property 2.

Maternal antibodies[a]	Number of sheep showing immune response γ-interferon			ELISA	
	Positive	Negative		Positive	Negative
2 months pv					
Positive	9	5	*	4	10
Negative	161	23		90	94

[a] Positve in ELISA at the time of vaccination, 3 months of age

* Significant difference (P<0.05, Fisher exact test)

Table 13. Association of maternal antibodies with subsequent OJD status on Property 2.

Maternal antibodies[a]	Shedding status		Number of sheep Infected			OJD Mortality	
	Positive	Negative	Positive	Negative		Positive	Negative
Vaccinates							
Positive	1	13	4	10		0	14
Negative	16	169	22	163		5	180
Controls							
Positive	3	9	3	9	*	2	10
Negative	85	102	106	81		37	150

[a] Positve in ELISA at the time of vaccination, 3 months of age

* Significant difference (P<0.05, Fisher exact test)

4.6 Vaccination site lesions

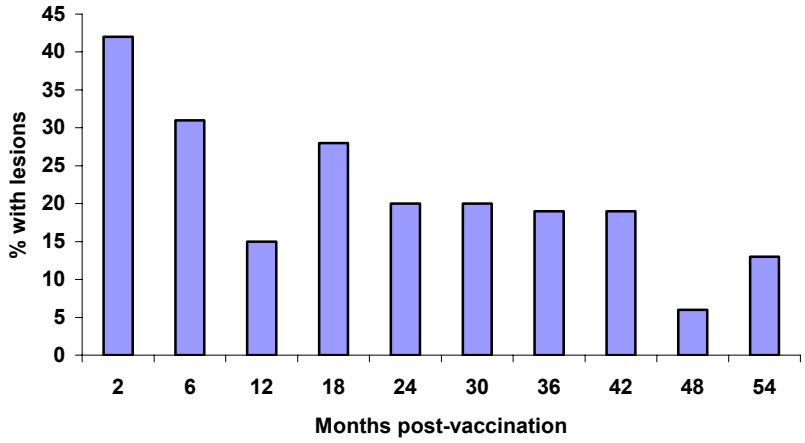
Injection site lesions were not identified in any control sheep, but 42% of vaccinated lambs developed lesions as assessed 2 months post vaccination (Table 14, Figure 9). The incidence of lesions decreased to a minimum 12 months pv and then remained steady at approximately 20% of vaccinates having palpable lesions until 3-4 years of age. The low percentage of palpable lesions at 12 months pv was most likely associated with the sheep carrying lots of wool, so that lesions were harder to detect. They were shorn as hoggets between 12 and 18 months pv, and so at the 18 month pv sampling were freshly shorn, and any lesions were more readily detected. The diameter of lesions ranged from 3 to 45 mm, however the average diameter of palpable lesions did not decline with time. The mean diameter (mm) of all remaining lesions was 18.2, 22.6, 18.4, 21.0, 23.5, and 25.8 at 2, 6. 12, 18, 24 and 30 months pv, respectively.

Table 14. Percentage of vaccinates with palpable lesions at the site of vaccintion

	Months post vaccination									
	2	**6**	**12**	**18**	**24**	**30**	**36**	**42**	**48**	**54**
Property 1	49	39	23	33	23	31	19	25	na	13
Property 2	41	35	9	23	12	14	na	na	6	
Property 3	37	20	14	28	24	15	18	7		
All farms	42	31	15	28	20	20	19	19	6	13

Only small numbers of vaccinated sheep (<9%) developed lesions that ruptured and discharged – 16,11 and 18 sheep on P1, P2 and P3 respectively.

Figure 9. Percentage of vaccinated sheep on all farms with palpable lesions at the site of vaccination



Association of post-vaccinal lesions with subsequent OJD status (excretion, infection status and OJD-mortality)

The presence or absence of local post-vaccinal lesions may be another means of assessing a positive response to vaccination. The presence or absence of a post-vaccinal nodule (2mths pv), was compared to OJD status on each farm (Table 15), and stratified analysis was done across all farms. No association was found with excretion of *M. a. paratuberculosis* nor with OJD-mortality on any farm, nor for stratified analysis across all farms. There was a negative association of post-vaccination lesions with infection status on property 3 only ($P<0.01$). Thus the presence or absence of a pv lesions was of little use to predict efficacy of vaccination in individual sheep. The presence of lesions in a proportion of the flock, however, may be a useful indication that the flock has been vaccinated, for up to 3 years after vaccination.

At both the hogget cull and final slaughter, post-vaccinal lesions did not result in significant losses on the slaughter floor. No extra labour was needed for trimming, and no carcasses were downgraded. More detailed assessment is the subject of a separate study (Monitoring vaccination site lesions at slaughter, OJD.032).

Table 15. Association of post-vaccination (2 mths) lesions with subsequent OJD status

		Number of sheep						
		Shedding status		Infected			OJD Mortality	
	Lesions	Positive	Negative	Positive	Negative		Positive	Negative
Property 1								
	Positive	2	96	7	91		1	97
	Negative	3	99	6	96		1	97
Property 2								
	Positive	6	77	12	71		1	82
	Negative	11	106	14	103		4	113
Property 3								
	Positive	0	75	0	75		0	75
	Negative	6	124	16	114	**	1	129

* Significant difference (P<0.05, Chi-square or Fisher exact test)

** Significant difference (P<0.01, Chi-square or Fisher exact test)

Comparison of IFN-γ, ELISA and post-vaccinal lesions as indicators of immune response

The presence or absence of a post-vaccinal nodule (2mths pv) in vaccinated sheep, and negative or positive IFN-γ and ELISA results (2 mths pv, P2, 3 and 12 mths pv, P1) were compared using the kappa statistic (Table 16). Although all three may be considered as indicators of vaccine response, there was no significant correlation between them, except for IFN-γ and ELISA on P3 (P<0.01).

Table 16. Comparison of IFN, ELISA and pv lesions as indicators of vaccine response

		Actual agreement	Expected	Kappa	SE	Prob(Z)
Property 1						
	Elisa v IFN	0.816	0.77	0.202	0.131	0.061
	Elisa v Lesion	0.551	0.56	-0.021	0.081	0.602
	IFN v Lesion	0.541	0.567	-0.062	0.081	0.774
Property 2						
	Elisa v IFN	0.543	0.553	-0.028	0.079	0.636
	Elisa v Lesion	0.563	0.506	0.115	0.072	0.055
	IFN v Lesion	0.457	0.538	-0.175	0.077	0.989
Property 3						
	Elisa v IFN	0.851	0.768	0.359	0.128	0.003
	Elisa v Lesion	0.455	0.526	-0.149	0.074	0.978
	IFN v Lesion	0.426	0.531	-0.223	0.075	0.999

4.7 Production measurements

Tables 17, 18 and 19 show summary data for liveweight, condition score and fleece characteristics respectively. These data are from surviving sheep at each sampling time, and loss of production due to mortalities has not been included in this analysis.

