final report

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Johne's Disease Harvest Year 2011

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Introduction

It gives Meat and Livestock Australia great pleasure to present you with a copy of our second 'Harvest Year' publication. Since the last document, published in December 2005, MLA has invested heavily in research into Johne's disease in Australia and has committed to a further 5 years of funding for ongoing projects. We recognise that Johne's disease is a difficult disease to work with and that many of the requisite tools and basic understanding of the disease have been missing. We have had a sustained relationship with the Sydney University team and other Australian researchers and fostered research to address these deficiencies. Following is a summary of the excellent research funded by Australian livestock producers and the Federal government, conducted on behalf of the red meat industry and Australian consumers of livestock products.

Jim Rothwell,
Program Manager Animal Health Welfare and Biosecurity,
Meat and Livestock Australia,
November 2011.

Ovine Johne's disease

1 Pathogenesis of OJD – Strategic Research on Diagnosis and Prevention (Project OJD.031, 2004-2007)

Principal Investigator: Richard Whittington
Research Agency: University of Sydney

Johne's disease is a significant international problem because of the direct impact on animal health and welfare and its possible public health implications. It is essential that Australia has the capacity to deal with diseases such as Johne's disease and this necessitates maintenance of expertise to work at international levels of competence. This project identified new types of tests that will provide new ways to tackle Johne's disease in the future. At the same time, it provided direct and indirect training for eight PhD students and four post-doctoral fellows, to help strengthen Australia's capacity for international standard animal health research. New diagnostic test methods and advanced immunological, molecular biological and proteomic technologies were developed and applied to sheep for the first time.

In order to control Johne's disease, more information is needed about the biology or pathogenesis of the disease. The biggest knowledge gaps are in the early stages of disease. The research program was planned following review of current research into tuberculosis, which was the closest relevant example. Throughout biology there is a basic pattern of information encoded in DNA and translated into proteins, which carry out instructions. The latest techniques in genomics and proteomics were applied to study the gene signals and protein effects, respectively. This was done for both the causative bacterium, and the sheep, in order to try to understand the interaction between the two. To enable research in the early stage of infection, a reliable method for experimentally inducing Johne's disease in sheep was developed, a world first.

Important outcomes of this research include:

- The development of a realistic experimental infection model in sheep. From the basic research program a method was proven for creating experimental ovine Johne's disease in a flock under tightly controlled conditions, leading to realistic and natural outcomes. This approach was a world-first development and will be invaluable for diagnostic test and vaccine development studies. This infection model has already been adopted by overseas researchers and we expect that Australian producers will benefit from such collaborative international studies.
- Cell mediated immune responses play an important role in protecting sheep against *Mycobacterium paratuberculosis* (*Mptb*). Many types of cell mediated responses were studied using newly developed methods. The key findings were:
  - improvement to the existing gamma-interferon test is possible by extending an incubation period
  - newly developed tests (Cell-ELISA and ELISPOT) for interferon gamma were more sensitive than the existing gamma-interferon test
  - a new assay to measure the cytokine IL-10 found responses as early as 4 months post infection, especially in sheep that had been exposed to *Mptb* but not succumbed to disease.

- Apoptosis was measured in sheep for the first time; apoptosis is believed to be important in the development of mycobacterial disease. Although some sheep did have strong apoptosis test results, there was no consistent difference between infected and uninfected sheep.
- Using a new flow-cytometry based test, a strong cell proliferative response was detected in the blood as early as 4 months following exposure to *Mptb*. This has the potential to detect infection in sheep within a few months after exposure.
- Proteins in serum were studied in order to find new biomarkers for Johne's disease using mass spectrometry. Analysis using these biomarkers achieved diagnostic sensitivities of 90-93% and specificities of 83-91% in discriminating between infected and unexposed animals.
    - the specificity of many of these tests will depend on the antigens used in them. To improve this aspect over 60 new proteins of *Mptb* were discovered using state-of-the-art proteomic approaches. The proteins were specifically associated with the stress/dormancy response of *Mptb*, which is thought to enable the organism to persist in the sheep for long periods. Some of the proteins were shown to stimulate an immune response during early infection which might have diagnostic significance.
    - Antibody mediated immune responses can also be used to detect Johne's disease. The new proteins also have potential application in tests of the humoral response.
- Research was also conducted on genomics of the sheep, and the genes that respond during infection:
    - new methods were developed for sheep to enable gene discovery
    - many genes in the intestine and associated lymph nodes were differentially regulated
    - in a conceptual breakthrough, the genes that code for the molecules on the surface of cells which first recognize invading *Mptb* (Toll-like receptors, TLR) were found to be switched on during early infection. These are engaged by microbial pathogens to initiate innate and adaptive immune responses.
- Direct detection of *Mptb* remains a cornerstone of diagnosis, providing proof of infection. New methods for detection of the organism in faecal and blood samples were developed:
    - a new approach for direct detection of the organism in faecal samples was developed and published. The sensitivity and specificity of this method appear to be similar to faecal culture.
    - Methods for culture of *Mptb* from blood were also developed, but few sheep were found to have the organism in their blood in the early stages of the infection.

New antigens and the tests identified as part of this research and applicable to blood samples are being further investigated to ascertain levels of sensitivity and specificity in sheep and cattle in subsequent projects (P.PSH.0297 and P.PSH.0311). The new proteins may also have application in new-generation vaccines for Johne's disease. They can be produced using genetic engineering and so would be much cheaper than the existing commercial vaccine. Cost is now emerging as a major impediment to widespread use of Gudair ® vaccine.

Further development and field evaluation of the direct faecal PCR test for rapid detection of *Mptb* in faeces is also continuing for both sheep and cattle in the follow-on projects.

As a result of this project Johne's disease potentially can be detected early in life and removed from a flock before causing harm or spreading. This is a dramatic change in thinking as current tests are too insensitive to detect infection before it has spread. It may lead to renewed interest in control programs if animal health regulatory authorities and public health agencies deem this to be necessary. If public health concerns persist and impact markets, it will be possible to apply tests to determine prior exposure to *M. paratuberculosis.* This will certainly be possible at flock level and may also be possible at individual animal level with further research.

To achieve these advances, a group of young scientists was assembled at Camden after an international search. They joined technical officers, PhD research students and permanent research staff at the Faculty of Veterinary Science. The University of Sydney added additional scholarships to enable international PhD scholars to join the research program. Eight PhD students undertook studies through direct or indirect involvement with this project. Three have already completed their degrees and are now engaged in employment that services the livestock sector. This is a vital contribution to help redress the skills-shortage in Australia. Biological samples that had been archived from earlier research projects conducted under the National Ovine Johne's Disease Control Program were used. This added value to earlier projects and further reduced the cost of the new research.

Johne's disease remains a difficult problem and without ongoing basic research at an international level will not be successfully controlled. There are parallels between the needs in Johne's disease research and those in tuberculosis in humans, which is caused by a related bacterium, *M tuberculosis:* "the TB vaccine development program include an assortment of tasks such as identifying mechanisms of host defence, improving animal models and conducting Phase 1/II trials over a period of 20 years. There is little certainty in the time span chosen to achieve these goals, but there has been definite progress made in many of the tasks" (Izzo et al (2005) NIH pre-clinical screening program: overview and current status. Tuberculosis 85:25-28). With this background it is vital to note that the aims of Johne's disease research programs worldwide will have the same challenges and difficulties as those for tuberculosis.

2 Ovine Johne's Disease: Applications of basic research on enhanced diagnosis and prevention (Project P.PSH.0311, 2008-2011)

Principal Investigator: Richard Whittington
Research Agency: University of Sydney

2.1 Background

The diagnosis of ovine Johne's disease remains a problem because until recently there has been very little basic research conducted anywhere in the world, and most of the current knowledge is based on the study of human tuberculosis from the early 1900's. Practical applications of this include tests used today: culture, histopathology and intradermal skin tests, none of which currently are very sensitive. The lack of basic knowledge has severely limited the development of new diagnostic tests and vaccines. Therefore this project included components of basic research, as well as translational research aimed at delivering practical tools for industry in the near term.

This project was designed to use basic research and combine it with strategic elements to discover new test options and to improve existing tests. The project involved state of the art methods in microbiology, immunology, molecular biology and genomics in a multidisciplinary team with international collaborations to achieve its objectives.

2.2 Major outcomes

As a result of the project it is now known that:

- interferon gamma tests can be improved for use in sheep in Australia
- faeces can be tested quickly and accurately using direct PCR
- it is possible to reproduce the disease in a natural form in a controlled experimental situation, opening up options for test evaluation, vaccine development and other studies
- new antigens are available for evaluation to improve test specificity and sensitivity
- new cytokine-based tests appear to be useful
- immune suppression and weight loss during ovine Johne's disease may be explained by dysregulation of amino acid metabolism
- detection of the organism in blood is not a useful diagnostic approach
- a blood test based on cell proliferation may be predictive of disease susceptibility in sheep

A further objective of the project was to ensure that there is a credible team of researchers available to Australian sheep producers, and this also was achieved. Some of the findings of this project have been published already and the project team has an international reputation.

As a result of the project it is now clear that two existing diagnostic tests which had severe practical and technical limitations can be improved and may be of substantial benefit in the near future. These include a direct faecal PCR test which can provide results to producers within a few days instead of the current 3 months for culture and an interferon gamma blood test which can be made practical for use in sheep in Australia. In addition to these there are several new research-level tests of immune

function that require further development. Further information on the two most promising tests follows.

2.3 Direct faecal PCR test

The direct faecal PCR test is a breakthrough for the sheep industry. Previously, faecal samples were collected, sent to a laboratory and 3 months would elapse before negative test results could be confirmed. For sheep sales this meant considerable forward planning and great inconvenience for the producer. Where culture was used to confirm a suspected flock infection, the long delay caused considerable additional anxiety for the producer. The new test overcomes these problems because it can provide results within a few days of receipt of samples at a laboratory. It will cost no more than culture, and will be of similar accuracy. Of 65 culture positive samples, 62 were positive in the new test. Of 140 culture negative samples, 12 were positive in the new test. As no samples from flocks known to be free of OJD tested positive in either test, we believe that the new faecal test is slightly more sensitive than culture. Furthermore it is suitable for testing pooled faecal samples, which enables a cheap method of flock testing, either to detect infection or to show that it is not present in flocks in the Market Assurance Program. Additional validation of this test has been recommended by the JD Research Advisory Group, after which the data will be submitted to the SubCommittee on Animal Health Laboratory Standards (SCAHLS) for approval for use in the National Johne's Disease Program. A submission on the test will be made to SCAHLS in 2011.

