final report

Project code: P.PSH.0311

Prepared by: RJ Whittington, D Begg, K Bower, K de Silva, RB Gurung, I Marsh, K Plain and A Purdie

The University of Sydney

Date published: October 2011

ISBN: 9781741916478

PUBLISHED BY
Meat & Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

Ovine Johne's Disease: Applications of basic research on enhanced diagnosis and prevention

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication and acknowledges the contribution made by WoolProducers Australia and Sheepmeat Council of Australia, through Animal Health Australia.

This publication is published by Meat & Livestock Australia Limited ABN 39 081 678 364 (MLA). Care is taken to ensure the accuracy of the information contained in this publication. However MLA cannot accept responsibility for the accuracy or completeness of the information or opinions contained in the publication. You should make your own enquiries before making decisions concerning your interests. Reproduction in whole or in part of this publication is prohibited without prior written consent of MLA.

Abstract

The diagnosis of ovine Johne's disease remains a problem because until recently there has been little basic research conducted and this 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 used state of the art methods in microbiology, immunology, molecular biology and genomics in a multidisciplinary team with international collaborations to achieve its objectives.

As a result of the project it is clear that two existing diagnostic tests which had severe practical and technical limitations can be improved and may be of substantial benefit in the future. These include an interferon gamma blood test which can be made practical for use in sheep in Australia and a 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 new research-level tests of immune function that require further development.

A further objective of the project was to ensure that there is an experienced and internationally credible team of researchers available to Australian sheep producers, and this also was achieved.

Executive Summary

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.

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.

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.

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.

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.

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.

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.

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.

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.

Table of contents

1 Background 7
2 Project objectives 12
3 Methodology 12
4 Results 13
4.1 Subprogram 1. Diagnostics 16
4.1.1 Enhancement of IFN-γ blood test technologies 16
4.1.1.1 ELISPOT and Cell-ELISA 16
4.1.1.2 Improving the whole blood interferon gamma ELISA 16
4.1.2 Development of a direct faecal PCR test for commercialisation 19
4.2 Subprogram 2. Animal resources and experimental infection models 19
4.3 Subprogram 3. Fundamental research on host and pathogen genomics and host immune response 20
4.3.1 New antigens for assay development 20
4.3.2 A blood test based on the detection of IL-10 21
4.3.3 IgA isotype ELISA for detection of paratuberculosis using faecal samples 23
4.3.4 Detection of Mptb in blood samples 24
4.3.4.1 Culture of Mptb from blood samples 25
4.3.4.2 Detection of Mptb DNA in blood samples using PCR 25
4.3.4.3 Detection of free DNA from Mptb in blood samples 26
4.3.4.4 Summary 26
4.3.5 IDO, tryptophan catabolism and Mptb infection 27
4.3.6 Toll-like receptor (TLR)6 and TLR1 differentiation, and TLR9 and TLR10 gene expression studies of ovine Johne's disease 27
4.3.7 Immunological assays based on cell proliferation 27
5 Discussion/conclusion 28
6 Bibliography 31
Acknowledgements 34

1 Background

Ovine Johne's disease (OJD) caused by *Mycobacterium avium* subsp. *paratuberculosis* (Mptb) remains a significant issue for the sheep industries throughout southern Australia. It can be responsible for significant losses if left uncontrolled. Vaccination to reduce the prevalence of clinical disease within infected flocks has been very successful, with widespread adoption by affected producers. In addition, vaccination is used to improve flock status in a risk based trading scheme. However, vaccination does not prevent infection, rates of shedding of Mptb remain high on several properties where the vaccine has been used and there are injection site reactions in sheep and occupational health and safety risks for producers. Furthermore, some producers have ceased to vaccinate due to reductions in flock mortality rates, high cost of vaccine and reduced farm incomes under drought conditions. In addition, producers in some regions where OJD is uncommon or absent continue to exclude sheep from endemic regions because it is recognised that a percentage of vaccinated sheep from infected areas will be shedding bacteria, and this is impacting on trading options for producers. Food safety (milk and meat) is also a concern. Although controversial and still unproven, Mptb has been proposed as a potential cause of Crohn's Disease in humans. Thus, contamination of meat with this bacterium is of importance to both the sheep meat and beef industries. For these reasons it is necessary to maintain a research program on OJD to underpin future options for control of this disease.

Internationally, Johne's disease (JD) is considered to be a significant threat to the livestock sector, regardless of the species affected. Several studies have confirmed direct economic loss, but a greater threat exists because of a perceived link with Crohn's disease, and therefore concern exists about ensuring future market access for livestock products. Public health authorities in many developed countries have adopted a neutral position on the possible link between Mptb originating in livestock and the occurrence of the organism and disease in humans. Nevertheless, animal health authorities in many countries have introduced, or plan to introduce, control programs for JD. These will depend on accurate diagnostic tests, which are still lacking. Consequently there are large research programs on JD in the EU/Europe, Japan, North America and New Zealand and smaller research programs in many other places. These countries are trading partners and/or competitors of the Australian red meat industries. For market access insurance, Australia needs to be engaged with R&D at an international standard. Currently there is very little basic research on JD in Australia other than that in existing MLA projects. This research program has led to substantial Australian capacity in this field of research. The research needs for OJD are complementary to those for BJD.

There are parallels between the needs in JD research and those in tuberculosis in humans, which is caused by a related bacterium. Writing recently in the international journal Tuberculosis, Izzo and others (2005) noted that "The model established by the National Institute of Health to achieve their goals in the TB vaccine development program include an assortment of tasks such as identifying mechanisms of host defense, improving animal models and conducting Phase I/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 laudable aims of JD research programs worldwide will have the same challenges and difficulties as those for tuberculosis.

Mptb bacteria are spread via the faeces of infected sheep, and they may persist in the environment for prolonged periods given the right conditions. Young lambs are the most susceptible to infection, and most infections occur within the first 6-12 months of life. Bacteria ingested from the environment infect the lining of the gut, resulting in a slowly progressing, chronic inflammation. The consequences of this are an inability to absorb nutrients, resulting in chronic wasting and death. OJD is a very slow progressing disease with the average incubation period (time from infection to clinical signs) of 3 years (range 1.5-5 years). There are no effective treatments available. Infected animals usually start shedding bacteria into the environment prior to the development of any signs of clinical disease.