Liveweight

GLM analysis for liveweight, with weight at the time of vaccination included as a covariate, revealed significantly higher liveweight gain for control sheep compared to vaccinates at 2 ($P<0.5$), 6 ($P<0.5$) and 12 months ($P<0.001$) pv. The magnitude of the differences was small, however (0.22, 0.34 and 0.75 kg at each time period respectively). Samplings beyond 12 mths pv were analysed without vaccination weight as a cofactor, because the sheep had attained adult weight. There were no significant differences between the vaccinates and controls, except at 30 months pv, when the controls were again heavier than the vaccinates ($P<0.05$). The mechanism for the lower weight gains of the growing vaccinated sheep is unclear. It is tempting to suggest that the pv lesions may be painful and/or reduce the animals' enthusiasm for grazing, but when vaccinated sheep with lesions were compared to those without lesions, in a GLM, again with weight at vaccination as covariate, no significant differences in weights at 2, 6 or 12 months pv were found. Presumably a systemic reaction to vaccination, and/or some grossly unobservable local reaction was responsible. There were, not surprisingly, highly significant differences in weight gains between farms, but farm x vaccination interactions were not significant ($P>0.05$), except at 30 months pv ($P<0.01$).

GLM analyses were also performed to compare the liveweights of sheep shown to be infected with those of sheep not detectably infected (again with weight at vaccination as a covariate for weights up to 12 months). This reflects the effect of subclinical OJD at the time of measurement. Infected sheep were significantly lighter, 1-2 kg, ($P<0.05$) at 24, 30 and 42 months pv, but there were no differences at other times.

Table 17. Mean live weights (kg) with standard deviation (below) of experimental animals.

Farm	Treatment	Time (months post vaccination)										
		0	2	6	12	18	24	30	36	42	48	54
1	Control	19.4 3.2	26.2 4.0	27.9 3.5	44.7 4.8	38.7 3.7	48.3 6.2	46.4 4.7	51.3 6.3	39.5 4.2	na	39.6 5.2
	Vaccinate	19.7 3.2	26.0 3.9	28.2 3.3	44.3 4.1	38.4 3.4	48.4 6.6	46.0 5.0	51.3 6.2	39.8 4.4	na	40.6 5.0
2	Control	12.2 3.0	21.7 3.5	32.1 3.4	37.6 3.5	46.5 4.9	50.3 5.2	49.5 5.6	na	na	44.9 6.6	
	Vaccinate	12.5 3.1	21.8 3.6	31.8 3.4	37.4 3.3	46.8 4.8	51.1 4.8	50.0 4.5	na	na	44.9 5.9	
3	Control	17.9 3.8	23.8 4.1	32.1 4.4	36.4 4.4	43.0 5.3	46.5 5.5	45.9 5.9	46.6 5.8	52.9 4.8		
	Vaccinate	17.8 3.8	23.7 4.0	31.7 4.3	35.0 4.2	42.2 5.1	45.4 5.2	43.5 5.2	46.5 5.5	52.2 5.9		

Condition score

Analysis for condition score yielded similar findings to liveweight at 6 and 12 months pv, with controls scoring higher than vaccinates. Again, the magnitude of these differences was very small (0.1). In contrast, at 30 months pv, vaccinates had a higher conditions score than controls ($P<0.05$), and there was significant farm x vaccination interaction.

GLM analyses were also performed to compare the condition scores of sheep shown to be infected with those of sheep not detectably infected. This reflects the effect of subclinical OJD. Infected sheep had significantly lower scores at 18, 24, 30 and 36 months pv ($P<0.01$), and at 42 months pv ($P<0.05$). Again, the magnitude of the differences was small (0.06-0.21 units).

Table 18. Mean condition scores with standard deviation (below) of experimental animals.

Farm	Treatment	2	6	12	18	24	30	36	42	48	54
1	Control	3.2	3	3.9	3	2.6	3.1	3.1	2.4	na	2.5
		0.4	0.3	0.3	0.4	0.6	0.4	0.5	0.5		0.5
	Vaccinate	3.2	2.9	3.9	3	2.7	3.3	3.2	2.4	na	2.5
		0.4	0.4	0.3	0.4	0.7	0.5	0.5	0.5		0.5
2	Control	3.1	3.1	3.5	3.8	3.6	3.4	na	na	2.7	
		0.4	0.4	0.5	0.5	0.5	0.6			0.6	
	Vaccinate	3.1	3	3.4	3.9	3.6	3.5	na	na	2.6	
		0.3	0.4	0.5	0.4	0.5	0.5			0.6	
3	Control	3	3.1	2.9	3.6	3	3	2.9	3.3		
		0.4	0.4	0.5	0.6	0.3	0.5	0.5	0.5		
	Vaccinate	2.9	3	2.9	3.6	2.9	2.9	3	3.3		
		0.4	0.4	0.4	0.5	0.3	0.4	0.5	0.6		

Fleece characteristics

There were no differences between controls and vaccinates in average greasy fleece weight or fibre diameter at the hogget shearing across the three properties, nor on properties 2 and 3 at the 2002 shearing. At the 2003 shearing on farms 1 and 2, when sheep were 3-4 years old, greasy fleece weight for vaccinates was slightly higher (0.123 kg) than controls ($P<0.05$). There were no differences in fibre diameter at any sampling time.

GLM analyses were also performed to compare the fleece measurements of sheep shown to be infected with those of sheep not detectably infected. This reflects the effect of subclinical OJD. There were no significant differences in GFW or fibre diameter at any sampling time.

Table 19. Wool production characteristics (mean, with standard deviation below) of experimental animals.

Farm	Treatment	Shearing 2001		2002		2003	
		GFW	FD	GFW	FD	GFW	FD
1	Control	4.6	18.5			4.5	17.9
		0.6	1.2			0.7	1.5
	Vaccinate	4.7	18.6			4.6	17.9
		0.7	1.2			0.8	1.3
2	Control	4.4	18.9	4.7	17.6	4.3	17.2
		0.6	1.2	0.8	1.3	0.7	1.1
	Vaccinate	4.3	18.9	4.9	17.7	4.4	17.3
		0.6	1.3	0.8	1.2	0.7	1.0
3	Control	3.4	17	4.5	17.2		
		0.5	1.1	0.5	1.1		
	Vaccinate	3.4	17	4.5	17.2		
		0.5	1.1	0.6	1.1		

4.8 General discussion

This trial has been the first detailed, objective and controlled examination of the efficacy of vaccination of sheep against paratuberculosis. In overseas work to date, properly controlled studies have not been done, and it has been difficult to isolate the effects of vaccination from other aspects of control programs. Some of the most useful overseas insights can be gained from the experience in Iceland, where vaccination of sheep was introduced only after more traditional control measures, such as isolation and test and cull, had failed (Fridriksdottir et al. 2000). Vaccination has been an effective control measure and was shown to reduce mortality by 94%, but despite long term mandatory vaccination, OJD infection remains widespread. The parallels with the Australian situation are clear. Many results from the current trial have general relevance also to unvaccinated sheep – rarely have 200 sheep on each of three farms been examined in such detail over so many sampling times.