2.4 Whole blood interferon gamma assay

A whole blood interferon gamma assay which was developed to prototype stage in a previous project has been modified and improved in this project. Previously it was necessary to ship blood samples to a laboratory and test them within 8 hours of collection – something that usually was impossible and prevented validation of the test. Two blood additives were discovered which extend the life of the blood samples to 48 hours. Now it is possible to ship samples from most places in Australia to a laboratory in time to conduct the test. Additional research is now required to find a way to make the test more specific, as some uninfected sheep react, and this will be done in project P.PSH.0576, with a goal of validating the new procedure within the life of that project. Interferon gamma detection assays offer the potential to detect more infected animals at an earlier stage of the disease compared to an antibody ELISA or direct detection of Mptb in the faeces. This may provide opportunity for control strategies aimed at removal of young infected animals before they start shedding bacteria into the environment.

2.5 Experimental infection model

From the basic research program a method was proven for creating experimental ovine Johne's disease in a flock under tightly controlled conditions, leading to realistic and natural outcomes. This approach will be invaluable for diagnostic test and vaccine development studies and has already been adopted by overseas researchers – we expect that Australian producers will benefit from such collaborative international studies.

2.6 Mining the genome of Mptb for better tests

Also in the basic research program, computer-based methods were used to mine the DNA sequence of the causative bacterium Mycobacterium paratuberculosis (Mptb) to

identify new components to include in future diagnostic tests with the objective of greater accuracy than current tests.

2.7 Mptb is not found in blood very often

It was shown that Mptb does not circulate very often in blood at detectable levels, which is of great significance for diagnosis and has positive implications for public health.

2.8 Proliferating cells for detecting exposure to Mptb

The ability of white blood cells to remember contact with Mptb has been tested in a proliferation assay in experimentally infected sheep – the response in non-exposed controls remained low while it increased and remained elevated in exposed sheep. Furthermore, the proliferative response varied with disease status and may be predictive of resistance.

2.9 Mechanisms of disease progression and weight loss in OJD

Finally, an explanation for the weight loss that occurs in ovine Johne's disease may have been found – rather than intestinal malabsorption, it is possibly due to an amino acid deficiency induced by the infection. Blood levels of the amino acid tryptophan were shown to plummet as ovine Johne's disease develops, and this was due to a trick played by the mycobacterium to induce the sheep to destroy its own tryptophan. Further research will be conducted across these fundamental discoveries to maximise their potential.

3 Changes in Within-Flock Prevalence of *Mycobacterium Avium Paratuberculosis* Shedding Following Vaccination with Gudair in High & Low Prevalence Flocks (Project OJD.033, 2003-2009)

Principal Investigator: Peter Windsor
Research Agency: University of Sydney

Ovine Johne's disease (OJD) is a fatal enteric infection of sheep by *Mycobacterium avium* subspecies *paratuberculosis* 'S' strain (*Mptb*) that has proven difficult to both diagnose and control. Collaborative research in project OJD.009 demonstrated the efficacy of vaccinating lambs between 1 and 4 months of age with Gudair™, a killed whole cell vaccine imported from Spain, for controlling OJD in high prevalence Australian sheep flocks. Vaccination reduced mortality by 90%, delayed the onset of faecal shedding of *Mptb* by 12 months, and reduced the prevalence of shedders by 90% compared to unvaccinated lambs (Reddacliff *et al*, 2006). This study led to the registration of Gudair™ and it is now established as a key strategy to control the disease in infected flocks.

The original vaccine research in Australia (MLA project OJD.009) was conducted on the first cohort of vaccinates from 3 flocks that were considered to be heavily infected, with presumed exposure of intra-uterine and neonatal lambs of infected ewes to significant *Mptb* challenge. However following registration of Gudair™ many lower prevalence flocks also commenced vaccination as a precaution against increased mortalities and as a means to improve their ability to sell re-stocker sheep through the risk based trading Assurance Based Credit (ABC) point scheme. It was suggested that the efficacy of the vaccine might be superior in flocks with low OJD prevalence where later drops of vaccinated lambs were exposed to sheep of a much lower risk of developing OJD than in the original study (MLA project OJD.009). Modelling work suggested that the prevalence of mortalities and shedding would fall rapidly after the commencement of a vaccination control program depending on disease prevalence at the time of commencing vaccination. Validation of this work by field research was required.

In this study (OJD.033) we report on the changes in the prevalence of shedding over the first 6 cohorts of vaccinates in 12 infected flocks of variable prevalence when they commenced vaccinating. The study determined the changes in shedding of *Mptb* in the 3-4 year and 5-6 year old cohorts in 2003-4, 2005-6 and 2007-8 following initiation of vaccination with Gudair™ in 1-4 month old lambs in 2002.

Faecal samples for pooled faecal culture (PFC) were collected on 3 occasions from 11 flocks and on 2 occasions from one flock (which withdrew from the trial in 2007). Samples consisting of 7 pools of 50 faeces were collected randomly from each of the 3, 4, 5 and 6 year old female sheep from each flock and stored at –80°C until cultured. Faecal culture was by modified BACTEC 12B radiometric culture and direct confirmation by IS900 PCR using published methods (Whittington *et al*, 1998, 2000). The prevalence of sheep shedding *Mptb* in faeces was estimated from the total number of pools, number of pools positive and number of samples per pool using the pooled prevalence calculator (Sergeant, 2004). Prevalence calculations for this analysis used published methods which assume perfect test sensitivity and specificity but allow for variable pool size, which was important for this analysis (Williams and Moffitt, 2001).

3.1 Results

A significant decrease in shedding rates of *Mptb* following the introduction of vaccination was observed in the majority of flocks in the study. However shedding of *Mptb* was detectable in 10 of the 11 flocks that remained in the study until 2008 (see Table 1). Vaccinates had significantly lower prevalence (0.63%) than non-vaccinates (1.66%) as would be expected (P<0.001) from comparison of the data for vaccinates (shaded) with unvaccinated sheep (unshaded) in Table 1. No other variable was significant when added to the model of prevalence on vaccination status.

Table 1. Mean prevalence estimates for each age group for each sampling (note shading demonstrates appearance of vaccinated cohorts)

Sampling number	3-yr-olds	4-yr-olds	5-yr-olds	6-yr-olds	Total
1 (2004)	1.43%	2.74%	1.93%	2.28%	2.05%
2 (2006)	0.72%	0.99%	2.55%	1.59%	1.58%
3 (2008)	1.03%	1.05%	0.58%	0.90%	0.87%
Total	1.07%	1.69%	1.75%	1.73%	1.55%

There were also significant differences in prevalence over the years and in different age groups (P<0.001; as expected from visual examination of Table 1). Further, there was a significant reduction in prevalence across the three samplings, with the table of predicted means for year being 1.54%, 1.08% and 0.71% for samplings 1, 2 and 3 respectively. In general, prevalence was higher in older age groups, with the table of predicted means for age being 0.72% for 3-year-olds, 1.14% for 4-year-olds, 1.13% for 5-year-olds and 1.33% for 6-year-olds. Interestingly, the prevalence was not significantly lower in the same cohort at the next sampling (such as 5-year-olds at Sampling 2 compared to 3-year-olds at Sampling 1).

When analysed by status of individual faecal pools, non-vaccinates were 7.92 times more likely to be pool positive compared to vaccinates. The analyses of age and sampling year for pool status determined that there was not a significant reduction in the likelihood of a pool being positive in Sampling 2 compared to Sampling 1. However there was a significant reduction in the likelihood of a pool being positive in Sampling 3 compared to Sampling 2, plus a significant reduction in the likelihood of a pool to be positive in Sampling 3 compared to the Sampling 1.

3.2 Conclusion

The study provides evidence that Gudair™ vaccination is a valuable but imperfect tool in managing OJD. The study is being continued for a further 3 rounds of testing to evaluate the effect of long-term vaccination in infected flocks and to provide data on expected shedding rates in flocks composed entirely of 'second generation vaccinates', that is, sheep that are progeny of accredited vaccinates. The outcomes from the extension of these studies will greatly assist sheep producers to assess the risk of ceasing vaccination in their flocks and the risk of purchasing vaccinated re-stocker sheep.

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4 Evaluation of the effectiveness of Gudair™ vaccination for the control of OJD in flocks vaccinating for at least 5 years (Project P.PSH.0309, 2008-2011)

Principal Investigator: Peter Windsor
Research Agency: University of Sydney

Ovine Johne's disease (OJD) is an enteric infection of sheep by *Mycobacterium avium* subspecies *paratuberculosis* 'S' strain (*Mptb.*) that has proven difficult to both diagnose and control in Australia. Collaborative research in project OJD.009 demonstrated the efficacy of vaccinating lambs between 1 and 4 months of age with Gudair™, a killed whole cell vaccine imported from Spain, for controlling OJD in high prevalence Australian sheep flocks. Vaccination reduced mortality by 90%, delayed the onset of faecal shedding of *Mptb.* by 12 months, and reduced the prevalence of shedders by 90% compared to unvaccinated lambs. This study led to the registration of Gudair™. This vaccine and the Assurance Based Credit (ABC) Scheme are now established as the key strategic intervention to control the disease in Australia. Computer modelling suggested that the occurrence of mortalities and shedding would fall rapidly after the commencement of a vaccination control program, depending on disease prevalence at the time of commencing vaccination. However it was acknowledged at the time that validation of this modelling by field research was required, particularly as points for vaccination became incorporated into the ABC risk based trading scheme.

Following registration of Gudair™ many flocks with an apparent low prevalence also commenced vaccination as a precaution against increased mortalities and as a means to improve their ability to sell re-stocker sheep through the ABC scheme. In project OJD.033, we reported on the changes in the prevalence of shedding of *Mptb.* in the 3-4year and 5-6year old cohorts in 2003-4, 2005-6 and 2007-8 following initiation of vaccination of 1-4 month old lambs in 2002. The study found a significant decrease (1.66% to 0.63%; $p<0.001$) in shedding rates of *Mptb.* in the majority of flocks in the study. However we also identified that shedding was detectable in 10 of the 11 flocks that remained in the study until 2008 (range 0.13% to 1.29%) and it was recommended that a broader study of the current prevalence of OJD in flocks that had been vaccinating for 5 years or more was required. This led to project P.PSH.0309 as reported here. More recently it was also decided that evaluation of shedding in the remaining flocks in Project OJD.033 be continued for a further 3 rounds of testing to provide more accurate data on the decline of shedding rates in flocks composed entirely of 'second generation vaccinates' (sheep that are progeny of accredited vaccinates). This study has commenced as Project P.PSH.0565. Appropriate extension of the outcomes from these studies will greatly assist sheep producers to assess the risk of ceasing vaccination in their flocks and the risk of purchasing vaccinated re-stocker sheep.