This project is a program of basic and applied research that aims to develop new diagnostic tests for OJD and, through a program of basic research on the pathogenesis of the disease, increase understanding of the immune response to infection, dormancy of the bacterium and how this relates to chronic infection and transmission of the bacterium.

The project is part of MLA's commitment to the National JD Program. It is an extension of the basic research project OJD031 which was funded under the National Ovine Johne's Disease Control Program. Research breakthroughs in that project led to outcomes which were further evaluated and developed in this project.

Arguably the greatest knowledge gaps in OJD include:

- the means to ensure accurate early diagnosis
- how to differentiate resistant sheep from susceptible sheep
- the means for rapid, efficient detection of super-shedders which contaminate the environment
- the immune response and how this leads to resistance
- dormancy of Mptb and how this relates to chronic infection
- ways to induce strong immunity without injection site reactions in sheep and occupational health and safety risks

This project concerns the diagnosis and prevention of Johne's disease in the future. The two topics of diagnosis and prevention are linked – if the infection can be diagnosed effectively, its spread can be prevented by identifying all infected animals and intervening in a practical way. In a complementary approach, if the disease process within animals (termed pathogenesis) was understood fully, new vaccines that either prevent infection or drive an effective immune response towards recovery from infection could be developed. A brief review of both diagnosis and pathogenesis is needed to place this research project into context.

Current approaches to diagnose the infection

There are two overarching themes for diagnosis of Johne's disease – detection of the pathogen, and detection of host responses directed against the pathogen. These have not changed conceptually for decades.

Detect the pathogen

Pathogen detection by culture remains the gold standard test for JD. Ideally methods used for cultivation of Mptb should have: the capacity to support the growth of all strains of Mptb; high analytical sensitivity; low contamination rate; short incubation period; ease of identification of Mptb; low overall cost; low occupational health and environmental impact. It is difficult to meet all of these criteria with a single medium; there have not

been many advances in this field for many years but there are many constraints. In particular, problems with selectivity of media for certain strains, the disparity between sensitivity of various methods and the impacts of contamination of cultures need to be recognised. Finally, the prolonged delay between submission of samples to a laboratory, and the availability of results has been a major constraint to practical use of culture. For this reason the development of a rapid faecal test has been an objective internationally for many years.

Rapid detection methods for Mptb based on PCR are appearing in the literature and being commercialised, with increasing frequency, but validation data are uncommon. In Australia, a test cannot be used in a national control program unless it has been properly validated. This is fortunate because when new PCR tests for Johne's disease have been applied in the field for surveillance purposes, discrepancies with culture results have affected the classification of herd status and have aroused concern (Buckley and Cashman, 2009; Orpin and Sibley, 2009) with a call for "large scale examinations of the available tests". This issue was highlighted again in a recent publication about the Tetracore VetAlert TM Johnes real time PCR test: "Although a new Mptb detection method has been added to the list of approved Johne's test methods, the test has yet to be formally evaluated in the field" (Alinovi et al., 2009).

A further problem is the impact of international variation in culture protocols to the extent that we cannot readily evaluate or compare the many new molecular methods that are being developed, particularly their sensitivities. However, methods of this type have the potential to meet the characteristics of an ideal method and therefore replace culture.

Detect the host immune response

New discoveries about the first contact between host and pathogen have been described, and these offer the most likely opportunities for the early detection of infection. Using the genome sequence of Mptb, unique antigens have been identified and these provide new opportunities for inclusion in diagnostic tests to lift sensitivity and specificity of immunological tests.

Current antibody detection and interferon gamma ELISA tests are based on a range of poorly characterized crude protoplasmic or purified protein derivative antigens of Mptb. These tests are now acknowledged by most authorities to lack sensitivity and/or specificity, probably due to the nature of the antigen. It has long been thought that the humoral immune response (antibody response) of ruminants during paratuberculosis is not useful for diagnosis until relatively late in the pathogensis of the disease. By this time shedding of Mptb in faeces has occurred, and it is too late to stop spread of infection. This has recently been shown to be an over-generalisation because IgG1-specific responses to PPD antigen are detectable mostly in the clinical stage of paratuberclosis in cattle, but total immunoglobulin or isotype-specific responses to the defined antigens Hsp70-, Hsp65-, and LAM occur earlier (Koets et al., 2001). Several studies have now shown that there is an early antibody response to Mptb in cattle and also sheep (Begg et al., 2011; Koets et al., 2001; Waters et al., 2003). This offers impetus to research to develop a simple, inexpensive technology platform based on improved antigens in ELISA. This well accepted test could then be applied in new ways to control paratuberculosis.

Experiments to produce better "natural" antigens by physical or chemical extraction from Mptb have been reported (for example (Eda et al., 2006)) but such antigens have

not replaced crude antigens in commercial tests. Discovery of one or more pure and defined antigens with adequate sensitivity and specificity for Mptb infection remains an objective. Several rational approaches to discover defined antigens for cloning and production of recombinant antigens for inclusion in immunological tests for paratuberculosis have been devised in recent years. Examples of these include research on: secreted antigens (Cho et al., 2007; Leroy et al., 2007); surface antigens (Newton et al 2008) ; antigens unique to Mptb discovered through whole genome comparison (Bannantine et al., 2002; Bannantine et al., 2004; Hughes et al., 2008; Paustian et al., 2004) with in-silico epitope prediction (Leroy et al 2009) or comparative proteomics (Hughes et al., 2007; Santema et al., 2009); and stress-dormancy-associated antigens (Gumber et al., 2009a; Gumber et al., 2009b; Gumber and Whittington, 2009). Research on secreted antigens identified in Mptb culture filtrate has shown that some are immunogenic and must be secreted in vivo as cattle develop antibody responses against them during paratuberculosis (Cho et al., 2007). The general approach of antigen identification, followed by cloning, expression in *E. coli,* purification and incorporation in ELISA has been widely adopted. The major constraint is evaluation of the usefulness of the antigens because this requires the testing of large numbers of samples. Again, as with direct faecal tests, the validation phase of new test development has lagged.

The development or pathogenesis of disease

In order to devise new ways to diagnose and prevent paratuberculosis more information is needed about the disease process – current knowledge is decades-old and there do not appear to be any new ways to exploit the old concepts about disease development. Therefore the pathogenesis of paratuberculosis is being studied with renewed interest. Stages of disease from initial infection via M cells in the intestinal epithelium through to survival within macrophages have been the subject of many recent studies, in contrast to the situation 10 years ago when the research community was focused mainly on advanced disease.