Despite the rigour of the current trial, there were unavoidable limitations in the design, which must be considered when translating the findings from this trial into management and control recommendations for the wider industry.

Firstly, this trial investigated the effect of vaccination only against a very heavy challenge. All farms were experiencing OJD related mortality, which was readily observed by the farmer, and well above the “reasonable” background mortality expected in fine-wool Merino flocks. Moreover, clinically affected sheep from other mobs, rather than being culled immediately as normally recommended, were added to the trial mobs, further increasing the infectious challenge, especially in the early stages. The reason that the trial was conducted against such a heavy challenge is simple – the difficulty in reliably detecting *M. a. paratuberculosis* infection in low and even in medium prevalence flocks, where few or no OJD mortalities are recognised. Obviously any effect on mortality could not be demonstrated (unless full necropsy was done on every sheep that died, and that is just not feasible). In low prevalence flocks, it would also not be possible in the relatively short time frame of the current trial to determine whether vaccination reduced shedding levels of *M. a. paratuberculosis*, because the detection of

infection by PFC may be intermittent, and only one or a few pools may be positive at any given sampling. Statistically, this is not significantly different from no pools positive (the confidence limits for low levels of prevalence are very wide), and repeated samplings over many years would be necessary to be sure that any observed reduction in excretion was real. Such a trial, over several generations of sheep, is now underway, as a separate project (Monitoring shedding in high, medium and low prevalence flocks. MLA Project number OJD.033)

Secondly, all sheep in the cohort were not vaccinated. The unvaccinated control sheep were run with the vaccinates, and were excreting *M. a. paratuberculosis* by 12 months of age, with peak excretion at 2-3 years of age, thus providing a high and on-going level of challenge. In the real world situation, the whole of a cohort flock would be vaccinated, so that this additional challenge from cohorts would be reduced, and vaccination may be even more effective. Conversely, it is also possible that high environmental challenge may enhance cellular immune responses in vaccinates. In the current trial, the inclusion of the unvaccinated control sheep was essential to assess the effect of vaccination against controls that had had exactly the same *M. a. paratuberculosis* exposure under exactly the same management conditions. The alternative – having some wholly vaccinated and some wholly unvaccinated control flocks – is subject to bias as it is not possible to exactly replicate conditions in different flocks.

Finally, in the current trial we examined only a single cohort of sheep over 4 to 5 years. In the real world situation, a new crop of lambs would be raised and vaccinated each year. The amount of *M. a. paratuberculosis* exposure would be expected to be successively reduced, with a reduction in the opportunity for transmission of the disease to future generations. Any effect of the vaccination on successive generations of lambs over time is not reflected in the results from the main trial in the current project. This was, however, examined in a preliminary fashion in the "extended trial" in the current project. The results indicated a lower prevalence of shedding in third generation vaccinates compared to first generation vaccinates (Appendix 5). This effect will be properly tested in the on-going trial, Project OJD.033.

The above design limitations combine to give a "worst-case scenario" from the perspective of disease challenge, but did assure a high level of disease against which any reduction due to vaccination could readily be measured. Intuitively, one may expect that vaccination will be more effective when other management procedures are used concurrently to reduce *M. a. paratuberculosis* challenge, both in the first and subsequent generations, but this CANNOT be inferred from the current trial. We must await the results of the long term specific studies, and field experience over many years. Indeed, there are a number of potential problems for control programs highlighted by the current trial.

All of the vaccinates that died had severe multibacillary infection, and would have been excreting enormous numbers of *M. a. paratuberculosis* leading up to their death. While there were very few of these sheep (only 7 across the three farms), the contribution of just a single sheep with multibacillary infection to environmental contamination is enormous, and may outweigh many hundreds of sheep with less fulminant disease. If such a breakdown in OJD occurred at a critical time (eg in a ewe in the lambing paddock when pasture was short and many young lambs were beginning to graze) a whole cohort of young susceptible animals may be exposed to high levels of contamination.

Another important observation was that the effect of vaccination on subclinical disease, although significant, was less than the effect on clinical disease. Clinical disease (i.e. mortality) in vaccinates was reduced by about 90%. In the hogget culls, 17% of vaccinates had lesions, compared to 49% of controls - about a 65% reduction in subclinical disease. Among the sheep at final slaughter, the reduction in subclinical disease in the vaccinates was less than 50% (19% of vaccinates with detectable infection or lesions compared to 31% of controls). However, the fact that many of the controls had already died (probably the most susceptible sheep) does

confound the interpretation. In addition, vaccination did not decrease the proportion of subclinically affected sheep that had multibacillary lesions. This contrasts previous overseas reports (Garcia Marin et al. 1997). These findings are probably not a concern on a heavily infected farm viewed in isolation. But they do have relevance where vaccinated sheep are moved to areas of low prevalence for OJD. Because vaccination is less effective in reducing subclinical disease, and because subclinical disease is more difficult to detect, it follows that accurate quantification of the risk of vaccinated sheep to transmit OJD is not possible.

In this trial, vaccination was seen to provide life-long protection. However, because of the on-going high challenge with *M. a. paratuberculosis*, a natural boosting effect may have been operating. Whether vaccinated sheep in a low or zero challenge environment would still be protected if suddenly exposed after several years to high levels of *M. a. paratuberculosis* is unknown.

M. a. paratuberculosis excretion was detected in control sheep by 9 to 11 months of age, a little earlier than generally assumed. This is important information when young sheep are used to prepare low risk pasture for lambs and weaners. Vaccination extended the interval without excretion to 15 months on the worst affected property, and 21 months on the other two farms, thus providing a much larger window of safety for management of pasture contamination.

Considerable data on *M. a. paratuberculosis* excretion, assessed by both PFC and IFC on repeated occasions was generated by this trial. This data was pivotal in the development and validation of the web-based pooled prevalence calculator, a potentially important future management tool.

5 Success in achieving objectives

Gudair™ Vaccine was successful registered in early 2002, and this registration was predicated on interim findings from this trial. An assessment of success of the project must consider the expectations of both the rural and scientific communities, as well as the needs of regulators for sound data on which to base decisions (eg risk of vaccinated sheep to spread OJD). The objectives of OJD.009 as revised in 2002 are listed below with comments on their achievement:

1) The principal objective is to determine if vaccination with Gudair™ Vaccine can reduce the incidence of mortality in merino sheep due to Ovine Johne's Disease (OJD) under Australian conditions.

Clearly this objective was fully met. By project end there had been only seven confirmed OJD mortalities in vaccinates compared to 80 from the controls (a reduction of about 90%), and the mortalities in the vaccinated sheep were delayed by at least 12 months. Vaccination reduced mortalities over the productive life of the sheep and did not simply delay the onset of mortalities.