Project P.PSH.0309 was an observational cross-sectional study conducted in 40 selected trial flocks of varying initial OJD prevalence from the southeast of NSW and Victoria, examining the efficacy of Gudair™ vaccine in decreasing the prevalence of shedding of *Mptb* 5 years after the commencement of vaccination. OJD prevalence in these flocks after 5 years was determined by pooled faecal culture of 350 sheep (PFC350; 7 pools of 50) and was compared with estimates of OJD prevalence from various data estimating prevalence (including serology and culture) recorded prior to commencement of vaccination. Results show that 5 years or more after the

commencement of vaccination with Gudair™ there has been a noticeable decline from 14 to 4 flocks categorised as high prevalence flocks, with 7 flocks having no detectable shedders. However 82.5% of the 40 flocks still contained sheep that were shedding. By combining shedding data with other prevalence information by Bayesian modelling (resulting in elimination from analysis of 2 flocks with inadequate initial prevalence data), the results identified a significant decline in the median OJD prevalence pre-vaccination of 2.99% to 0.74% post-vaccination (95% PI 0.42, 1.29%). Despite 18.4% (7/38) of the flocks apparently not shedding currently, 47.4% (18/38) flocks had a cohort prevalence of >0 and ≤1% and 34.2% (13/38) had a cohort prevalence of >1% Seven of the 14 flocks with initial low prevalence had increased prevalence (medium or high) after 5 years vaccination.

To examine the sensitivity of the initial PFC350 and better understand infection in low-prevalence flocks, pooled faecal culture sampling was conducted on 600 sheep (PFC600; 12 pools of 50) from 4 of the 7 negative flocks from the initial survey and one was found to be shedding. This flock had ceased to vaccinate wethers. When the PFC 600 was conducted on 16 known infected flocks on Kangaroo Island in South Australia, including 6 flocks that had been recently found not to be shedding by PFC350, no subsequent shedding was found in 14 of the flocks. This indicates a decline in proportion of these 16 flocks as positive for shedding from 100% to a proportion of 12.5% of flocks shedding currently. It was later identified that both positive flocks on Kangaroo Island had introduced unvaccinated sheep in recent years. Further, it was noted that in addition to whole flock vaccination, a number of other farm management factors designed to minimize the spread of OJD had been introduced on KI.

These data indicate that despite a rapid decrease in OJD mortality in flocks following the commencement of a vaccination program, shedding is likely to have persisted for at least 5 years in a majority of infected flocks in NSW and Victoria and is of concern if sheep are to be traded from these flocks or vaccination ceases. However the data from Kangaroo Island are encouraging and suggest that the second generation vaccinates have greater protection from shedding than the first generation vaccinates and will likely present a substantially lower risk of transmission of the disease.

5 How well is OJD vaccine performing?

The following paper summarises recent OJD vaccine research in Australia and is reproduced with permission of the Australian Sheep Veterinarians.

Reference: P Windsor, A Masters, J Eppleston, N Dhand and R Whittington, 2011. How well is OJD Vaccine performing? In: *Proceedings of the Australian Sheep Veterinarians 2011 Conferences*. Australian Sheep Veterinarians, Brisbane, Australia. Pp 132-139.

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5.1 Summary

Since the registration of Gudair™ vaccine in 2002 for the control of ovine Johne's disease (OJD) is Australia, a number of projects have sought to gain information on the level of protection offered to our sheep populations. Experience has shown that vaccination against OJD quickly eliminates the significant mortalities in infected high prevalence flocks. However our research conducted in commercial flocks indicates that, at least in the first 6 years after commencing a lamb vaccination program, shedding persists in the majority of flocks (82.5% flocks shedding in Trial 0309). Shedding continues at a level that indicates cessation of vaccination (as has occurred with wethers in some flocks) and introduction of unvaccinated sheep would likely result in recrudescence of disease and sales of vaccinates would potentially transmit OJD. A risk factor study has shown that management factors such as biosecurity with attention to straying sheep in addition to vaccination may be required to optimize the protection offered by Gudair™. Re-examination of vaccinating flocks when they consist entirely of vaccinated sheep born from approved vaccinates (i.e. second generation vaccinated flocks) is required.

5.2 Introduction

In a large successful research project (Trial 009) conducted in single generations of lambs in 3 Ovine Johne's Disease (OJD) infected Merino flocks, Gudair™ vaccination of lambs was found to reduce by about 90%, both the prevalence of animals dying with the disease, and the prevalence of sheep shedding *Mycobacterium avium* subspecies *paratuberculosis (MPtb)* (Reddacliff et al, 2006). This research led to registration of Gudair™ and established vaccination, with risk-based trading (where points are allocated for vaccination), as the key strategies to control disease and manage the risk of transmission of OJD in Australia (Windsor, 2006). However as this study was conducted in high prevalence flocks with considerable mortality and extremely high bacterial challenge, concerns were raised that the efficacy of the vaccine might be different in flocks with less challenge. Further, as observations that high rates of shedding was persisting in vaccinating flocks (Eppleston et al, 2005) questions were raised on how long vaccination should continue in infected or at–risk flocks until risks of OJD transmission were mitigated (ie minimal or nil bacterial challenge).

We have been examining the efficacy of Gudair™ vaccination over a larger number of commercial flocks across a broad range of initial OJD prevalence. This includes a

long-term longitudinal study that has continued since 2004 (Trial 033) to monitor the shedding of *MPtb* over 4 drops of vaccinates in 11 OJD-infected flocks in NSW following the implementation of vaccination. We have also conducted a cross sectional trial (Trial 0309) that in 2008-2009, measured the shedding rates in 40 flocks from NSW and Victoria with different levels of initial prevalence of OJD following vaccination for at least 5 years. This latter trial was initially intended to include flocks from South Australia but as the regulatory and industry framework in this state was so very different, we conducted a small trial on Kangaroo Island (results to be presented separately).

Observations from these studies has shown that in some flocks, moderate rates of shedding persist in vaccinates. This is not surprising as in the initial field research project (Trial 009) the few vaccinated sheep that did develop OJD had multibacillary lesions and shed *Mptb* at very high levels. However we have been pleased to see that on Kangaroo Island, more progress has been made and flocks are now being found to be negative and released from quarantine. During these studies we observed that many producers had ceased to vaccinate their wether lambs. We presumed this was due to high costs of vaccine, drought-induced destocking and financial incentives from the sheep meat trade. There was an expectation that wethers will be sold by 2 years of age and prior the commencement of mortalities or shedding of *MPtb.* However Trial 009 identified that in heavily infected flocks, shedding of *MPtb* occurs in animals as young as 8 months of age with clinical losses from 15 months. To examine this issue we conducted a small study (Wether Trial) to investigate shedding rates in unvaccinated wethers (Eppleston et al, 2011).

The major outcomes from these investigations suggest that with the exception of Kangaroo Island, we need to dampen the widespread optimism that appears to exist in the rural community on the performance of the vaccine in reducing shedding rates in vaccinates and thus disease transmission risk. It is important that data from these trials be more broadly recognized and understood as we expect that information from these studies will assist sheep producers to make better informed assessments of both the risk of cessation of vaccination and the risk from the purchase of vaccinated sheep.

5.3 Material and Methods

Trial 033
Eleven self-replacing Merino flocks in N.S.W. were selected that had previously commenced a vaccination program where 1 and 2 year-old sheep were vaccinated as lambs. Available OJD surveillance data for each flock was examined to provide about 4 flocks in each of the high, medium and low prevalence categories. Each flock was sampled 3 times at 2-yearly intervals. At each sampling up to 7 pools of faeces (50 sheep per pool) were collected from the current generation of 3, 4, 5, and 6 year-old sheep (28 pools in total). At the first sampling, all 4 age groups had not been vaccinated and provided an accurate estimate of initial disease prevalence (Table 1). At the second sampling, the 3 and 4 year olds were vaccinates but not the 5 and 6 year-olds. At the third and final sampling, all four age groups were vaccinated.

Table 1.Trial 033, sampling schedule: vaccination status of each cohort at each sampling

Sampling		Age cohort (years of age at sampling)			
Number	Year	3	4	5	6
1	2004	Not vaccinated	Not vaccinated	Not vaccinated	Not vaccinated
2	2006	Vaccinated	Vaccinated	Not vaccinated	Not vaccinated
3	2008	Vaccinated	Vaccinated	Vaccinated	Vaccinated

Pooled faecal samples were cultured using a modified BACTEC radiometric method (Whittington *et al*. 2000) and the animal level prevalence of shedding was estimated from the proportion of pools that were culture-positive.

Trial 0309

Forty commercial self-replacing Merino flocks that had commenced vaccination at least 5 years previously, and had sufficient data available to establish OJD prevalence at the commencement of vaccination, were selected for this study. A summary of flocks by district, initial prevalence category and sampling progress is presented (Table 2). Note that each flock was categorised according to the following prevalence levels where 7 pools of 30-50 were available for PFC or 450 gel tests were available:

- High prevalence: ≥4 +PFC or ≥10 gel +ves
- Medium prevalence: 2-3 +PFC or 4-9 gel +ves
- Low prevalence: ≤1 +PFC or ≤3 gel +vs

Table 2. Classification of Trial 0309 flocks by location and initial prevalence.

District	*Initial Prevalence estimate**			*Total*
	High	Low	Medium	
Braidwood		1		1
Central Tablelands	1	3	1	5
Goulburn			3	3
Hume	4	2	3	9
Moss Vale			1	1
Victoria	4	4		8
Wagga	3	1	3	7
Young	2	3	1	6
Total	14	14	12	40

* categorised using 7 pools of 30-50 for PFC or 450 gel tests as above

Each flock was sampled by pooled faecal culture (PFC350 comprising 7 pools of faeces from 50 animals) from the 3 and 4 year old cohort. Comparisons were made between the current prevalence and the initial estimate of disease prevalence in each flock at the commencement of vaccination. An estimate of the expected change in prevalence following continuous vaccination for 5 years was established for a range of flocks with varying initial OJD prevalence levels. For 36 flocks, a questionnaire was used to obtain detailed information on OJD and other losses and Gudair™ vaccine use, plus ongoing and current flock management to identify risk factors that may have affected the effectiveness of the vaccine.

To examine the sensitivity of the PFC in low prevalence flocks, we conducted pooled faecal culture on 600 sheep (PFC600 comprising 12 pools of faeces from 50 animals) from as many flocks as possible that were found to be negative on the PFC350 (4 of 7 flocks). To extend this examination of sensitivity, we also conducted testing on Kangaroo Island as we were aware that a number of infected properties had recently been released from quarantine on the basis of negative results in the PFC following absence of disease on farm and failure to detect infection on abattoir surveillance. On Kangaroo Island, OJD is still regulated, whole flock vaccination had occurred initially and vaccine use had been subsidised by industry. The PFC 600 was conducted on 16 flocks on Kangaroo Island, including 6 negative flocks that had been recently found not to be shedding by PFC350.

Wether Trial
To examine the risk of leaving wethers unvaccinated, 6 OJD-infected self -replacing Merino flocks located between Gundagai and Bathurst were included in this trial. These flocks had been vaccinating all lambs since at least 2004 but had left their wether lambs unvaccinated in 2006/07. In each flock 1-2 year old unvaccinated wethers and their vaccinated ewe cohorts were sampled (7 pools of 50) and tested by PFC350. This enabled comparison of the prevalence of shedding in each cohort as an indicator of the risk of increased shedding of unvaccinated wethers compared to vaccinated ewes.