The first encounter of Mptb and the host probably occurs within epithelia and involves pattern recognition receptors such as Toll-like- receptors (TLR). Expression of these receptors may be upregulated in response to infection, as we have shown in sheep (Taylor et al., 2008). Subsequent immune activation, and successful control of the infection or otherwise may depend on the pattern of receptor encounter. Genetic predisposition to Mptb infection may be manifest at this early stage, as mutations in genes such as NOD2 and SLC11A1 are correlated with outcome in cattle (Ruiz-Larrañaga et al., 2010a; Ruiz-Larrañaga et al., 2010b). The next steps in disease progression are poorly understood, but the presence of the organism in tissues, detected by PCR or culture, precedes the development of a visible inflammatory response, i.e. precedes histopathological lesion development. However, shedding of Mptb DNA in faeces may precede the presence of visible histopathological lesions. It is unknown whether this is due to restricted distribution of lesions which by chance are not included in a histological assessment.

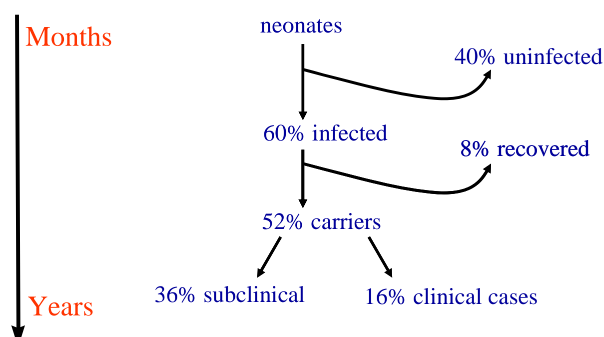
It is commonly assumed that infection with Mptb leads to JD, but this is simplistic. We hypothesised that the process is not necessarily linear, may involve periods of latency or dormancy of Mptb, and that some animals may recover from infection, and we have been seeking evidence for this using several approaches: demonstrating the potential of Mptb to enter a latent or dormant phase when exposed to unfavourable environments; longitudinal studies of immune responses in a sheep infection model; longitudinal studies in naturally infected sheep using surgical biopsy.

Mptb appears to have a stress response with expression of a range of proteins that confer resistance to noxious environments: heat, nutrient deprivation, hypoxia, nitrosative and oxidative stress. Some of these proteins are expressed in vivo, evidenced by some sheep in early stages of infection developing an antibody response against the proteins (Gumber et al., 2009a; Gumber et al., 2009b; Gumber and Whittington, 2009; Kawaji et al., 2010). In some way this stress response may allow Mptb to withstand conditions within host macrophages, and lie dormant until signals are appropriate for proliferation and stimulation of granulomatous inflammation, which leads to clinical expression of Johne's disease. This work commenced in project OJD.031 and continued in the current project.

We developed an experimental model for paratuberculosis based on low dose inoculation with a pure culture of Telford 9.2 Mptb from a lyophilized seedstock (Begg et al., 2010). This model results in infection outcomes that closely resemble natural infection in terms of infection rates determined by faecal culture, incidence of clinical cases and prevalence of histopathological lesions. The model enables the study of immune responses over time under controlled conditions, with the time of infection known.

In a prior project OJD.020 we used repeated surgical biopsy to enable histopathological and tissue culture assessments over time to gain greater insight into these processes. A flock of 77 sheep were continuously exposed to Mptb from shedding sheep at pasture. Biopsies were collected from the terminal ileum and associated lymph nodes at 12, 18 and 24 months of age while necropsy was performed at 36 months of age (Dennis et al., 2011; McConnel et al., 2004). The outcomes are summarised in Figure 4.1. About 60% of sheep became infected during the trial, based on the results of culture and histopathology of terminal ileum or mesenteric lymph node. New infections were detected at about the same rate (0.01 to 0.03) at each time point during the trial – proving that sheep remain susceptible to infection after the perinatal period. Six sheep recovered from infection, including one that had histopathological lesions at 18 months of age but not at 24 or 36 months of age. The rate of development and progression of intestinal pathology in the 24 sheep with intestinal lesions was variable. Severe multibacillary lesions developed over 6-12 months without prior lesions in 8 sheep, while in 1 there was mild multibacillary disease and in 3 there were mild paucibacillary lesions at earlier time points. In the group of 24 sheep with intestinal lesions, 12 had clinical disease; 10 of these had severe multibacillary lesions while two had mild to moderate paucibacillary lesions. Of the 12 with subclinical infection, all but two had mild to moderate paucibacillary lesions and one recovered from infection. Overall these findings suggest that about 40% of sheep were resistant to infection, 8% had the capacity to recover from infection while in the remainder there was a general progression from mild paucibacillary to multibacillary disease, but at variable rates. Clinical signs were usually associated with progression of lesions. If this is expanded, this study showed that individual sheep follow different pathways including: resistance to infection per se; recovery from infection prior to development of histological lesions; recovery after development of histological lesions; arrested lesion development, and; progression of infection from paucibacillary to multibacillary disease.

Figure 4.1 The outcomes of infection in a mob of 77 Merino lambs which were exposed naturally to OJD on a farm near Goulburn NSW.



The implications for the diagnosis of paratuberculosis from these studies are profound: early signs of exposure to Mptb and infection can be detected; it is currently impossible to predict the final outcome for an individual animal based on current tests applied at early stages in the pathogenesis of the disease; application of tests too early, with a view to culling infected animals, may lead to removal of resistant animals from the herd or flock; there is opportunity to discover the triggers for disease progression, and biomarkers for resistance. This is challenging research but could lead to substantial breakthroughs leading to new tools to manage the occurrence of paratuberculosis in herds.

Conclusion
In order to develop, validate and apply new diagnostic approaches and prevent Johne's disease through vaccination or other control measures we need far greater understanding of the pathogenesis of Johne's disease, which means the events leading to initiation of infection because not all animals get infected; progression of disease; regression of disease; dormancy/latency of infection during the long incubation period; and clinical breakdown to produce the disease seen by producers on their farms. This project is a step towards the goal of control and prevention of Johne's disease.