2) To secondarily assess, quantitatively via faecal culture, any reduction in bacterial shedding of *M. a. paratuberculosis* that may occur in vaccinated sheep in comparison with non-vaccinated controls.

Again, the project has answered this question. The results from farms P1 and P3 clearly demonstrated a delay and reduction in faecal excretion of *M. a. paratuberculosis*, with maintenance of the protection for the economic life of the sheep. On these farms, there was no detectable excretion of *M. a. paratuberculosis* by the vaccinated groups until 18 months pv (about 21 months of age), compared to 6 or 8 months pv (9 -11 months of age) in the controls. The reduction in prevalence of excreters averaged about 90%. The results for P2 – this was the property with the highest prevalence of infection – differed slightly, but only in degree, and mainly as the sheep aged. Excretion of *M. a. paratuberculosis* by vaccinates was delayed, but only by about 4 months. Results at 12-36 months pv were similar to the other two farms, with reduction in prevalence in the vaccinates of about 90%. However, at 42 and 48 mths pv there was little difference in prevalence between the vaccinated and control groups, but the control group was by this time much smaller due to the large number of OJD mortalities. Moreover, the peak prevalence for excreters over the life of the sheep was very much lower, and delayed, in the vaccinated group, and at the final sampling was lower than at 42 months, indicating maintenance of protection.

3) To investigate the cellular and humoral immune response following vaccination with Gudair™ Vaccine, and to attempt to correlate this with any reduction in OJD mortality or in faecal *M. a. paratuberculosis* shedding.

This objective was also met. Development of immune responses was demonstrated following vaccination, and protection in terms of reduced mortality and reduced shedding was shown. Both IFN-γ and ELISA responses post-vaccination were negatively correlated with subsequent faecal shedding, mortality and subclinical disease.

4) To assess the field safety of Gudair™ Vaccine in vaccinated flocks.

There was no indication of any short term adverse clinical effect of vaccination. Vaccination site lesions were persistent, but mainly due to the local abattoir practices are not currently a major concern to processors. More detailed assessment is the subject of a separate study (Monitoring vaccination site lesions at slaughter, OJD.032). The current project has been of particular importance as an education tool, in emphasising the importance of correct vaccination

technique to limit the impact of an inherently irritant adjuvanted vaccine product.

5) To determine changes in productivity parameters associated with either vaccination or subclinical disease.

Within the limits of project design, this objective was achieved. Production analyses were conducted on surviving sheep at each sampling time. Thus the findings for vaccinates vs controls do not include prior losses due to OJD mortality, and the findings with regard to infection status are for subclinically infected sheep. A small, but consistent and statistically significant reduction in the weight gains of vaccinates over the first 12 months pv was seen. Among adult sheep, there were contradictory findings with vaccinates at 30 mths pv having higher condition scores but lower liveweight than controls. Vaccinates cut slightly more wool than controls at the 2003 shearing. With regard to OJD infection status, subclinically infected sheep were lighter and had lower condition scores than uninfected sheep between 18 and 42 months pv. No differences were detected in growing sheep, nor in the fleece parameters at any time. The project did not examine reproductive performance.

6 Impact on Meat and Livestock industry

Interim results from this project were keenly sought by industry, producers and policy makers, and have been presented in many fora throughout the trial (see Appendices 1 and 2). Ongoing, informal, one-on-one advice was provided on a continuing basis.

Data from this trial enabled the registration of Gudair™ in Australia in 2002, and the vaccine now plays a pivotal role in the management of OJD in Australia.

The findings from this project were critically important in the development of the Assurance Based Credits (ABC) scheme, developed for use when many regulatory controls were removed in 2004.

Prior to vaccination, OJD in Australia was a politically sensitive and emotive issue. Economic losses for individual producers flowed not just from the direct effects of the disease, but from the regulatory controls and the stigma attached to a positive diagnosis. The expense, low sensitivity (especially in the early stages) and prolonged time for results of existing diagnostic tests for OJD further compounded the problem, and control programs were always 12 months behind the disease. A sense of doom and uncertainty prevailed. The inclusion of vaccination in control and management programs for OJD has had an enormous positive influence. Producers now feel that there is something they can do about the problem, and OJD has become just another disease and management issue.

7 Conclusions and recommendations

7.1 Conclusions

In flocks with a high prevalence of OJD, vaccination with Gudair™ stimulated specific immune responses and significantly reduced and delayed both OJD-related mortality and the excretion of *M. a. paratuberculosis.* Protection was maintained for the economic life of the sheep.

Thus vaccination will provide immediate benefit (within one generation of sheep) to producers who are experiencing significant OJD-related mortality.

The delay in excretion of *M. a. paratuberculosis* by vaccinates provides producers with a larger window of safety for management of pasture contamination. This and the reduction in excretion levels will reduce environmental contamination with *M. a. paratuberculosis*, with expected benefits in subsequent generations of sheep.

Ignoring the considerable OJD-related mortality, there were minimal effects of either vaccination or subclinical OJD on production parameters. Vaccination was associated with a slight reduction in weight gain in young sheep, but with a small increase in greasy fleece weight in older sheep. Subclinical OJD was associated with reduced liveweight and condition score in adult sheep.

This trial examined sheep in only one generation in only high prevalence flocks, and unvaccinated control sheep were present. This was necessary to facilitate measurement of the effects of vaccination. In the real world, whole cohorts of lambs are vaccinated over successive years in flocks of low, medium or high prevalence, and benefits of vaccination may accrue over many years. To assure the best use of vaccination in control programs, it is essential that on-going investigations into the efficacy of vaccination under these more typical farm conditions are continued.

This project was a “worst-case scenario” from the perspective of disease challenge. Intuitively, one may expect that vaccination will be more effective when other management procedures are used concurrently to reduce *M. a. paratuberculosis* challenge, but this CANNOT be inferred from the current trial. We must await the results of the long term specific studies, and field experience over many years.

High levels of excretion by vaccinates on some occasions, multibacillary disease in the few vaccinates that died from OJD, and persistence of subclinical OJD in a proportion of vaccinates indicate risk that some vaccinated sheep could transfer the disease. This has implications for translocation of vaccinated sheep to very low prevalence areas, and implies that sustained vaccine use will be necessary to avoid recrudescence in heavily infected flocks.

A figure of 90% efficacy for Gudair for control of OJD is reasonable. Mortalities, prevalence of sheep excreting *M. a. paratuberculosis* and levels of excretion are all reduced by vaccination. This figure indicates that the estimates used in the modelling work to date were reasonable, even conservative, and underscores the incorporation of vaccination into assurance programs.

7.2 Specific recommendations

1. Continue on-going research into efficacy of vaccination over several generations of sheep on farms of varying OJD prevalence.

2. Consider research into the duration of immunity in low challenge situations.

3. Maintain awareness of the limitations of vaccination.

4. Because of the limitations of vaccination, improved diagnostic tools for subclinical OJD will be necessary if control programs are to reduce the prevalence of the disease.