5.4 Results and Discussion

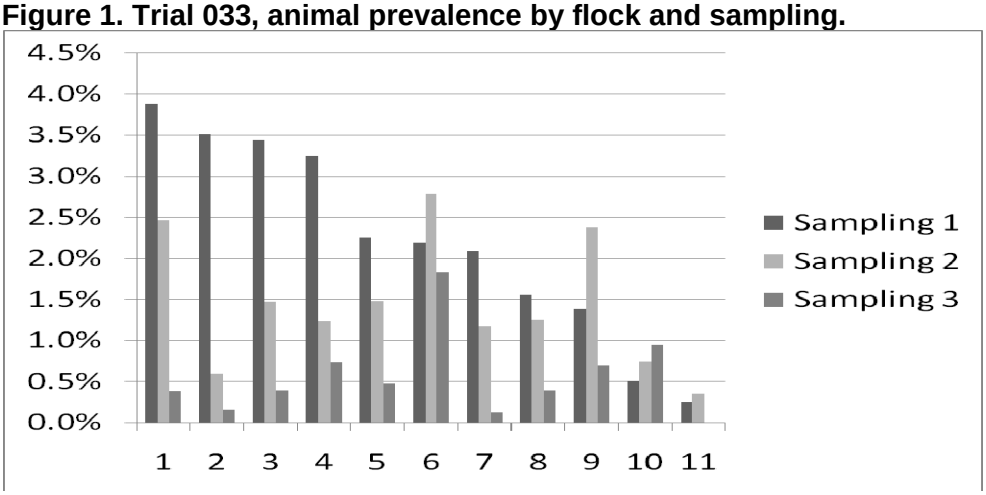
Trial 033
Vaccination reduced the prevalence of shedding by 62% (1.66% vs. 0.63%; P<0.001), and non-vaccinates were 7.9 times more likely to be pool positive compared to vaccinates. As a result there was a significant reduction in whole flock shedding from Sampling 1 when all sheep were not vaccinated, to Sampling 3 when all sheep were vaccinated (Table 3). The shedding response to vaccination varied between flocks (Figure 1). The estimated prevalence of shedders at Sampling 1 across all 4 ages of non-vaccinates was 2.1% and ranged from 0.2% to 3.8%. At sampling 3, when all sheep were vaccinates, 10 of the 11 flocks had detectable levels of shedding (range 0.1 to 1.8%).

Table 3. Mean animal level prevalence estimate for each age and sampling group (shaded cells represent vaccinated cohorts)

Sampling number	3yr olds	4yr olds	5yr olds	6yr olds	Total
1 (2004)	1.43%	2.74%	1.93%	2.28%	2.05%
2 (2006)	0.72%	0.99%	2.55%	1.59%	1.58%
3 (2008)	1.03%	1.05%	0.58%	0.90%	0.87%
Total	1.07%	1.69%	1.75%	1.73%	1.55%

Across the 11 flocks, prevalence fell in 7 flocks, rose then fell in 3 flocks, and continued to rise in 1 flock. There was no obvious relationship between the rate of decline in shedding and either the initial disease prevalence or management factors identified by survey

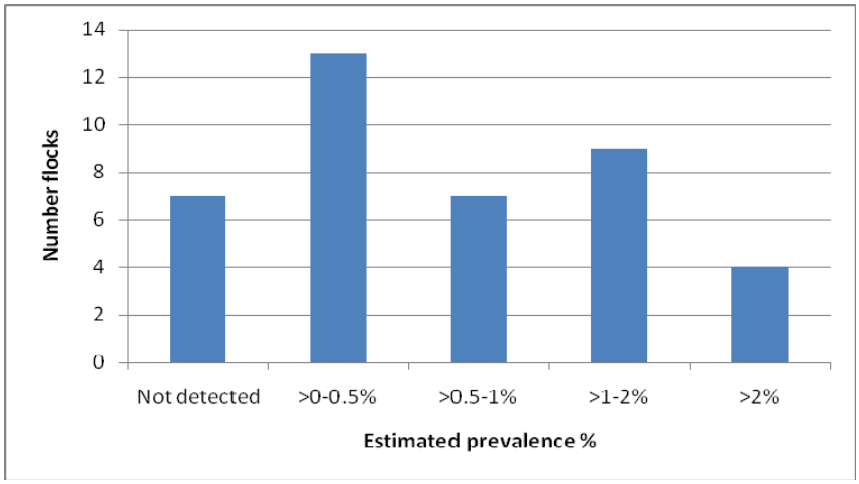
Figure 1. Trial 033, animal prevalence by flock and sampling.



Trial 0309

The estimated OJD prevalence in 2009 for the 40 NSW and Victorian flocks as based on the proportion of positive pools and then submitted to the pooled prevalence calculator, is presented in Figure 2. As can be seen, no shedding was detected in 7 flocks and the prevalence exceeded 2% in 4 flocks.

Figure 2. Trial 0309, number of flocks by estimated prevalence 5yrs after commencing vaccination.



The change from the initial prevalence categories as documented in Table 2 when re-examined with current PFC data, is presented in Table 3, representing the change in prevalence category after 5 years of vaccinating with Gudair™. There was a reduction in number of flocks in the high prevalence category from 14 to 8 over the 5 years since commencement of vaccination. Despite 17.5% (7/40) of the flocks apparently not shedding currently, 50.0% (20/40) flocks had a cohort prevalence of >0-1% and 32.5% (13/40) had a cohort prevalence of >1%.

Table 3. Changes in estimated prevalence of sheep shedding *Mptb.* after 5 years of vaccination

Initial prevalence*	Current Prevalence				
	Not Detected	Low	Medium	High	Total
Low (14)	5	1	4	3	13
Medium (12)	0	5	5	2	12
High (14)	2	7	3	3	15
Total	7	13	12	8	40

* Initial number of farms in each category is provided in brackets

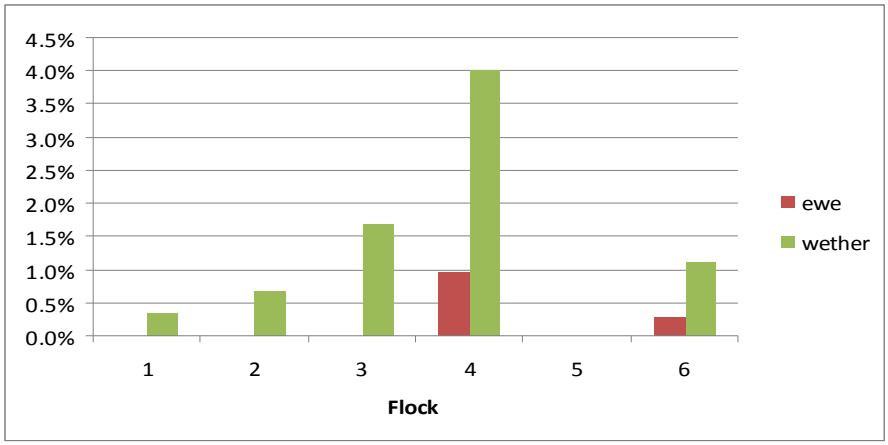
This risk factor study that examined management factors on 36 of the 40 properties that were sampled in Trial 0309, sought to identify factors that may be affecting the efficacy of the Gudair™ vaccine in flocks vaccinating for at least 5 years. The results of the PFC, with 72% of the pools tested negative and only 28% positive, confirmed that the majority of the flocks had a current cohort OJD prevalence of <1% with 6 of the 36 flocks having undetectable levels. However 13 of the 36 flocks still had a cohort OJD prevalence of ≥1%. Management factors found to be associated with OJD prevalence exceeding 1% in flocks vaccinating with Gudair™ included having sheep stray and the introduction of new sheep on farms. Having a concurrent cattle enterprise was shown to be protective. The findings of this study suggest that while

Gudair™ vaccine does decrease the OJD prevalence levels in vaccinating flocks, it does not negate the continuing need for farm biosecurity in the control of OJD.

In the examination of sensitivity of the PFC, we were able to conducted the PFC600 (12 pools of 50) from 4 of the 7 flocks that had been found to be negative on the recent PFC350 (Table 3). One was found to have shedding, although this flock had ceased to vaccinate wethers. When we extended this regimen to Kangaroo Island, we were able to conduct the PFC600 on 16 flocks, including 6 negative flocks that had been recently found not to be shedding by PFC350. No subsequent shedding was found in 14 of the 16 flocks. A significant decline in OJD prevalence ($P<0.001$) from initial cohort OJD prevalence range (0.24-1.94%) to the current cohort OJD prevalence range (0.00-2.17%) was found. It was also found that both positive flocks on Kangaroo Island had introduced unvaccinated sheep in recent years. It was also noted that in addition to whole flock vaccination and subsidised vaccine, a number of other farm management factors designed to minimize the spread of OJD had been introduced in Kangaroo Island flocks

Wether Trial
Shedding of *Mptb* was detected in 5 of the 6 flocks in the unvaccinated wether cohort and in only one flock in the vaccinated ewe cohort (Figure 3). We found that the estimated prevalence of shedding sheep in wethers left unvaccinated at marking was 7 times greater than in their vaccinated female siblings, with faecal pools collected from unvaccinated wethers being 19 times more likely to be positive than from the vaccinated ewes. Overall this practice would result in increased levels of pasture contamination and greater exposure in subsequent drops of lambs.

Figure 3. Wether Trial, animal prevalence by flock and gender.



5.5 Conclusions

In Trial 033, in 8 of the 11 flocks we observed a substantial reduction in prevalence of shedding of at least 75% following vaccination. This finding is similar to the reduction reported previously (Reddacliff *et al*. 2006). However, in the remaining 4 flocks this reduction was not evident. At this stage it is uncertain what factors contributed to the failure of vaccination to achieve the expected substantial reduction in shedding of *MPtb* and a decision has been made to continue the monitoring of these flocks (Trial 0565). It may be of interest that the 4 flocks with disappointing reductions in prevalence were from the moderate or low initial prevalence groups, suggesting that the high expectations of vaccine performance identified in Trial 009 may partly reflect the high prevalence of OJD in these flocks.

Results from Trial 0309 also included flocks with a broad range of initial OJD prevalence, and supports the finding that shedding persists for at least 6 years in the majority of flocks following commencement of vaccination with Gudair™. The findings from the risk factor study suggest that management factors such as biosecurity and attention to straying sheep in addition to vaccination may be required in some flocks to reduce the prevalence of infection to an acceptable level of disease risk. Examination of the sensitivity of the initial PFC350 by re-sampling negative flocks using the PFC600, confirmed that the PFC350 adequately detects flocks of low prevalence. These data indicate that despite a rapid decrease in OJD mortality in flocks following the commencement of a vaccination program, shedding is likely to have persisted for at least 6 years in a majority of infected flocks in NSW and Victoria and is of concern if sheep are to be traded from these flocks or vaccination ceases. However the data from Kangaroo Island are encouraging and suggest that the second generation vaccinates have greater protection from shedding than the first generation vaccinates and will likely present a substantially lower risk of transmission of the disease.