2 Project objectives

At the completion of the Project, the University will have completed the following to MLAs satisfaction:

1. Conducted a research program over 3.5 years to explore current international advances in OJD diagnosis in the Australian context
2. Developed new tools to close some key knowledge gaps
3. Exploited advances in OJD research and validated and commercialised a new rapid test for OJD
4. Ensured that Australian sheep producers have access to respected researchers and relevant research findings on OJD

3 Methodology

The project required a multidisciplinary approach using state of the art techniques in microbiology, immunology, molecular biology and genomics. They were applied in three inter-related subprograms of research. Each sub-program required methods of

varying technical complexity and/or novelty, as well as farm-based flock management. These approaches are described in detail in the appendices which link to the specific sections of the results.

4 Results

The results of this project flow from three inter-related subprograms of research. Each sub-program contained a body of work of great depth and complexity. In order to describe this work succinctly the following approach was adopted:
A brief summary is provided in tabular form (Table 1)
The results mentioned in Table 1 are presented in more detail under sub-headings for each of the subprograms
Detailed methods and results for each section of work are provided in appendices
As the project included a substantial component of basic research many of the findings are of potential future value. Consequently Table 1 provides a guide covering what has been discovered, whether it was useful or potentially useful, and the outcomes that were addressed.

Table 1. Summary of results of project P.PSH.0311

Sub project Name	Status	What it has found out	Implication of this	Outcomes it will/has addressed
Subprogram 1	**Development and commercialisation of discoveries in diagnostics**			
Enhancement of IFN-γ blood test technologies	Active	Two new methods (ELISPOT and Cell ELISA) were not useful but adding IL-12 and IL-7 to blood samples was highly beneficial. As a result, blood transport between farm and lab can be extended from 8 to 48 hours, which makes a test practical for most of Australia.	Useful. The investigation is continuing	Need for new blood test. This advance overcomes a previous hurdle to get blood to a lab within 8 hours of collection. This provides a practical basis for a new IFN-γ test for sheep.
Development of a direct faecal PCR test for commercialisation	Active	A new high-throughput method has been developed. The JD RAG recommended further validation on a larger number of samples before commercialisation.	Useful. The investigation is continuing.	Need for new faecal test. This test will enable rapid (1 week) testing and reporting of Mptb in faeces at a cost no greater than culture. The test appears suitable for pooled samples.
Subprogram 2	**Animal resources and experimental infection models**			
Infection trials	Active	Disease has occurred in some animals in a paddock-based experimental infection trial; the disease appears to be very natural, as it would occur on a farm with OJD	Useful. The investigation is continuing.	The trial underpins all other subprograms and is a breakthrough because it will allow us to compare breed susceptibility, develop new tests and evaluate new vaccines under controlled conditions.
Subprogram 3	**Fundamental research on host and pathogen genomics and immune response**			
New antigens for assay development	Active	New antigens have been produced ready for testing.	Useful. The investigation is continuing	Need for new blood test. The use of new antigens in the ELISA or IFN

Sub project Name	Status	What it has found out	Implication of this	Outcomes it will/has addressed
				assays may enable earlier detection with greater accuracy.
New cytokine assays	Active	The IL-10 cytokine response measured in a new test increases a few months after exposure of sheep to Mptb.	Useful. The investigation is continuing	Need for new blood test. The IL-10 assay, and the use of new antigens in it, may enable earlier detection with greater accuracy.
Assays for immunoglobulin isotypes	Active	IgA specific for Mptb can be detected in the faeces of some sheep with Johne's disease	Useful. The investigation is continuing	Need for a new diagnostic test. This is a novel approach which may have practical application.
Detection of Mptb in blood	Completed	Mtpb was detected in the blood of some animals with clinical disease. However, detection of Mptb from blood using culture or PCR is not a useful diagnostic assay. The organism is rarely present in blood, except in advanced cases of OJD.	Useful. Complete.	Need for new diagnostic test and recommendations for public health. The findings call into question many studies of Crohn's disease in humans, suggesting low specificity of tests used in those studies.
Novel gene expression by host	Completed	Sheep with OJD have elevated TLR and IDO gene expression in blood cells and depressed serum tryptophan levels - supplementation might be an option to treat loss of body condition.	Useful. Complete.	Need for new diagnostic test and vaccine. The TLR and IDO expression results will be used in conjunction with other parameters to determine a disease signature for OJD.
Assays based on cell proliferation	Active	Sheep exposed to Mptb have elevated white blood cell proliferation responses, which vary at an early stage according to later disease outcomes.	Useful. The investigation is continuing	Need for a new diagnostic test that may predict the susceptibility of individual sheep

4.1 Subprogram 1. Diagnostics

4.1.1 Enhancement of IFN-γ blood test technologies

There is a need for a new blood test for Johne's disease. Detection of interferon gamma (IFN-γ) is thought to be a good way to diagnose paratuberculosis in the early stages of infection. IFN-γ is a factor that is produced by blood cells as part of the cell-mediated immune response to Mptb infection. Assays can be devised to detect IFN-γ when white blood cells are stimulated by components of Mptb. Cell-mediated immunity is critical to early protection against intracellular Mptb infection. IFN-γ 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. However, an obvious limitation to the widespread application of IFN-γ assays for the diagnosis of paratuberculosis has been the logistical difficulty as the assay needs to be done in a laboratory within 8 hours of blood collection. This is impossible across much of Australia. In this project new methods for detecting IFN-γ were evaluated while the existing whole blood interferon gamma assay was improved.

4.1.1.1 ELISPOT and Cell-ELISA

Two new methods (ELISPOT and Cell ELISA) for the detection of IFN-γ were developed and trialled but were not useful in sheep and research on their commercial application has ceased. Detailed results are presented in Appendix 1.

In project OJD.031 it was found that these new methods were as good if not better at the diagnosis of Mptb infected animals than the traditional IFN-γ ELISA. The IFN-γ responses from the ELISPOT and Cell-ELISA assays could be detected earlier after infection. The ELISPOT measures the number of white blood cells producing IFN-γ, the Cell-ELISA measures the total amount of IFN-γ produced by the white blood cells while the traditional whole blood interferon gamma ELISA measures the amount of IFN-γ released into the plasma. While the ELISPOT and Cell-ELISA assays looked promising in the animal trials of OJD.031 they were not commercially viable as diagnostic tests. This was due to the high cost of the preparing the white blood cells for the assays. The cells had to be isolated using density gradient centrifugation which is both expensive and labour intensive. Consequently the aim of this research was to evaluate an alternative method of cell isolation, red blood cell lysis, which has shown promising results in pilot trials (Begg et al., 2009). This method is far less expensive and labour intensive but it was not suitable for these assays. For this reason both ELISPOT and Cell-ELISA are useful only in research trials.