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Ian Lugton (NSW DPI) was responsible for initial design and establishment of the trial in 1999. Subsequently, Peter Windsor (NSW DPI) took over as Principal Investigator and the field aspects of the trial were subcontracted out to the Bathurst Rural lands Protection Board under the control of Jeff Eppleston. At this time, the trial was also reassessed in great detail, and modified to better reflect the needs of vaccine registration and provide data on individual animal responses. CSL undertook to perform the lab-based ELISA and stage 2 of the IFN-γ testing. Stephen Jones and Ivan Jasenko from CSL were responsible for the immunological assay processing. Faecal cultures were done by the dedicated team of technical staff in the Microbiology research laboratory at EMAI under the direction of Richard Whittington (NSW DPI). In May 2002, following the move to Sydney University by both Peter Windsor and Richard Whittington, Leslie Reddacliff (NSW DPI) took over as Principal Investigator, supported by a management team comprising also Jeff Eppleston, Peter Windsor and Richard Whittington. Kevin Thornberry (NSW DPI at Orange) assisted during sample collection, and the Orange Agricultural Institute Laboratory team performed initial sample processing for the IFN-γ testing. Histopathology and AGID testing was done initially at the Orange laboratory, and later at EMAI.

The contributions of all the above, and others not specifically mentioned, are reflected in the considerable presentations and publications from this project to date (Appendices 1 -5).

10 Appendices

Appendix 1. Public and other presentations of interim results from this project

MLA fora: Ovine Johne's Disease, An update of Australian and International Research, Sydney University, July 2002; Ovine Johne's Disease, An update of Australian research, North Sydney, March 2004

Vaccine Producer Field Days: Interim results of this project were presented to several hundreds of interested producers at a series of Field Days on OJD control held by RLPB's throughout the Management Area from 2002 through 2004 - Bigga, Brewarinna, Cowra, Forbes (2 occasions), Goodooga, Hill End, Lithgow, Lyndhurst, Palmer's Oakey, Wagga, Wallenbeen, Walmeringle, Woodstock.

Successive annual conferences of the Australian Veterinary Association from 2001 through 2004, in Melbourne, Adelaide, Cairns, and Canberra.

Australian Society for Microbiology Annual Conference, Melbourne, 2002

ISVEE10 Conference, November 2003. Viña del Mar, Chile.

Seventh International Colloquium on Paratuberculosis, June 2002, Bilbao, Spain

Eighth International Colloquium on Paratuberculosis, August 2005, Copenhagen, Denmark (planned).

Appendix 2. Publications arising from this project

Information from this project has been keenly sought from industry in real time. The interim results of the project were pivotal for the registration of Gudair™ in 2002, and in the development of regulatory policy since that time. It was considered also important to detail these interim findings formally, and many scientific articles, often as conference proceedings, have been written. A final publication in the peer reviewed literature, which will bring together all the major results over the 5 years of this trial is planned. Scientific publications to date, that have arisen directly or indirectly from this project, are listed below in reverse chronological order. Where an abstract was available, it has been included.

- Toribio J-ALML, Sergeant ESG. Estimation of prevalence of Johne's disease in sheep from the culturing of pooled faecal samples. *Australian Veterinary Journal.* 2005. submitted.

- Eppleston J, Reddacliff L, Links I. Preliminary observations on the prevalence of sheep shedding *Mycobacterium avium* subsp. *paratuberculosis* after 3 years of a vaccination program for ovine Johne's disease (OJD). *Australian Veterinary Journal.* 2005. submitted

- Windsor PA, Bush R, Woodward K, Eppleston J. Accidental self-inoculation with Gudair™ vaccine for the control ovine paratuberculosis. *Australian Veterinary Journal.* 2005. in press.

  Objective: To document the occurrence of accidental self-inoculation by producers using the recently registered Gudair™ vaccine for the control of ovine paratuberculosis in Australia.
  Design: A survey of producers and case reports of 3 incidents requiring medical assistance.
  Procedure: A survey of the first 50 producers permitted to use the vaccine in sheep and a description of 3 case histories of accidental self-inoculation where medical attention was sought.
  Results: The survey estimated that of 37 producers vaccinating 155,523 sheep, there was an exposure rate to the vaccine of 1/7406 and needle stick rate of 1/9720 doses used respectively. There were no reports of self-inoculation in this survey, however at least 4 cases have been reported that required medical intervention, 3 in males and one involving a female. Three cases involved injection of whole or part of a 1ml dose of vaccine into the leg, with a single case involving the hand. Several of these cases progressed in severity of the local chronic inflammation to require surgery to remove the vaccine product. One case required extensive surgery due to apparent movement of the vaccine up to 15cm within muscle tissue and formation of multiple fistulating ulcers. Even with surgery, recovery periods as long as a year following severe self-inoculation may be expected.
  Conclusions: Gudair™ vaccine is a valuable tool in the control of ovine paratuberculosis in Australia however the mineral used in the vaccine formulation as an adjuvant can cause prolonged granulomatous inflammation if inadvertently injected into human tissue. In some cases, surgical debridement of the inflamed tissue and mineral oil prior to progression to necrosis is advised.

- Eppleston J, Reddacliff L, Windsor P, Whittington R, Jones S. Field studies on vaccination for the control of OJD in Australia: an overview. *Proceedings of the Australian Sheep Veterinary Society 2004 – Canberra Conference*, Volume 14, 56-59.

  The most recent results from the Intensive Vaccination field trial 009 are presented.

These data continue to indicate that vaccination can reduce mortalities and shedding by approximately 90% and that vaccination will offer industry a valuable control tool in its fight against OJD. However this trial has also identified 2 potentially negative outcomes: i) that shedding continues at a level that is likely to be infective, and ii) that persistent vaccination site lesions occur in around 25% of vaccinates. Two additional field trials designed to monitor the rate of reduction in shedding in vaccinating flocks with different initial disease prevalence, and the prevalence and economic impact of vaccination site lesions are described. Together these field trials will supply industry with further information on the full impact of controlling OJD by vaccination.

- Reddacliff LA, Eppleston J. Field evaluation of OJD control using Gudair™. Ovine Johne's Disease, *An update of Australian research. Meat and Livestock Australia, March 2004*, 7-11.

- Toribio J-ALML, Reddacliff L, Eppleston J, Windsor P, Whittington R. Epidemiological considerations on the use of pooled faecal culture in an on-farm trial of the Gudair® vaccine in Australia. *ISVEE10 Conference, November 2003*. Viña del Mar, Chile.

  Pooled faecal culture (PFC), in an on-farm trial to assess the efficacy of Gudair® vaccine for ovine Johne's disease in Australia, is being used to provide reliable measures of the prevalence of faecal shedders over 4 years in a cost-effective manner. Reasons for variation between prevalence estimates from PFC and individual faecal culture performed on positive pools and the need for this work to be extended to a Bayesian approach at project end are discussed.