While vaccination against OJD virtually eliminates clinical disease the results of these trials in commercial flocks indicates that, at least in the first 6 years after commencing a lamb vaccination program, shedding is substantially reduced but is negative in only a small proportion of flocks (17.5% negative and 82.5% shedding in Trial 0309) and continues at a level that would suggest cessation of vaccination would result in recrudescence of disease and sales of vaccinates would potentially transmit OJD. Leaving wethers unvaccinated and introducing unvaccinated sheep were identified as high risk practices. It should be noted that vaccine has been available for a relatively short period and most of the flocks monitored would have only recently sold off unvaccinated sheep. Re-examination of vaccinating flocks when they consist entirely of vaccinated sheep born from approved vaccinates (i.e. second generation vaccinated flocks) is highly desirable.

Acknowledgements
The contribution of Dr Evan Sergeant, John Seaman, Deb Lehmann, Andrew Ewars and Peter Whyte and the many producers and field and laboratory support staff working for and collaborating with the research institutions is gratefully acknowledged. Trials 009 and 033 were funded by industry through Meat and Livestock Australia. Trial 0309 was funded through the industry partnership program with thanks to Meat and Livestock Australia plus WoolProducers Australia and Sheepmeat Council of Australia through Animal Health Australia courtesy of Lorna Citer. The Wether Trial was supported by the McGarvie Smith Institute Fund. Studentships for two final year BVSc Honours projects in Trial 0309 conducted by Josie Gollan and Amy Masters, were provided by the Australian Wool Education Trust.

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6 OJD in cattle case study 1 (Project P.PSH.0206, 2006-2008)

Principal Investigator: Sally Ridge
Research Agency: Department of Primary Industries, Victoria

Johne's disease is a chronic granulomatous enteritis of ruminants and camelids that is caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*). In cattle, the disease typically presents as intractable diarrhoea and severe wasting in mature animals.

The observation that cattle do not become clinically affected by Johne's disease when grazing with infected sheep for extended periods and the fact that culture media required for laboratory growth of isolates from sheep or cattle strains are different, support the hypothesis that there are distinct "strains" of *Mptb*; a cattle (C) strain that predominantly affects cattle but is also capable of infecting goats, deer and alpaca, and a sheep (S) strain that predominantly affects sheep. Recent Australian studies using polymorphism in the IS1311 element and other DNA techniques have confirmed this general proposition (Whittington *et al*, 2000; Whittington *et al*, 2001, Cousins *et al*, 2000).

However, it is now clear that this species-strain adaptation or preference is not absolute. S strain *Mptb* was retrospectively diagnosed from archived histological sections collected in the late 1990s from three individual cattle from three NSW properties (Whittington *et al*, 2001) and investigation of clinical Johne's disease cases in Victorian cattle has identified further cases of cross-infection.

Detection of S strain *Mptb* in cattle raises the possibility that control of Johne's disease using current strategies which assume there is no cross-infection between cattle and sheep could, on occasions, be compromised.

This project was undertaken to determine the within herd distribution of S strain *Mptb* infection in cattle in a single beef cattle herd in the Ballarat region of Victoria, to provide a better understanding of the epidemiology of S strain *Mptb* infection in cattle and assist in the development of control strategies for other similarly affected herds.

Blood, faecal and tissue samples from all the cattle, at or before, the time they were slaughtered were obtained and samples were processed and examined for evidence of *Mptb* and for S strain in particular. Herd management information was collected to enable a profile of the Johne's disease situation within the herd to be developed. This included purchase records, grazing history, calving records, age, sex and exposure to infected sheep or sheep faeces as calves.

6.1 Results

In total, 73 head of cattle were sampled before and after slaughter with 15 animals (20.5% of the herd) returning positive results to at least one of the non-serological tests for *Mptb* infection applied in this herd. Fourteen animals (19.2% of the entire herd) returned positive results to at least one of the definitive, post-mortem (histology or tissue culture) tests for *Mptb* infection applied. The positive animals ranged in age from 15 months to 6 years.

Initial (ante-mortem) testing in the herd using ELISA and faecal culture suggested that infection with S strain *Mptb* in the study herd was confined to cows in the 5 and 6-year old age groups. Further investigation including thorough post-mortem examination and testing revealed that infection was widespread throughout the herd. All infected cattle from which organisms could be cultured were shown to be infected with S strain *Mptb*.

The sensitivity of each of the tests employed in this study was determined by comparing the number of animals detected by the test with the total known to be infected. No combination of ante-mortem tests provided a test regime with more than 36% sensitivity (see Table 1).

Table 1. Sensitivity of diagnostic tests (AGID, ELISA, faecal culture, histopathology and bacteriological culture of tissues following slaughter) when applied to 14 animals from a beef herd in the Ballarat region deemed to be infected* with S strain *Mycobacterium paratuberculosis* (*Mptb*).
*** Infection with *Mptb* was deemed to be confirmed if tissue samples collected at slaughter were found to positive on histological examination or on bacteriological culture.**

Diagnostic test	Number of cattle test positive	Test sensitivity	95% Confidence interval
AGID	0	0	–
ELISA	4	28.6%	5% - 52%
Faecal culture	4	28.6%	5% - 52%
ELISA and faecal culture in parallel	5	35.7%	10.6% - 60.8%
Histopathology	10	71.4%	47.8% - 95.1%
Tissue Culture	11	78.6%	57.1% - 100%

In addition to determining the prevalence of disease in the study herd we sought to examine the relationship between possible risk factors for disease transmission to cattle from sheep. Co-grazing of cattle (in particular calves) and ovine Johne's disease (OJD) infected sheep, direct exposure of cattle to infected pastures (cattle following infected sheep) and indirect exposure of cattle to infected pastures (cattle grazing paddocks contaminated by run-off from OJD infected neighbouring flocks) were all occurring on the study property from at least 2002. It is likely that increasing severity (number of clinical cases or high mortality), prevalence and duration of OJD infection in the resident and neighbouring flocks increases the likelihood of cattle acquiring S strain infection.

Other factors such as drought and hand feeding sheep and cattle (leading to grazing close to the ground for both cattle and sheep) are thought to increase *Mptb* transmission to cattle. These factors were present in the recent history of the study herd.

This study has implications for the current Johne's disease control and accreditation programs in south eastern Australia. Historically, it has been assumed that cattle do not become clinically affected by Johne's disease when grazing with *Mptb* infected sheep for extended periods and that cattle to cattle transmission of S strain *Mptb* does not occur. Many properties undertaking OJD control programs run cattle as an additional or alternative enterprise. The owners and managers of OJD infected flocks (and their advisors) should consider the risk factors that create favourable circumstances for cross species transmission of *Mptb*. Specifically, the results of this study support recommendations that:

- When cattle are being reared in OJD endemic areas care should be taken to ensure that they are not exposed directly or indirectly to infected sheep or manure (including contaminated run-off from neighbouring properties) until the cattle are at least 12 months old.
- Care should be taken when hand feeding cattle to avoid areas of high sheep manure build up.

- Diagnostic testing of suspect clinical cases should routinely involve strain typing of any cultivated *Mptb*.
- Disease control and accreditation programs for both OJD and for bovine Johne's disease should include consideration of the possibility that S strain *Mptb* may be transmitted to cattle

Although S strain *Mptb* infection of cattle is currently a sporadic and relatively rare event, changes to the current use of alternative species (cattle) for pasture decontamination of S strain *Mptb* are warranted.

6.2 Conclusions

- Diagnostic testing of suspect clinical cases of bovine Johne's disease should routinely involve strain typing of any cultivated *Mptb*.
- Existing diagnostic tests may be used to detect cattle infected with S strain *Mptb*. Strain typing is required to distinguish C and S strain infections.
- This study provided the first documented estimates of the sensitivity of diagnostic tests for S strain *Mptb* infection in cattle.
- Ante-mortem tests (ELISA and faecal culture) have poor sensitivity except when applied to cattle in the latter stages of disease pathogenesis (5 years old and older).
- The true prevalence and distribution of S strain *Mptb* infection in a herd is therefore difficult to estimate using existing ante-mortem diagnostic tests.
- The on-going source of infection of cattle in this study remains unclear but could include infected and shedding adult cattle, infected and shedding sheep resident on the study property or organisms in the environment originating from contaminated water draining from neighbouring OJD affected properties.
- Disease control and accreditation programs for both OJD and for bovine Johne's disease should include consideration of the possibility that S strain *Mptb* may be transmitted to cattle
- Taking the most cautious approach, only adult cattle should be grazed on areas recently grazed by known OJD infected sheep or contaminated by run-off from adjacent land holdings that may have grazed OJD infected sheep.

6.3 References

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7 OJD in cattle case study 2 (Project P.PSH.0301, 2007-2008)

Principal Investigator: Nicky Stone and Iain McLaren
Research Agency: Department of Primary Industries, Victoria

Johne's disease (JD) is a chronic gastrointestinal wasting disease of some importance in cattle and sheep populations, caused by *Mycobacterium avium* subsp. *paratuberculosis* (*Mptb*), which can survive in the environment for months.

There are distinct strains of *Mptb*; a cattle (C) strain that affects predominantly cattle (but is also capable of infecting goats, deer and alpaca), and a sheep (S) strain predominantly affecting sheep. Retrospectively, *S* strain *Mptb* has been diagnosed in cattle on at least eight New South Wales (NSW) properties, and in 1999 and 2005 cattle with clinical signs were diagnosed with *S* strain *Mptb* in Victoria. These properties had known ovine Johne's disease (OJD) infection in sheep.

This study was undertaken following the diagnosis of *S* strain *Mptb* infection a seven year old cow in a MN2 stud beef herd in the Ballarat region of Victoria that had been managed in accordance with the National Market Assurance Program for Cattle (CattleMAP) on a property without sheep.

The Australian Johne's Disease Market Assurance Program, an audited quality assurance program incorporates animal health risk assessment, testing and movement control that provides low risk animals for the various industry sectors.

To determine the within herd distribution of *S* strain *Mptb*, to improve understanding of S strain *Mptb* infection, and to examine possible risk factors for disease transmission, the remaining 55 head of cattle were slaughtered and samples collected and examined for *S* strain *Mptb* .

The index case was the only animal found to be infected in the herd, and the route of infection could not be determined given the results. There was no evidence of within herd spread of infection, which is more significant as time increases since initial infection of the index case.