4.1.1.2 Improving the whole blood interferon gamma ELISA

There are two problems recognised with the whole blood IFN-γ ELISA when applied to paratuberculosis: poor specificity and lack of practicality. The latter is due to an absolute requirement to test samples within 8 hours of collection. Given that most samples take 24-48 hours to reach a laboratory via a courier after collection on farm, such a test is unsuitable for use in Australia. Two approaches were attempted to improve the whole blood IFN-γ ELISA and make it more practical: the addition of an antibody against IL-10, and the addition of two cytokines, IL-12 and IL-7 to samples.

Addition of anti-IL-10

Low IFN-γ secretion in some animals with Johne's disease may be due the immunosuppressive effects of IL-10, which is a chemical (cytokine) produced in the

body by certain types of cell (Buza et al., 2004). In addition, the results from an earlier project OJD.031 showed that there was an increase in IL-10 in sheep exposed to Mptb (de Silva et al., 2011). Therefore it was hypothesised that detection of infected animals by the conventional whole blood IFN-γ ELISA assay could be improved by removing the effects of IL-10 by adding substances to blood (specific antibodies) that would neutralise this cytokine. However, the results indicated that the IFN-γ assay for sheep cannot be enhanced by the addition of anti-IL-10 antibodies. Detailed results are provided in Appendix 2.

Addition of the cytokines IL-12 and IL-7
The addition of IL-12 to tests to potentiate the response of cattle has been attempted (Jungersen et al., 2005). A protocol to potentiate IFN-γ cellular responses in culture of sheep blood was developed and optimised, involving supplementation with IL-12 and IL-7 at the time the culture was set-up in the laboratory (Appendix 3). This protocol, termed the IFN-γ^{Plus} assay, had sensitivity comparable to the standard assay run within 8 hours in naturally infected sheep. The cytokines added to the culture do not activate the cells by themselves, as the addition of IL-12 and IL-7 did not increase background readings in control samples stimulated with media. The advantage of this assay in comparison with the standard IFN-γ assay that needs to be performed within 8 hrs of blood collection, is that this assay can be performed after 2 days, allowing for practical shipment of blood samples to the laboratory. A practical and easily implemented assay format (IFN-γ^{Plus} assay) to extend the shipping time from farm to laboratory for IFN-γ testing of blood samples to detect OJD has been developed. Studies on naturally infected sheep showed great promise, though specificity was identified as a potential issue in our infection trial with best results obtained early post-infection. The potentiation protocol was tested using our in-house IFN-γ ELISA but may be applicable to commercial Bovigam® assays for paratuberculosis or potentially also tuberculosis and other cell mediated immune assays (e.g IFN-γ ELISPOT, IFN-γ cell-ELISA).

An example of the results obtained using this assay is provided in Figure 7.1.1. This was an on-farm trial conducted on 29 sheep from a vaccinated property near Bathurst, NSW (trial P.PSH.0311.A1). Blood was collected and then transported to Camden and stored at room temperature. Mptb-specific responses were enhanced in the majority of sheep with no detrimental effect on specificity (unstimulated controls were still at background levels). The addition of IL-12 and IL-7 increased the sensitivity of detection of these animals. With no additives in the culture, only 8/29 (28%) were detected as having a positive IFN-γ response. When IL-12 and IL-7 were added to the cultures, 24/29 (83%) were detected as having a positive IFN-γ response. The responses observed were likely due to vaccination, but the individual infection status of these sheep was not determined.

The test requires further validation, particularly of specificity as poor specificity has been an issue in sheep and cattle in other research trials. Future studies would include examining Mptb-specific antigens for stimulation of the cultures to improve specificity, and a large scale validation trial to test for practicality and performance (sensitivity and specificity) for field samples.

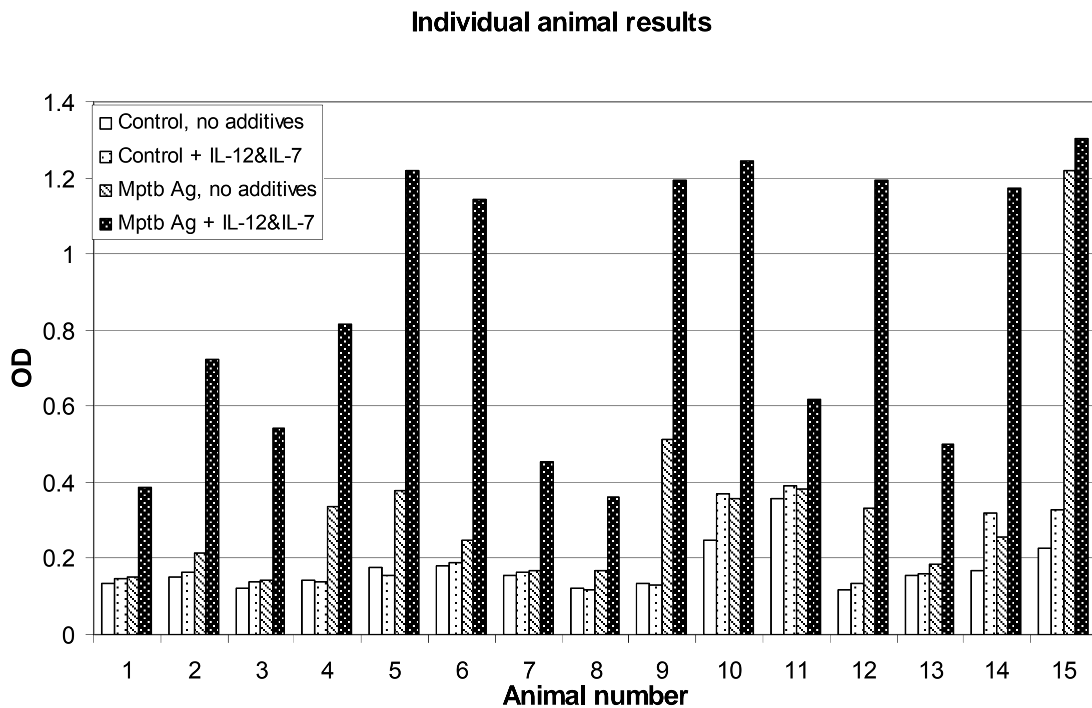


Figure 7.1.1. IFN-γ response of sheep from trial P.PSH.0311A.1 to Mptb antigen with or without the addition of IL-12 and IL-7. Significant enhancement of sensitivity was seen (dark bars). The graph is representative and shows 15 of the 29 animals included in the trial.