- Eppleston J, Reddacliff L, Windsor P, Whittington R and Jones S. An update on the efficacy of GudairTM OJD Vaccine in Australia. *Proceedings of the Australian Sheep Veterinary Society 2003 – Cairns Conference*, Volume 13, 107-113

  This paper will present the latest information on the impact of vaccination against OJD on immunity, faecal shedding of *M.ptb*, mortality rate, animal productivity, and vaccine injection site lesions. On each of three properties experiencing significant OJD losses (5 to 15% per annum), 200 Merino lambs (age 2-3 months) were vaccinated with Gudair™, and 200 lambs were sham vaccinated with saline. Results to date indicate that vaccination significantly delays and reduces, but does not prevent, faecal shedding and deaths attributable to OJD. No significant differences have been observed in wool productivity of surviving sheep. Vaccine injection site lesions were detected in almost 50% of sheep at 2 months pv, reducing to less than 25% by 2 years pv. The vaccine stimulates both CMI and ELISA responses in a high proportion of vaccinated lambs, which tends to decline over time, accompanied by a significant increase in the proportion of unvaccinated animals with positive immune reactions. The trial continues to indicate that vaccination will be an important tool for the control of OJD in Australia.

- Windsor P, Eppleston J, Sergeant E, Reddacliff L, McGregor H, Bush R, Toribio J and Whittington R. Monitoring the efficacy of Gudair™ OJD vaccine in Australia. *Proceedings of the Australian Sheep Veterinary Society 2003 – Cairns Conference*, Volume 13, 114-120

- Eppleston J. An Australian evaluation of Gudair™ OJD vaccine. *Ovine Johne's Disease, An update of Australian and International Research, Meat and Livestock Australia, July 2002,* 68-70.

- Reddacliff L, Windsor P, Whittington R, Eppleston J, Jones S and Britton A. Vaccination for ovine Johne's disease in Australia. *Australian Society for Microbiology Annual Conference, Melbourne, 2002*, Abstract

  Ovine Johne's disease (OJD) is a significant cause of mortality of adult sheep in parts of Australia and vaccination with Gudair™, a killed *Mycobacterium avium* subsp. *paratuberculosis* preparation, is being investigated as a disease control tool. This paper presents preliminary data on the impact of vaccination on faecal shedding of *M. paratuberculosis*, mortality rate, lamb growth, condition score and wool productivity, vaccine injection site lesions and cellular (BOVIGAM™) and humoral (PARACHEK™) immunity. On each of three properties in New South Wales experiencing significant OJD losses (5 to 15% per annum), 200 Merino lambs (age 1-4 months) were vaccinated with Gudair™, and 200 lambs were sham vaccinated with saline. Animal assessments and sample collections are being conducted twice yearly. Results to date indicate that Gudair™ significantly delays faecal shedding for the first year post-vaccination (pv) and when shedding commences, it is at a significantly lower rate than in unvaccinated animals. There has been just one OJD attributable death in vaccinated sheep compared to 19 confirmed OJD mortalities in control sheep. No significant differences have been noted in live weight, condition score and wool productivity of surviving sheep. Vaccine injection site lesions were detected in almost 50% of sheep at 2 months pv, reducing to 10-30% by 2 years pv. The vaccine stimulates both CMI and ELISA responses in a high proportion of vaccinated lambs which tends to decline over time, accompanied by a significant increase in the proportion of unvaccinated animals with positive immune reactions, presumably reflecting an increasing prevalence of OJD in this group. This data has enabled registration of Gudair™ in Australia and it is expected that the vaccine will have an important role in OJD control in Australia.

- Eppleston J, Windsor P, Whittington R, Britton A, Jones S. Australian trial to evaluate the efficacy of Gudair™ OJD Vaccine. *Proceedings of the Australian Sheep Veterinary Society 2002 – Adelaide Conference*, Volume 12, 21-26.

  This paper presents the latest results from an ongoing evaluation trial of the Gudair™ OJD vaccine. At two years into the trial, vaccination of lambs has delayed and reduced mortalities due to OJD. Similarly a delay and reduction in shedding of *Mycobacterium paratuberculosis* by vaccinates continues to be observed although some bacteria have now been detected in the faecal pools from several groups of vaccinated animals. Immunological data indicate significant stimulation of the immune systems in the majority of vaccinates, however, the persistence of these responses is variable. The incidence of vaccination injection site lesions has decreased from initial levels to around 20% of vaccinates retaining lesions 18 – 24 months after vaccination. No difference between control and vaccinated sheep has been detected in the productivity traits of growth, body condition, greasy fleece weight or fibre diameter. The field trial to date has supported the Gudair™ vaccine registration claims for the control of paratuberculosis and reduction of faecal M ptb shedding in sheep.

- Windsor P, Whittington R, Eppleston J, Jones S, Britton A. Efficacy of a killed *Mycobacterium paratuberculosis* vaccine for the control of OJD in Australian sheep flocks. *Proceedings of the Seventh International Colloquium on Paratuberculosis, June 2002, Bilbao, Spain*

  Ovine Johne's disease (OJD) is a significant cause of mortality of adult sheep in some parts of Australia and vaccination for sheep with OJD with Gudair™,

a killed *Mycobacterium paratuberculosis* preparation, is being investigated as a disease control tool. This paper presents preliminary data on the impact of vaccination on faecal shedding of *M. paratuberculosis* (as assessed by pooled and individual faecal culture), mortality rate, lamb growth, condition score and wool productivity, vaccine injection site lesions and cellular (BOVIGAM™) and humoral (PARACHEK™) immunity. On each of three properties New South Wales experiencing significant OJD losses (5 to 15% per annum), 200 Merino lambs (age 1-4 months) were vaccinated with Gudair™, and 200 lambs were sham vaccinated with saline (1 property in December 1999 and 2 in June 2000). Animal assessments and sample collections are being conducted twice yearly. Data to date indicates that Gudair™ significantly delays faecal shedding for the first year post-vaccination (p.v.) and when shedding commences, it is at a significantly lower rate than unvaccinated animals. There have been no OJD attributable deaths in vaccinated sheep compared to 19 confirmed OJD mortalities in control sheep. No significant differences have been noted in live weight, condition score and wool productivity. Vaccine injection site lesions were detected in almost 50% of sheep at 2 months p.v., reducing to 10-30% by 2 years p.v. The vaccine stimulates both CMI and ELISA responses in a high proportion of vaccinated lambs which tends to decline over time, accompanied by a significant increase in the proportion of unvaccinated animals with positive immune reactions, presumably reflecting an increasing prevalence of OJD in this group. This data has been used to seek registration of Gudair™ in Australia and it is expected that the vaccine will have an important role in OJD control in Australia.