Bovine Johne's disease

8 Bovine Johne's Disease: Basic and Applied Research for Improved Diagnosis and Prevention (Project P.PSH.0297, 2008-2011)

Principal Investigator: Richard Whittington
Research Agency: University of Sydney

8.1 Background

The diagnosis of bovine Johne's disease remains a problem because until recently there has been very little basic research conducted anywhere in the world, and most of the current knowledge is based on the study of human tuberculosis from the early 1900s. Practical applications of this include tests used today: culture, histopathology and intradermal skin tests, none of which currently are very sensitive. The lack of basic knowledge has severely limited the development of new diagnostic tests and vaccines. Therefore this project included components of basic research, as well as translational research aimed at delivering practical tools for industry in the near term.

This project was designed to use basic research and combine it with strategic elements to discover new test options and to improve existing tests. The project involved state of the art methods in microbiology, immunology, molecular biology and genomics in a multidisciplinary team with international collaborations to achieve its objectives.

8.2 Major outcomes

As a result of the project it is now known that:

- interferon gamma tests can be improved and made practical for use in cattle in Australia, but specific antigens are required to obtain adequate specificity
- faeces can be tested quickly and accurately using direct PCR
- immune suppression and weight loss during bovine Johne's disease may be explained by dysregulation of amino acid (tryptophan) metabolism
- new antibody and cytokine-based tests on blood appear to have limitations
- a blood test based on cell proliferation may be predictive of infection in cattle
- it is possible to reproduce the disease in a natural form in a controlled experimental situation, opening up options for test evaluation, vaccine development and other studies
- gene expression studies can reveal the dominant features of the early immune response, opening up new avenues for research on diagnosis and prevention

A further objective of the project was to ensure that there is a credible team of researchers available to Australian beef cattle producers, and this also was achieved. Some of the findings of this project have been published already and the project team has an international reputation.

As a result of the project it is now clear that a diagnostic test which had severe practical and technical limitations can be improved and may be of substantial benefit in the near future. This is the direct faecal PCR test which can provide results to producers within a few days instead of the current 3 months for culture. In addition

there are several new research-level tests of immune function that require further development.

8.3 Direct faecal PCR test

The direct faecal PCR test will be a breakthrough for the beef cattle industry. Previously, faecal samples were collected, sent to a laboratory and 3 months would elapse before negative test results could be confirmed. For cattle sales this meant considerable forward planning and great inconvenience for the producer. Where culture was used to confirm a suspected herd infection, for example after positive or suspect ELISA test results, the long delay caused considerable additional anxiety for the producer. The new test overcomes these problems because it can provide results within a few days of receipt of samples at a laboratory. It will cost no more than culture. Furthermore it is suitable for testing pooled faecal samples, which enables a cheap method of herd testing, either to detect infection or to show that it is not present in herds in the Market Assurance Program. The test will also be suitable for environmental testing. Additional validation of this test was recommended by the JD Research Advisory Group to provide better estimates of sensitivity and specificity. This was due to there being only a low number of samples of suitable quality from infected herds, and few culture positive samples, despite appropriate effort to obtain these. An unexpected finding of the research was a requirement for faecal samples to be stored at -80°C prior to PCR – many samples obtained from infected herds had not been stored appropriately at a commercial laboratory. A request for funding to obtain appropriate samples has been submitted to MLA; faecal samples will be obtained from a Financial non Financial Assistance Program beef herd in Tasmania which has a high prevalence of ELISA reactors. Final data will be submitted to the SubCommittee on Animal Health Laboratory Standards (SCAHLS) for approval for use of the test in the National Johne's Disease Program later in 2011.

8.4 Whole blood interferon gamma and lymphocyte proliferation tests

A whole blood interferon gamma assay which was developed to prototype stage for the detection of OJD in a previous project has been modified for cattle in this project. Previously it was necessary to ship blood samples to a laboratory and test them within 8 hours of collection – something that usually was impossible. Two blood additives were trialled to extend the life of the blood samples to 48 hours. This would make it possible to ship samples from most places in Australia to a laboratory in time to conduct the test. The additive may have worked, but there was concomitant loss of specificity. Additional research is now required to find a way to make the test more specific, and this will be done in project P.PSH.0576, with a goal of validating the new procedure within the life of that project. Interferon gamma detection assays offer the potential to detect more infected animals at an earlier stage of the disease compared to an antibody ELISA or direct detection of Mptb in the faeces. This may provide opportunity for control strategies aimed at removal of young infected animals before they start shedding bacteria into the environment.

Also in the basic research program, the ability of white blood cells to remember contact with Mptb has been tested in a proliferation assay in experimentally infected cattle – remarkably the response in non-exposed controls remained low while it increased and remained elevated in exposed cattle.

8.5 Gene expression studies

Gene expression studies in infected cattle in the early stages of infection revealed a remarkable pattern of regulation of genes responsible for immunological processing.

This uncovered pathways that previously have not been suspected to play much of a role in the early development of Johne's disease. This new knowledge will be applied in a future project.

8.6 Mechanism of disease progression

A contributing factor in the weight loss that occurs in bovine Johne's disease may have been found – in addition to intestinal malabsorption and diarrhoea, an amino acid deficiency induced by the infection may contribute to wasting. Blood levels of the amino acid tryptophan were shown to plummet as Johne's disease develops in a sheep model. Expression of the enzyme that breaks down this amino acid was shown to be elevated in the blood of cattle exposed to Mptb. This relates to manipulation of the host by the mycobacterium, which induces the cow to destroy its own tryptophan, and impacts the way the immune system of the animal functions.

Further research will be conducted across these fundamental discoveries to maximise their potential.

8.7 New experimental infection model

From the basic research program a method was proven for creating experimental bovine Johne's disease in a herd under tightly controlled conditions, leading to realistic and natural outcomes. Surgical biopsy of the intestine on two occasions confirmed that the animals had become infected, were developing Johne's disease at different rates, and did not have unrealistically severe infection which has been so common in overseas studies. This approach will be invaluable for diagnostic test and vaccine development studies and has already been adopted by overseas researchers – we expect that Australian producers will benefit from such collaborative international studies.

9 Pooled faecal culture for low-shedding cattle (Project P.PSH.184, 2005-2006)

Principal Investigator: Graeme Eamens
Research Agency: NSW Department of Primary Industries
Reference: Eamens GJ, Walker DM, Porter NS, Fell SA (2008). Radiometric pooled faecal culture for the detection of Mycobacterium avium subsp. paratuberculosis in low-shedder cattle. *Aust Vet J*. 86: 259-265

Whole herd faecal culture, based on individual culture of samples, is recognised as a sensitive, but expensive diagnostic tool to evaluate herd infection rates of *M. avium* subsp. *paratuberculosis* (*Mptb*) in cattle. Pooled faecal culture (PFC), based on radiometric (Bactec) culture procedures with confirmation by IS*900* PCR and REA, has been proven to offer cost savings in detecting and evaluating infection rates in sheep flocks, and prior studies at EMAI indicated this technique was of merit in cattle. In the earlier studies, samples from cattle that were shedding moderate to high levels of *Map* were investigated. To augment that work, this study used similar radiometric culture procedures and confirmatory testing steps to investigate pooling rates suitable to low shedder cattle. In addition, since prior work with sheep samples by Reddacliff *et al* (2003a) showed *Mptb* concentrations in inocula for Bactec culture correlate with their growth rate in the culture media, this approach was adopted to quantify the *Mptb* shedding rate of the animals under test.

The case definition in the selection of "low shedder" cattle was based on slow growth of *Mptb* on initial radiometric faecal culture. Such samples were selected on the results of their initial diagnostic culture, if initial growth (as a growth index measured weekly after inoculation) was only evident at 5 or more weeks of an 8 week incubation period. From 14 faeces which met this criterion, and had been stored for up to 17 months at -80^{o}C, eight were found to yield *Mptb* on subsequent culture, including evaluation studies when samples were mixed with normal cattle faeces at pooling rates of 1:5, 1:10, 1:20, 1:25. 1:30 and 1:50. All samples were processed using procedures similar to those employed for OJD PFC. This included a 12 week incubation period, but all subcultures were made on Herrold's egg yolk medium instead of modified 7H10 media, since the former is more suitable for growth of cattle strains of *Mptb*.

Since the growth of sheep (S) strains of *Mptb* from ovine faeces (Reddacliff *et al* 2003a) may differ from cattle (C) strains from bovine faeces, regression equations were developed to define the relationship between the number of *Mptb* cells in the bovine faecal culture inoculum and the number of days to reach a cumulative growth index of 1000 (cgi1000). Our prior study of moderate to heavy shedders, and based on two representative animals (1085 and 38) in that study had defined such a regression equation. To augment that information, six samples from the original 14 were selected at random and their processed culture inocula subjected to a 10-fold dilution series in Bactec broth (replicated 5-fold per dilution) to determine the relationship for low shedder cattle. Of these six, *Mptb* growth occurred in four animals, but only one (sample 15) yielded sufficient growth at multiple dilutions to determine a reliable regression equation between the $\log_{10}$ inoculum (as determined by the Most Probable Number or MPN method) and the number of days to cgi1000 (dcgi1000). This data was added to that already established from prior results for animals 1085 and 38 to produce a final regression equation to describe the relationship between the rate of Bactec growth and the number of *Mptb* in the inoculum as follows:

$$\log_{10} \text{inoculum} = 6.55 - 0.121 \text{ dcgi1000}$$

This equation was then used to estimate, from the rate of growth in both the 10 fold dilution series and the growth in the PFC dilution series, the number of organisms inoculated from the original faeces prior to processing. Since Reddacliff *et al* (2003b) estimated a 1.7 log (50 fold) loss in viable cell concentration of S strains of *Mptb* due to routine decontamination procedures as used in this study, and allowing for dilution

steps to reach the final inoculum to Bactec, the original numbers of viable cells of *Mptb* per gram of faeces prior to culture of each positive animal were estimated. These methods indicated that the samples from the eight low shedder cattle generally contained between 10^2 and 10^5 viable *Mptb* cells per gram of faeces.

At pooling rates greater than 1:5, PFC sensitivity was found to be low in the low shedder cattle, especially those shedding ≤ 10^4 *Mptb* organisms/g of faeces. In addition, an incubation period of 10 weeks was necessary to maximise detection of low shedder cattle at a dilution rate of 1:5.

These results indicate that, for optimal results from pooling of bovine faeces, a dilution of 1:5 is recommended to detect cattle shedding low levels of *Mptb.* At higher dilutions, only animals shedding 10^4 *Mptb*/g or higher would be detected. Based on current laboratory fees, the laboratory costs for whole herd testing of infected herds where clinical signs of Johne's disease are not apparent can be reduced by approximately 35%.