Protocol for transport of blood samples to the laboratory for the IFN-γ^{Plus} assay

The results in the previous section on the IFN-γ^{Plus} assay had been from samples stored at room temperature (23^{0}C) for the entire 48 hour period between sample collection and conducting the laboratory test. This work was extended by investigating protocols for the transportation of blood from farms to the laboratory to prove that the blood cells were still viable and able to respond in an IFN-γ assay. There were some assumptions made: that the blood would require motor vehicle/air transportation and that the inside of vehicles may either get hot or cold depending on the season and method of transport. Therefore some form of buffering would be required to mitigate these temperature fluctuations. The transportation protocol tested initially involved a 10 L foam cooler box/esky (Willow) with one or more frozen ice bricks, which is the conventional approach. The blood tubes were wrapped in newspaper to protect against direct contact with the ice brick. To determine the temperature inside the package, a temperature probe was placed inside. This recorded the temperature at 5-10 minute intervals. The blood was 'transported', including a simulated flight (1 hr at 4°C) and simulated courier delivery. We found that the use of an esky with 2 ice bricks sandwiching the blood tubes kept the samples at a reasonably constant temperature and the blood samples were successfully tested 2 days later. Detailed results are provided in Appendix 4. The results suggested that specificity of the test might be a problem, consistent with prior observations.

4.1.2 Development of a direct faecal PCR test for commercialisation

Following an extensive development phase a new high-throughput method has been developed to detect Mptb in sheep faeces using quantitative PCR. The test is called HT-J which stands for "high throughput test– Johne's". Validation of the HT-J test was undertaken using 211 faecal samples from exposed and unexposed properties in New South Wales. Many of these were pooled faecal samples previously collected as part of other MLA funded projects (OJD.033 and P.PSH.0309) which were archived at -80°C at the University of Sydney. Although the results from these samples did not require reports to be sent to submitters, laboratory reports for samples could be issued within a 7 day timeframe, confirming the potential of the test to provide rapid results to submitters. Rigorous criteria were applied to the testing process and to the assessment of test results.

None of the 43 samples from unexposed sheep were positive in the HT-J test or the culture test. Therefore specificity of the HT-J test relative to both property disease status and faecal culture was 100% (95% confidence limits 91.8 to 100).

An idea of sensitivity can be obtained relative to faecal culture. Of 65 culture positive faecal samples, 62 were positive by HT-J. Therefore the sensitivity of HT-J relative to culture was 95.4% (95% confidence limits 87.1 to 99.0). However, a further 12 culture negative samples from exposed flocks were positive in HT-J, suggesting that it may be more sensitive than culture.

Considering all samples, it is reasonable to conclude that the specificity of the HT-J test is extremely high (approaching 100%) and its sensitivity is equivalent to or greater than that of faecal culture. Estimates of the sensitivity of the HT-J test relative to culture appear to be underestimates of the true sensitivity of the test.
The JD RAG recommended further validation before commercialisation – this will occur as part of a new project in 2011-2012. This test will enable rapid (1 week) testing and reporting of Mptb in faeces with similar sensitivity to culture. The test appears suitable for the testing of pooled samples. It is hoped that the test will be offered commercially to producers in 2012.

Detailed methods and results are provided in Appendix 5.

4.2 Subprogram 2. Animal resources and experimental infection models

The aim of this subprogram was to supply tissues and other samples for the other research subprograms in this project from experimentally and naturally infected sheep, and to examine in more detail the early stages of infection (i.e. first six months).

Trials P.PSH.0311.A1 and P.PSH.0311.A2 provided significant samples for the further understanding of Johne's disease in sheep. The experimental infection trial P.PSH.0311.1 is on-going, and will examine why some animals can overcome the infection or delay its onset, while others succumb earlier. It is known from previous experimental inoculation trials that sheep will develop clinical disease at different times, some succumbing early while other can take years. There have been 2 distinct periods (14-17 months, and 21 months post inoculation) when animals have developed clinical disease in this experiment, others may yet develop disease. The timeline for this trial is shown in Figure 7.2.1.

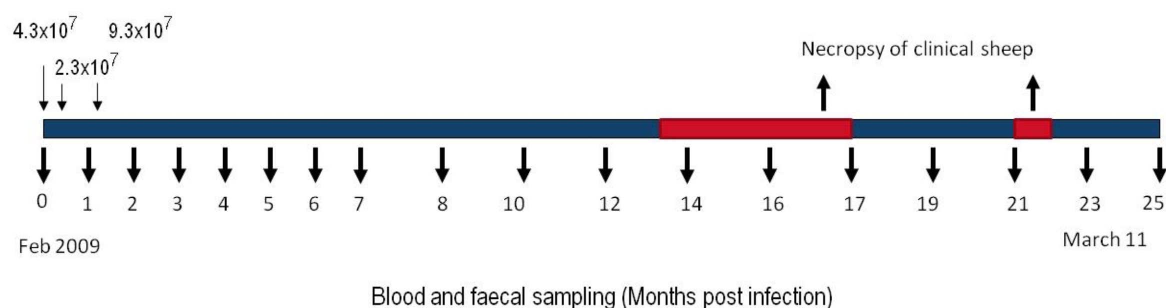


Figure 7.2.1. Time points post infection for blood and faecal sampling in trial P.PSH.0311.1. Twenty sheep were inoculated orally with the dose of Mptb shown above the line. Arrows below the line indicate the blood and faecal sampling time points with months numbered. The areas in red indicate times when some sheep were necropsied due to weight loss.

Both the antibody and IFN-γ responses in the sheep increased relatively soon after inoculation. By using a monthly sampling regime it was possible to see that the surviving animals had a higher antibody and IFN-γ response soon after infection than those animals that later developed clinical disease.

Another important point is that some animals with OJD may be able to eliminate the infection. Although we have surviving sheep in trial P.PSH.0311.1 it is not known if they are infected – this will be determined at necrospy. In trial P.PSH.0311.A2, even though all the animals were antibody positive, not all had the infection as determined by tissue culture and histology, the gold standard tests for the diagnosis of Johne's disease. Assuming that the ELISA is specific, this is an indication that animals may have recovered from infection.

Further details about the experimental design and results are provided in Appendix 6.