- Eppleston J, Britton A, Windsor P, Hall D, Whittington R, Jones S. Progress in a field trial to determine the effectiveness of a killed *Mycobacterium paratuberculosis* vaccine for the control of OJD in Australian sheep flocks. *Proceedings of the Australian Sheep Veterinary Society 2001 – Melbourne Conference*, Volume 11, 64-67.

  The effectiveness of vaccination with Gudair™, a killed Mycobacterial preparation, for the control of OJD is being investigated in Australia as part of the National OJD Program. Three properties in the Central Tablelands of NSW that have been experiencing significant losses from OJD have each vaccinated 200 Merino lambs with GudairTM at approximately 2 months of age. In addition another 200 lambs on each property were sham vaccinated with saline and will act as controls. Information on the impact of vaccination on the immune response, *Mycobacterium paratuberculosis* bacterial shedding, mortality and injection site lesions is being collected at regular intervals and growth and wool production will be recorded and assessed. Experimental animals were vaccinated on one property in December 1999 and on the other 2 properties in June 2000. Preliminary data on immunity, faecal bacterial shedding, lamb growth and injection site lesions are presented.

Appendix 3. AGID response post-vaccination – report on small supplementary trial

Background: There was a need from industry for a "quick and dirty" way to check the vaccination of flocks. Use of the AGID was suggested, since it is in routine use in the NSW labs, and results for the ELISA from the intensive vaccination trial (OJD-009) indicated high rates of seroconversions which persisted for years. Thus a small sample of a flock could be tested, and most would be expected to be positive if the flock was properly vaccinated. But the AGID had not been used on vaccinates, (except on some clinically ill with OJD from 009 which were culled – most were AGID positive) so a quick confirmation was sought. Field aspects of this study were organised by Marilyn Evers (NSW DPI, Young) with veterinary staff from the Young Rural Lands Protection Board.

Method: Samples were collected for AGID from 50 approved vaccinated sheep from each of 8 flocks, where the Young RLPB vets had full confidence in the vaccination history of the sheep. Two flocks each were 2mths, 6 mths, 12 months and 24 months post-vaccination (PV). We intended that one each of the flocks for each age would be a high and low prevalence flock respectively, but in practice, this was not possible, and only one flock had any observed mortalities due to OJD. Blood samples from these flocks were also sent to CSL, Parkville, where the Parachek ELISA was done by Stephen Jones. See excel file for details.

Results

		Serology results			OJD history	
Age	Mths pv	% AGID +ve	% ELISA +ve	PV lesions of 50	Pools +ve	Probable level of infection
36	24	2	50	40	na	low
36	24	4	82	33	na	high, owner suspects that he was losing up to 10% to
12	12	4	92	46	1/7	low
15	12	0	88	37	5/7	moderate (possibly low at time of vaccination)
8	6	0	92	25	5/7	moderate
8	6	10	92	18		
4	2	10	42	38		
6	2	10	90	6		

In summary, very few sheep were gel-test-positive post-vaccination. Highest rates of seroconversion in the AGID (but still only a low 10%) were seen in both 2 mth flocks and one 6mth flock. All the rest had $\leq$ 4% positive. In contrast, seropositivity rates in the ELISA ranged from 42 to 92%.

Discussion

It appears that the AGID behaves very differently from the ELISA after vaccination.

AGID will not be useful to confirm vaccination status of flocks, and the ELISA results from this trial confirm the 009 findings of high seroconversion rates after vaccination when measured by ELISA. The Parachek ELISA would be useful for confirmation of flock vaccination status, but note that this test is not used routinely in our DPI laboratories.

Also it seems that vaccination may not preclude most sheep from export where negative AGID results are required. (Note, however, that there was a small rate of seroconversion in the AGID post-vaccination, so if the testing requirement was that all sheep in the flock were serologically negative, vaccination could preclude export.)

Appendix 4. Use of data from OJD-009 in other projects

Use of PFC and IFC data from OJD-009 in the development of better methods for the estimation of prevalence from PFC results:

This project was undertaken as a component of a project under the Australian Biosecurity CRC for Emerging Infectious Disease. The main project (undertaken by AusVet Animal Health Services) was to develop an internet-based calculator to implement methods for estimating animal-level prevalence from pooled samples. Because both MLA and the University of Sydney are also participants in the CRC, data from the vaccine trial (OJD.009) was used for a small project to demonstrate and evaluate the methods implemented in the pooled prevalence calculator.

A scientific article has been submitted for publication (Toribio J-ALML, Sergeant ESG. Estimation of prevalence of Johne's disease in sheep from the culturing of pooled faecal samples. *Australian Veterinary Journal.* 2005). The abstract as submitted follows:

> *Objective* To develop a user-friendly computer program for estimation of animal-level prevalence from pooled testing and to compare prevalence estimates and confidence intervals between methods and with prevalence estimates based on individual culture.
> *Procedure* Seven methods for estimating animal-level prevalence were identified from the scientific literature and implemented using the R® statistical environment, including methods for imperfect test sensitivity and specificity and for variable pool size. A Bayesian method, which allows for imperfect tests and incorporation of prior knowledge about test performance and prevalence was also included. These methods were then used to analyse pooled sampling data from a field trial of a killed vaccine for the control of OJD, undertaken on three farms in New South Wales. Testing results for one observation per farm at approximately 30 months post-vaccination were analysed and results compared between methods and with the results of IFC.
> *Results* Prevalence estimates for methods assuming a perfect test were close to the IFC estimate, whereas for methods assuming an imperfect test estimated prevalence was generally higher than the IFC estimate, although the IFC estimate was within the 95% confidence limit on all occasions. For some methods, lower asymptotic confidence limits were negative when prevalence was close to zero, while frequentist methods assuming imperfect tests could not be applied in higher-prevalence situations, where the number of positive pools was not consistent with the assumed sensitivity of the test. Bayesian methods produced biased estimates in several cases, where the prior estimate of prevalence was not consistent with the observed prevalence and resulted in estimates substantially higher than the IFC estimate.
> *Conclusion* Despite the limitations of some methods, two methods provided accurate and reasonable estimates of true prevalence (as measured by IFC) in all instances, and are appropriate for further analysis of the vaccine trial data. One of these methods also has the advantage of allowing for variable pool size, as was the case in this trial. However, further research is needed to develop methods that will account for variation in pool size and test sensitivity and specificity as well as to determine suitable approaches for identifying appropriate prior distributions for prevalence.

The pooled prevalence calculator can be accessed at http://www.ausvet.com.au/pprev/.

Studies into host genetics of immune responses to M. a. paratuberculosis

Cell pellets left over from blood sampling have been sent to Massey University in New Zealand, where PhD student Rao Dukkipati under Alan Murray will use them for studies into the genetics of OJD immunology/resistance in sheep. This work builds on preliminary studies done by LR in her PhD.

Appendix 5. Extensive trial – preliminary findings

When wider use of Gudair™ was available in 2002, formal data collection for the "extensive trial" was abandoned. However, PFC data on seven properties was collected from 2 year-old sheep in the first and third years of vaccination on these farms, to provide a preliminary assessment of the effect of vaccination over several generations in a real world situation. This work was co-funded from the current project, and by DPI.