References

Reddacliff LA, Nicholls PJ, Vadali A, Whittington RJ (2003a). Use of growth indices from radiometric culture for quantification of sheep strains of *Mycobacterium avium* subsp. *paratuberculosis*. *Appl Environ Microbiol.* 69: 3510-3516

Reddacliff LA, Vadali A, Whittington RJ (2003b). The effect of decontamination protocols on the numbers of sheep strain *Mycobacterium avium* subsp. *paratuberculosis* isolated from tissues and faeces. *Vet Microbiol.* 95: 271-282

10 Validation of Pooled Faecal Culture for Caprine Johne's Disease (Project AHW.080, 2005-2006)

Principal Investigator: Graeme Eamens
Research Agency: NSW Department of Primary Industries
Reference: Eamens GJ, Walker DM, Porter NS, Fell SA (2007). Pooled faecal culture for the detection of Mycobacterium avium subsp paratuberculosis in goats. *Aust Vet J*. 85: 243-251

Whole herd faecal culture, based on individual culture of samples, is recognised as a sensitive, but expensive diagnostic tool to evaluate herd infection rates of *M. avium* subsp. *paratuberculosis* (*Mptb*) in goats. Pooled faecal culture (PFC), based on radiometric (Bactec) culture procedures with confirmation by IS*900* PCR and REA, has been proven to offer cost savings in detecting and evaluating infection rates in sheep flocks at a 1:50 pooling dilution, but this technology has not been validated in goats. This study evaluated PFC at dilutions ranging from 1:5 to 1:50 in goats shedding a wide range of concentrations of *Mptb*. Since prior work with sheep samples by Reddacliff *et al* (2003a) showed *Mptb* concentrations in inocula for Bactec culture correlate with their growth rate in the culture media, this approach was adopted to quantify the *Mptb* shedding rate of the animals under test. Faeces from 17 goats naturally infected with the cattle (C) strain and four goats naturally infected with the sheep (S) strain of *Mptb* were evaluated.

Of 21 faeces from goats previously confirmed culture positive for *Mptb*, and stored at -80^{o}C for up to 4 years, 14 were found to yield *Mptb* on subsequent culture, including evaluation studies when samples were mixed with normal goat faeces at pooling rates of 1:5, 1:10, 1:20, 1:25. 1:30 and 1:50. An additional two samples yielded *Mptb* only on undiluted samples, and both were only intermittently positive in undiluted faeces. All samples were processed using procedures similar to those employed for OJD PFC, including a 12 week incubation period. Depending on the infecting strain, subcultures were made on Herrold's egg yolk medium (for C strain) or modified 7H10 media (for S strain), as the former is more suitable for growth of cattle strains of *Mptb,* and S strains only grow on modified 7H10 medium and not Herrold's.

Since the growth of S strains of *Mptb* from sheep faeces (Reddacliff *et al* 2003a) may differ from that of C or S strains from goat faeces, regression equations were developed to define the relationship between the number of *Mptb* cells in the caprine faecal culture inocula and the number of days to reach a cumulative growth index of 1000 (cgi1000). To quantify the shedding rate, endpoint titrations (Most Probable Number or MPN method) were undertaken from 10 samples from the original 21, selected at random. Their processed culture inocula were subjected to a 10-fold dilution series in Bactec broth (replicated 5-fold per dilution) to determine the relationship for goats. Of these 10, *Mptb* growth occurred in 9 animals, but only 5 (samples 2, 5, 8, 9, 21) yielded sufficient and repeatable growth at multiple dilutions to determine a reliable regression equation between the $\log_{10}$ inoculum (as determined by the MPN method) and the number of days to reach cgi1000 (dcgi1000). This data produced a final regression equation to describe the relationship between the rate of Bactec growth and the number of *Mptb* in the caprine faecal inoculum as follows:

$\log_{10}$ inoculum = 7.3 – 0.121 dcgi1000

This equation was then used to estimate, from the rate of growth in both the 10 fold dilution series and the growth in the PFC dilution series, the number of organisms inoculated from the original faeces prior to processing. Since Reddacliff *et al* (2003b) estimated a 1.7 log (50 fold) loss in viable cell concentration of S strains of *Mptb* due to routine decontamination procedures as used in this study, and allowing for dilution steps to reach the final inoculum to Bactec, the original numbers of viable cells of *Mptb* per gram of faeces prior to culture of each positive animal were estimated. These methods indicated that the samples from the 16 culture positive goats generally

contained between 10^2 and 7 x 10^6 viable *Mptb* cells per gram of faeces, and those that were culture positive when diluted with normal faeces were estimated to have contained > 1.5 x 10^3 *Mptb*/g.

At pooling rates up to 1:25, PFC was able to detect 13 of the 14 goat samples from goats shedding > 1.5 x 10^3 *Mptb*/g of faeces. In addition, an incubation period of 10 weeks was necessary to maximise detection of goats at a dilution rate of 1:25.

These results indicate that, for optimal results from pooling of caprine faeces, a dilution of 1:25 is recommended to detect goats shedding low to moderate levels of *Mptb.* At a 1:50 dilution, only animals shedding 10^5 *Mptb*/g or higher would be detected. Based on current laboratory fees, the laboratory costs for whole herd testing of goat herds for Johne's disease can be reduced by approximately 40% relative to serology and 75-90% relative to individual faecal culture.

References

Reddacliff LA, Nicholls PJ, Vadali A, Whittington RJ (2003a). Use of growth indices from radiometric culture for quantification of sheep strains of *Mycobacterium avium* subsp. *paratuberculosis*. *Appl Environ Microbiol.* 69: 3510-3516

Reddacliff LA, Vadali A, Whittington RJ (2003b). The effect of decontamination protocols on the numbers of sheep strain *Mycobacterium avium* subsp. *paratuberculosis* isolated from tissues and faeces. *Vet Microbiol.* 95: 271-282

11 Epidemiology and control of bovine Johne's disease in beef cattle herds (Project P.PSH.0204, 2006-2008)

Principal Investigator: John Larsen
Research Agency: Mackinnon Project, University of Melbourne Veterinary School

Bovine Johne's disease (BJD) is a chronic wasting disease caused by the bacterium *Mycobacterium paratuberculosis*. Infection usually occurs in calfhood, with a significant age-related resistance to infection apparent after 12 months of age. Over time, the immune response to the presence and multiplication of the bacteria causes progressive thickening of the intestinal wall. This reduces absorption of nutrients by an infected animal, leading to a gradual loss of body condition, persistent diarrhoea and eventually death.

This project was initiated by The Cattle Council of Australia, the peak industry body representing cattle producers, to limit the potential impact of BJD on beef production, domestic and international trade and food safety. The project aimed to identify risk factors for the introduction and establishment of BJD into affected beef herds. This information could then be used to guide policy development to minimise the risk of spread of BJD infection to beef herds and reduce the contamination of beef product with *M. Paratuberculosis*.

Herds included in this study had their BJD infected status allocated between 1991 and December, 2005. There were 49 herds from Victoria, 59 from NSW and one from South Australia included in the analysis. This reflected the number of beef herds with usable records, but may not be an accurate estimate of the prevalence of BJD in beef herds within each state. Herds were excluded if they contained less than 30 breeding cows or if they also operated as a dairy farm.

In two-thirds (73) of the herds, the first detected case (the 'index' case) was an introduced animal and 36 herds had an index case that was bred on the farm. Of the homebred index cases, infection was most likely associated with dairy cattle in 14 cases (39%) and with introduced beef cattle in 10 cases (28%). However, the likely source of infection was unable to be identified in 12 of these cases (33%).

Overall, the index case was recorded as a beef breed in 77% and a dairy breed in 23% of cases (84 and 25 herds, respectively). This meant that the index cases were 3.4 times more likely to be beef derived cattle, although detailed records were not known for 64% (46 of 72) of introduced index cases. Thus, in addition to the introduction of infected beef cattle, dairy breeds, or a prior association with dairy breeds, were important risk factors for the introduction of BJD infection (see Tables 5, 8 and 9). For example, where the source farm of an introduced index case was known, 57% (15 of 26) were associated in some way with dairy cattle (Table 8).

An important implication of this is that beef herds may be exposed to the risk of introducing infection whilst the prevalence of BJD remains high in dairy herds. Consequently, programs that aim to control and eradicate BJD in beef herds will need to be undertaken on a continuing and long-term basis, creating a potentially large and ongoing financial demand on the beef industry. However, Beef Only sales and effective on-farm biosecurity practices are measures that can potentially control this exposure. For example, the vendor declaration required for Beef Only sales would have excluded the 24 dairy or dairy cross animals identified as index cases from Beef Only sales, plus many of the 17 beef cross bred index cases that were identified (see Table 2).

Cattle from most of the beef breeds that form the basis of the Australian beef industry were identified as being infected in the survey. Importantly, because of the insidious nature of the disease and the way it spreads between herds, there is the potential for

any beef breed to become a secondary risk for the introduction of BJD if it is allowed to establish and spread by uncontrolled trading within that breed.

About two thirds of infected herds were first identified through veterinary investigation of clinically sick animals. This emphasises an important role for the beef industry in funding disease monitoring programs aimed at detecting changes in the pattern of existing diseases and the emergence of new diseases.

In general, the control programs that were implemented were successful in progressing infected herds back to the equivalent of a 'non-assessed' (NA) herd status in each state. The exceptions were 'test and cull' programs, which were less successful unless they were combined with the culling of known high risk animals. However, no state has an ongoing program to assess the success of control and eradication programs currently being implemented in infected herds. This is an obvious deficiency, and so the apparent success of eradication programs within previously infected herds should be investigated in more detail.

The major factors that motivated producers to eradicate BJD were the high probability that the program would be successful, and that this would then lead to the removal of restrictions on the sale of animals and land. The social stigma of being the owner or manager of an infected herd was of less importance, but was still rated as very important by 51% of respondents. Access to financial assistance, the possibility of increased mortality rates in infected herds and the potential impact of BJD on the quality of products produced by a beef herd were all rated as unimportant when producers were making a decision about whether to eradicate BJD. However, many of the producers surveyed would not have had access to financial assistance because their herds were infected with BJD in the 1990s, whereas financial assistance was not available until 2003.

Modelling of the financial impact of BJD in infected herds showed that the impact of BJD within commercial herds will vary with the management and marketing strategies adopted. In general, the financial impact of BJD is associated with attempts to eradicate the disease and restrictions on stock sales, rather than production losses. For commercial producers, those relying on store markets will be most affected, whereas the impact within herds selling cattle direct to feedlots or finished cattle for slaughter will be insignificant, in terms of discounts on the value of sale stock and decreased farm income. However, for some producers the reduced flexibility of their sale options, through having to sell stock only for slaughter, may be more significant.

Results of financial modelling demonstrated that the impact of BJD within commercial beef herds can be reduced if affected producers change enterprises to target finishing systems. However, not all properties have suitable land class and pasture quality to successfully implement profitable finishing systems.