The animals in this study have provided an important resource for all subprograms in project P.PSH.0311. They have also in themselves provided new insights into disease pathogenesis and the performance of our current diagnostic assays.

4.3 Subprogram 3. Fundamental research on host and pathogen genomics and host immune response

4.3.1 New antigens for assay development

The current blood tests for paratuberculosis lack sensitivity and some, like the whole blood IFN-γ assay, are not sufficiently specific. One potential solution is to discover new antigens for inclusion in the existing test platforms. The antigen which is included in the test matches a part of the bacterium which is seen by the animal's immune system. Antigen discovery is difficult, and newly discovered antigens must be validated – this is a multi-step process. In this project we continued work that commenced in the previous project OJD.031. New methods were applied in an attempt to speed up the process.

Scanning Mptb fragments in search of an antigen through laboratory-based experiments is extremely time and resource consuming. However, identification of antigens specific to Mptb has been facilitated by the availability of the genome sequence for the organism. Bioinformatics, a multidisciplinary approach using

information technology, mathematics, statistics and biology has become a vital tool. This approach allows systematic selection of antigens and provides a basis for laboratory experiments that may avoid the high cost of cloning and testing all of the proteins from the Mptb genome. Prediction tools are being continuously updated and are available for predicting both B and T-cell epitopes (i.e. the protein structures recognised by either B or T cells that result in an immune response).

T-cell epitopes are identified based on major histocompatibility complex (MHC) binding motifs. These epitopes are bound to the surface of cells and serve as a target for T-cell recognition; the T-cells become the active players in the immune response. Epitopes with high binding affinity to MHC molecules are known to be immunogenic and epitope prediction tools can be used to identify the small number of epitopes that may be biologically significant.

B-cell epitopes are defined by their complementarity to B-cell receptors. Complementarity is determined by the physio-chemical properties of amino acids in epitopes. B-cell epitopes can be identified as continuous (linear) or discontinuous (conformational). Linear epitopes are generally not readily accessible to antibodies since many of the epitope sequences remain buried inside the globular or three dimensional (3D) structure of the protein. Methods of overcoming this limitation have been developed which predict discontinuous epitopes formed by separated amino acid residues that are brought into close contact when an antigenic protein is folded to form a 3D structure. Methods are available to predict conformational B-cell epitopes from the most relevant 3D structural model of a protein.

Another type of antigen is the secreted protein – these can also be predicted.

In project OJD.031 we use hypothesis-driven research to find antigens which might be useful for diagnosis. We identified a large group of proteins that were expressed by Mptb under stressful conditions, as part of the survival strategy of the organism, reasoning that these might be expressed in sheep during infection. The stresses were hypoxia, starvation, temperature fluctuation, nitrosative and oxidative stress (Gumber et al., 2009a; Gumber et al., 2009b; Gumber and Whittington, 2009; Kawaji et al., 2010). Some of the antigens identified were recognised by serum antibodies but did not stimulate cell mediated responses from peripheral blood mononuclear cells (PBMC) under experimental conditions (Kawaji, 2009). As many other antigens in this collection remained to be examined, the aim of this work was to use prediction tools to select those with theoretical greatest potential.

A total of eight proteins were selected for future cloning and expression of recombinant proteins. These will be evaluated as antigens in new diagnostic tests in a future project. The detailed methods and analysis are provided as Appendix 7.

4.3.2 A blood test based on the detection of IL-10

The immune response in Johne's disease is traditionally gauged in terms of antigen-specific IFN-γ production, cell proliferation or antibody production. IFN-γ and cell proliferation are markers of the cell-mediated immune response and are considered to be essential for effective clearance of mycobacteria. As disease becomes established, this response tends to be curbed and there is evidence that the immunosuppressive cytokine IL-10 may become predominant. IL-10 is known for its role in maintaining a controlled immune response. Results from a previous project (OJD.031) demonstrated that the IL-10 response in peripheral blood cells could be

detected by four months after exposure to Mptb in experimentally challenged sheep (de Silva et al., 2011). This was the first time point tested in that study. In the current study, sheep were sampled for testing in the IL-10 assay prior to infection as well every 4 weeks for 7 months to improve our knowledge of the immune response soon after exposure and in the very early stages of Johne's disease.

Although the IL-10 response tended to be elevated with time, it was lower than expected when compared to results from the OJD.031 project. Since purified blood leukocytes (PBMC) were used in the previous project, PBMC were compared with whole blood and RBC-lysed cells. These results clearly showed that the Mptb-specific IL-10 response can be seen when using PBMC and it is recommended that PBMC be used in the IL-10 assay for ovine Johne's disease.

A striking observation was that the sheep that go on to develop clinical disease with multibacillary lesions by about a year after exposure tended to have an elevated early IL-10 response (3-11 weeks post infection) compared to animals having clinical disease with paucibacillary lesions. The former were also more likely to shed Mptb in faeces in the first six months after exposure.

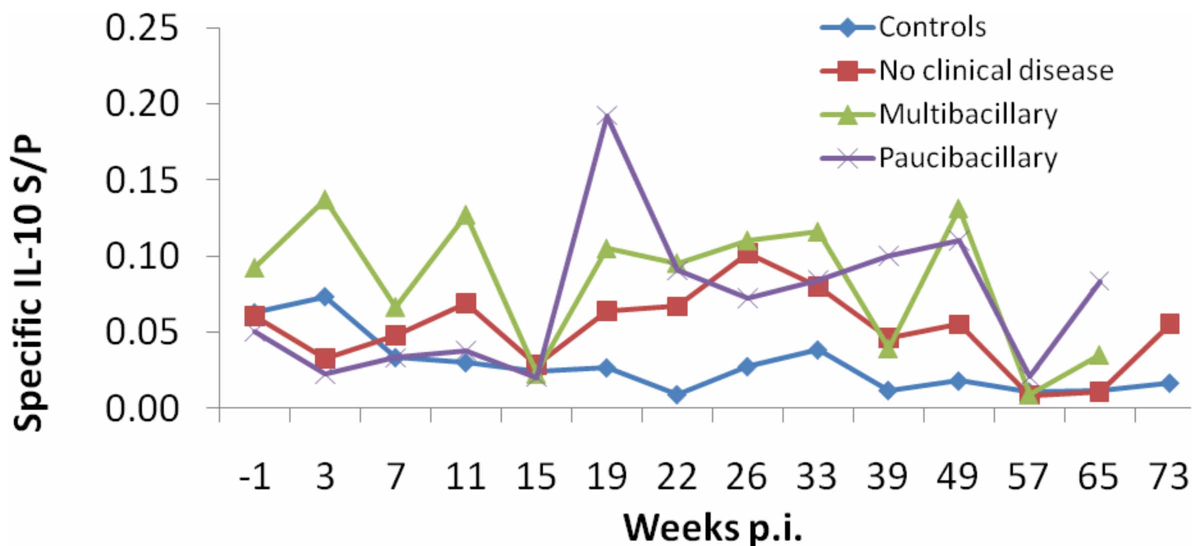


Figure 7.3.2.1 IL-10 response based on disease outcome
The IL-10 response in whole blood in non-exposed controls, sheep with no sign of clinical disease at 73 weeks p.i., and sheep sacrificed due to weight loss which were subsequently found to have multibacillary (Perez 3b) or paucibacillary lesions (Perez 3a+, 3a-c).