The preliminary findings have been summarized in a short communication for the peer reviewed literature. (Eppleston J, Reddacliff L, Links I. Preliminary observations on the prevalence of sheep shedding *Mycobacterium avium* subsp. *paratuberculosis* after 3 years of a vaccination program for ovine Johne's disease (OJD). *Australian Veterinary Journal.* 2005) The paper as submitted is reproduced below.

A long-term prospective trial (Monitoring shedding in high medium, and low prevalence flocks. MLA Project number OJD.033) has begun to monitor the rate of reduction in shedding from successive generations of vaccinated sheep in flocks with varying levels of infection, but these results will not be available for several years.

SHORT CONTRIBUTION

Preliminary observations on the prevalence of sheep shedding *Mycobacterium avium* subsp. *paratuberculosis* after 3 years of a vaccination program for ovine Johne's disease (OJD)

J EPPLESTON, L REDDACLIFF, I LINKS, P WINDSOR, R WHITTINGTON

Vaccination against Ovine Paratuberculosis with Gudair™, a killed *M avian* subsp *paratuberculosis* (*Map*) vaccine, has been shown to reduce the incidence of OJD-related mortalities and the prevalence of sheep shedding *Map* in heavily infected Merino flocks by around 90% [1]. Despite this reported reduction in prevalence, the total output of *Map* from the vaccinated groups was sometimes high, probably because of the very high excretion levels from particular sheep. The studies were conducted in single cohorts of vaccinates in very high prevalence flocks and no attempt was made to monitor the effect of vaccination on successive crops of lambs. The high levels of shedding in some vaccinates could result in further pasture contamination and reinfection of subsequent generations.

Recently the Australian sheep industry has embarked on an OJD control program based on risk based trading and the widespread use of vaccination in at-risk flocks and it is important to confirm a continued reduction in disease prevalence as vaccination control programs progress. A long-term prospective trial has begun to monitor the rate of reduction in shedding from successive generations of vaccinated sheep in flocks with varying levels of infection[1], but these results will not be available for several years. This paper reports preliminary evidence from 7 industry flocks of a lower prevalence of shedding in two year-old sheep vaccinated as lambs after three years of a recommended vaccination program, compared to two year old sheep vaccinated as lambs in the first year of the vaccination program.

Seven self-replacing merino flocks located in the central tablelands of NSW, that had been previously diagnosed with OJD and were experiencing OJD-mortality rates in excess of 5% per annum were selected for the investigation. These flocks commenced a vaccination control program when Gudair™ first became available in Australia under an NRA permit in early 2000.

In the first year, spring born 1999-drop lambs were vaccinated at an average age of 16 weeks (range 10 to 20 weeks), and 1998-drop hogget sheep were vaccinated at around 16 months of age, in an attempt to curtail the high level of mortalities being experienced in these flocks. These 7 flocks continued to vaccinate lambs annually at or soon after marking. The 2001-drop lambs were vaccinated at an average age of 11 weeks (range 8 to 12 weeks). All vaccinations were subcutaneous, high on the neck with 1 ml of product using the manufacturer's recommended procedures.

Faecal samples were collected per rectum in pools of 50 sheep from the 1999-drop and 2001-drop sheep when they were approximately 2 years of age. The faecal pools were cultured radiometrically as previously described. [4] Growth in Bactec medium was confirmed as *M. a. paratuberculosis* by PCR and REA, and by subculture onto solid media. [5] The approximate number (log_{10}) of *M. a. paratuberculosis* per gram of faeces in each infected pool was determined from the growth indices in the Bactec cultures.[2].

The raw data on the proportion of pools culture positive were analysed by binary logistic regression while the data on the mean excretion/g in each pool were analysed using a quasi-poisson model. Individual flock, year of drop and their interaction were included in both analyses.

Results are shown in Table 1. The prevalence of *Map* excretion at 2 years of age as assessed by the proportion of culture positive pools varied between flocks but was significantly lower in the 2001-drop compared to the 1999-drop vaccinates (26.4 ± 5.9% vs 58.8 ± 6.2%, respectively; $P < 0.01$). The amount of *M. a. paratuberculosis* excreted was also lower in the 2001-drop ($P < 0.01$).

The prevalence of OJD infection and the amount of excretion can be considered a consequence of the degree of exposure to *Map,* particularly exposure at an early age, and the immune status of the sheep at the time of exposure. In this study, environmental challenge during the early life of the 2001-drop cohort sheep was likely to be less than for the 1999-drop cohort. In 1999 no sheep had been vaccinated and *Map* excretion would have likely been near peak levels for each flock. In 2001 however the level of excretion from the flock and hence environmental contamination should have been reduced, as 3 drops (1998 to 2000 drops) had been vaccinated, although up to 3 drops (1995 to 1997) of unvaccinated sheep remained. While there may have been little benefit from vaccinating the 1998-drop as hoggets, excretion from the 1999 and 2000 drop treated as lambs would be expected to be significantly lower. In addition most flocks had preferentially culled older age groups in favour of retaining a greater proportion of younger vaccinated sheep.

Unfortunately, because of the timing in Gudair™ becoming available to the industry in early 2000 the 1999-drop animals were on average 5 weeks older when vaccinated than the 2001-drop lambs. Hence vaccination at an earlier age could also have contributed to reduced prevaccination exposure to *M. a. paratuberculosis* in the 2001-drop lambs compared with the 1999-drop lambs. Because the confounding effect of age of vaccination could not be eliminated in the analysis, the findings from this preliminary investigation should be interpreted cautiously pending the results from a more comprehensive study in several years time. [1] The current study indicates a fall in the prevalence of shedding of around 50% after 3 years of a vaccination control program. These results support recent modelling work [3]suggesting that the prevalence of OJD should fall rapidly after the commencement of a vaccination control program in heavily infected flocks and provides encouraging preliminary support for the current industry policy of advising vaccination as an important tool in reducing the OJD-risk of traded sheep.

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Tables

Table 1. Proportion of pools positive and mean excretion of M a paratuberculosis at 2 years of age from the 1999 and 2001-drop sheep vaccinated as lambs in 7 infected merino flocks

	1999 drop				2001 drop			
Flock	Pools sampled	Pools +ve	% pools +ve	Mean excretion/g[a] (log_{10})	Pools sampled	Pools +ve	% pools +ve	Mean excretion/g (log_{10})
1	6	6	100	4.4	10	8	80	4.4
2	7	5	71	3.7	8	3	38	3.3
3	7	5	71	4.1	8	1	13	5
4	7	3	43	3.3	7	0	0	0
5	5	2	40	1.3	4	0	0	0
6	7	2	29	3.1	8	3	38	2.5
7	7	2	29	2.1	7	1	14	0.3

[a] (mean excretion/g of all positive pools) x (proportion of pools positive)