Further, based on the assumptions made in the financial spreadsheet model it was found that if there was no price discount on sale stock, BJD would need to cause death rates in excess of 5% before either partial or total de-stocking was warranted. Alternatively, if sale cattle attracted a 10% price discount, then the death rates from BJD would need to exceed 1% before partial or total de-stocking was justifiable. Based on the data collected in this survey, mortality rates were negligible and the price discounts and death rates identified by the model were never exceeded. However, in some circumstances price discounts may exceed 10%, and so the cost of living with BJD will be more severe.

In other scenarios, where BJD was not eradicated using a test and cull strategy combined with partial de-stocking, trading restrictions were removed after 5 years, or after 2 years with total de-stocking. In these circumstances, the net present value (NPV) with total or partial de-stocking was still worse over 10 years, and significantly worse if the cost of restocking was increased by 10%. In addition, there are significant risks associated with de-stocking. These include the value received for sale stock,

decreased profitability of any intermediate enterprise, increased costs of restocking, failure to eradicate BJD and, ultimately, ending up with less profitable replacement stock and/or enterprises.

Although doing nothing in a commercial herd was initially more attractive from a cash flow perspective, the larger the discount on sale prices, the more important it was to eradicate BJD (assuming that BJD was effectively eradicated by the chosen program).

Data collected for this project pre-dates the availability of the Financial and Non-Financial Assistance Package which is currently available from industry, and owners of BJD affected herds who opted for herd disposal were currently clearly severely affected if sale cattle were sold at discounted prices, making the subsequent enterprise less profitable and restocking more expensive. However, the impact on individual herds is highly variable and this needs to be quantified on a case-by-case basis, as the losses are highly dependent on the existing productivity and sale strategies of each herd. In stud herds the detection of BJD and restrictions on cattle sales will severely compromise the viability of that enterprise and often cause the failure or indefinite suspension of the stud business.

Vaccination programs were not considered in this analysis, but if they become available they may be a realistic option to help improve trading options and limit the financial impact of BJD in the future, especially if attempts to regulate the disease continue.

Finally, it is important to clearly understand that the current programs and industry strategy, designed to provide assistance to affected producers and reduce the prevalence of BJD, will not eradicate BJD from the beef industry due to the constant potential for re-infection from introduced dairy or dairy cross-breed cattle, or when beef cattle are closely associated with dairy herds. However, as already noted, Beef Only sales and effective 'Biosecurity' practices on individual farms are measures that can potentially control this exposure.

12 Bibliography from projects OJD.031, P.PSH.0311 and P.PSH.0297

The following section lists publications and conference presentations arising from the projects OJD.031, P.PSH.0311 and P.PSH.0297 during the period 2007-2011:

12.1 Refereed publications

Begg, D. J., K. de Silva, et al. (2009). "Enzyme-linked immunospot: an alternative method for the detection of interferon gamma in Johne's disease." J Vet Diagn Invest **21**(2): 187-196.

Begg, D. J., K. de Silva, et al. (2011). "Does a Th1 over Th2 dominancy really exist in the early stages of Mycobacterium avium subspecies paratuberculosis infections?" Immunobiology **216**(7): 840-846.

Begg, D. J., K. de Silva, et al. (2010). "Experimental infection model for Johne's disease using a lyophilised, pure culture, seedstock of Mycobacterium avium subspecies paratuberculosis." Vet Microbiol **141**(3-4): 301-311.

Begg, D. J. and R. J. Whittington (2008). "Experimental animal infection models for Johne's disease, an infectious enteropathy caused by Mycobacterium avium subsp. paratuberculosis." Vet J **176**(2): 129-145.

Begg, D. and R. Whittington (2010). Paratuberculosis in sheep. Paratuberculosis: Organism, Disease, Control. M. A. Behr and D. M. Collins. Oxfordshire, U.K., CAB International**:** 157-168.

Bosward, K. L., N. K. Dhand, et al. (2010). "Optimization of a whole blood gamma interferon assay for the detection of sheep infected with Mycobacterium avium subspecies paratuberculosis." J Vet Diagn Invest **22**(2): 210-217.

Bower, K., D. J. Begg, et al. (2010). "Optimisation of culture of Mycobacterium avium subspecies paratuberculosis from blood samples." J Microbiol Methods **80**(1): 93-99.

Bower, K. L., D. J. Begg, et al. (2011). "Culture of Mycobacterium avium subspecies paratuberculosis (MAP) from blood and extra-intestinal tissues in experimentally infected sheep." Vet Microbiol **147**(1-2): 127-132.

de Silva, K., D. Begg, et al. (2010). "The early lymphocyte proliferation response in sheep exposed to Mycobacterium avium subsp. paratuberculosis compared to infection status." Immunobiology **215**(1): 12-25.

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Gumber, S., D. L. Taylor, et al. (2007). "Protein extraction from Mycobacterium avium subsp. paratuberculosis: Comparison of methods for analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis, native PAGE and surface enhanced laser desorption/ionization time of flight mass spectrometry." J Microbiol Methods **68**(1): 115-127.

Gumber, S., D. L. Taylor, et al. (2009). "Evaluation of the immunogenicity of recombinant stress-associated proteins during Mycobacterium avium subsp. paratuberculosis infection: Implications for pathogenesis and diagnosis." Vet Microbiol **137**(3-4): 290-296.

Gumber, S. and R. J. Whittington (2007). "Comparison of BACTEC 460 and MGIT 960 systems for the culture of Mycobacterium avium subsp. paratuberculosis S strain and observations on the effect of inclusion of ampicillin in culture media to reduce contamination." Vet Microbiol **119**(1): 42-52.

Gumber, S. and R. J. Whittington (2009). "Analysis of the growth pattern, survival and proteome of Mycobacteriumavium subsp. paratuberculosis following exposure to heat." Vet Microbiol **136**(1-2): 82-90.

Gumber, S., G. Eamens, et al. (2006). "Evaluation of a Pourquier ELISA kit in relation to agar gel immunodiffusion (AGID) test for assessment of the humoral immune response in sheep and goats with and without Mycobacterium paratuberculosis infection." Vet Microbiol **115**(1-3): 91-101.

Kawaji, S., D. J. Begg, et al. (2011). "A longitudinal study to evaluate the diagnostic potential of a direct faecal quantitative PCR test for Johne's disease in sheep." Vet Microbiol **148**(1): 35-44.

Kawaji, S., S. Gumber, et al. (2011). "Evaluation of the immunogenicity of Mycobacterium avium subsp. paratuberculosis (MAP) stress-associated recombinant proteins." Vet Microbiol.

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Marsh IB, Bannantine JP, Paustian ML, Tizard ML, Kapur V and Whittington RJ (2005). Genomic comparison of Mycobacterium avium subsp. paratuberculosis sheep and cattle strains by using microarray hybridization. Journal of Bacteriology 188: 2290-2293.

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Plain, K. M., A. C. Purdie, et al. (2010). "Toll-like receptor (TLR)6 and TLR1 differentiation in gene expression studies of Johne's disease." Vet Immunol Immunopathol **137**(1-2): 142-148.

Purdie, A. C., K. M. Plain, et al. (2011). "Candidate gene and genome-wide association studies of Mycobacterium avium subsp. paratuberculosis infection in cattle and sheep: a review." Comp Immunol Microbiol Infect Dis **34**(3): 197-208.

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Zhong, L., L. Di Fiore, et al. (2009). "Identification of differentially expressed genes in ileum, intestinal lymph node and peripheral blood mononuclear cells of sheep infected with Mycobacterium avium subsp. paratuberculosis using differential display polymerase chain reaction." Vet Immunol Immunopathol **131**(3-4): 177-189.

Zhong, L., D. Taylor, et al. (2011). "Biomarker discovery for ovine paratuberculosis (Johne's disease) by proteomic serum profiling." Comp Immunol Microbiol Infect Dis **34**(4): 315-326.

12.2 Conference Presentations (2005-2011)

9th International Veterinary Immunology Symposium,Tokyo Japan (2010)
Richard Whittington, Douglas Begg, Kumudika de Silva, Karren Plain and Auriol Purdie. Mycobacterial infection: lessons from tuberculosis and paratuberculosis.

Richard Whittington, Douglas Begg, Kumudika de Silva, Karren Plain and Auriol Purdie. Immunological characteristics of paratuberculosis as a mycobacterial infection.

Kumudika de Silva, Douglas Begg, Satoko Kawaji, Karren Plain, and Richard Whittington. Immune profile and faecal shedding of Mycobacterium avium paratuberculosis vary with lesion severity in Johne's disease.

Karren Plain, Kumudika de Silva, John Earl, Douglas Begg, Auriol Purdie and Richard Whittington. The role of indoleamine 2,3-dioxygenase (IDO) in response to Mycobacterium avium subspecies paratuberculosis infection.

Douglas Begg, Kumudika de Silva, Karren Plain, Auriol Purdie and Richard Whittington. Does a Th1 over Th2 dominance exist in the early stages of Mycobacterium avium subspecies paratuberculosis infections?

10th International Colloquium on Paratuberculosis in Minnesota, USA (2009)
Begg D, de Silva K, Di Fiore L, Talyor D, Bower K, Zhong L, Kawaji S, Emery D, and Whittington R. Development of an experimental infection model for Johne's disease using a lyophilised pure culture of Mycobacterium avium subspecies paratuberculosis

Bower K, Begg D, & Whittington R Detection of Mycobacterium avium subspecies paratuberculosis (MAP) using culture from blood during early, subclinical and clinical stages of disease.

Plain K, Begg D, Waldron A, de Silva K, & Whittington R. Development of an experimental infection protocol in cattle using a low passage cultured strain of MAP.

de Silva K, Begg D, Taylor D, & Whittington R. The early IL-10 response in ovine Johne's disease.

Australasian Society for Immunology Conference, Gold coast (2009)
de Silva K, Begg D, & Whittington R. Interleukin 10 response to mycobacterial challenge.

Australasian Society for Immunology Conference, Canberra (2008)
Begg D, de Silva K, and Whittington R. 2008. The IFN-γ to antibody switch – does it really happen in *Mycobacterium paratuberculosis* infections?

Plain KM, Begg D and Whittington RJ. 2008. Role of IDO in the response to *Mycobacterium avium* subspecies *paratuberculosis* infection.

9th International Colloquium on Paratuberculosis, Tsukuba, Japan (2007)
Begg D, de Silva K, Di Fiore, Taylor D, and Whittington R. Seeing spots, developing an IFN-gamma ELISPOT assay to detect ovine *M. avium* subspecies *paratuberculosis* infection.

Begg D, Taylor D, de Silva K, Di Fiore, and Whittington R. Experimental infection of sheep with *M.avium* subspecies *paratuberculosis*: a brief review and introduction to an Australian ovine challenge model.

Bower K, Begg D, Di Fiore L, Taylor D, and Whittington R. Culture of *M. paratuberculosis* from blood.

Browne S, de Silva K, Begg D, Whittington R and Emery D. Apoptosis of mononuclear cells in experimental and natural ovine Johne's disease

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