This study is the first to report on the IL-10 response from pre-infection to clinical Johne's disease and beyond two years post inoculation in surviving sheep. While whole blood can be used for the IL-10 assay, PBMC is recommended as the preferred cell source for this assay. Distinct patterns in the early IL-10 response were noted in animals that are likely to develop multibacillary lesions and clinical disease. The early IL-10 response is likely to be important when assembling disease signatures for ovine Johne's disease.

4.3.3 IgA isotype ELISA for detection of paratuberculosis using faecal samples

The sensitivity of antibody ELISAs for the detection Johne's disease is usually low. Sensitivities range from 15%-34% and this is dependent on the stage of the infection. Typically antibody diagnosis works best for animals with clinical disease (Gumber et al., 2006; Robbe-Austerman et al., 2006). Sheep with subclinical infections or paucibacillary lesions are thought to be less likely to be detected (Gumber et al., 2006). The issues associated with failure of these ELISAs are multi-factorial including the nature of antigens and detection antibodies (conjugates) used in the assays. Another factor that may be of importance is the isotype of antibody that is detected. Some reports have shown that diagnosis can be improved by the use different antigens (Bannantine et al., 2008a; Bannantine et al., 2008b), while detecting different isotypes of antibody may be beneficial in diagnosis (Griffin et al., 2005).

In response to infection the host will typically produce antibodies to assist with the overall immune mediated destruction of the pathogens. In mammals there are 5 categories or isotypes of antibody. Most of the current commercial ELISA assays for Johne's disease detect IgG antibodies specific to Mptb. Sub-isotypes of IgG (IgG1 and IgG2) have also been evaluated, with IgG1 identified as diagnostically more relevant (Griffin et al., 2005). IgA which is found predominantly in mucus secretions, IgE associated with parasitic infections and IgD whose function is unknown but may be associated with pregnancy have also been considered. The IgM isotype, is generally a short term precursor to the other isotypes.

The aim of this research was to examine different isotypes to see if the diagnosis of Johne's disease was possible. Full details are provided in Appendix 9. This work included the examination of IgG1 and IgG2. IgG2 was not useful as the results indicated that only very low amounts of this antibody could be detect in Mptb infected animals. IgG1 was briefly examined but due to problems finding a suitable monoclonal antibody that detects it in sheep, this work could not be completed.

Johne's disease in sheep is primarily an infection of the gut, while most of the current ELISAs examine IgG which is predominantly a serum-associated antibody. IgA is primarily found in mucus secretions and in mammals this is mainly saliva, vaginal or gut associated. Blood typically carries low amounts of IgA, but faecal samples contain gut associated mucus and therefore possibly IgA. Therefore we attempted the detection of IgA from faeces of sheep for the detection of Mptb infection.

The results of this assay are shown in Figure 7.3.3.1, where most of the animals at 16 months post infection had detectable levels of faecal IgA specific for Mptb. Samples from animals that went on to develop clinical disease (mostly within the next month) are shown in red and had high responses. Samples from animals with faecal shedding at this time point are shown in the green bars (one animal went on to develop clinical disease and was faecal shedding, shown in red and green) and tended to have lower responses. Animals shown in blue bars had not shown evidence of infection at the time of sampling, but some also had high responses.

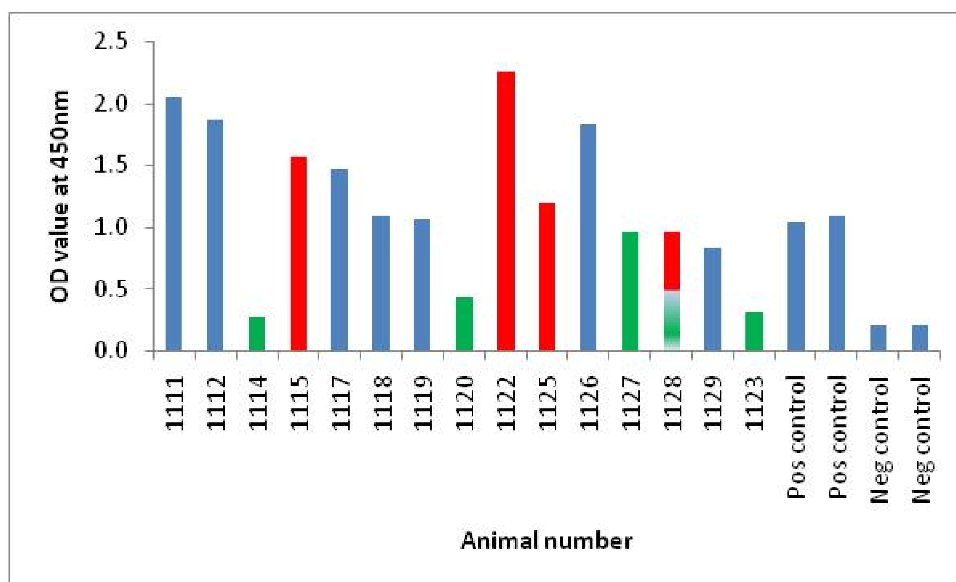


Figure 7.3.3.1. Faecal IgA detection in sheep in trial P.PSH.0311.1. Inoculated animals at 16 months post infection. Red bars indicated animals that went on to develop clinical disease, green bars indicate animals with faecal shedding, while a red/green bar indicates an animal with faecal shedding that went on to develop clinical disease. Blue bars indicate animals with no faecal shedding or clinical disease.

The assay was then used on samples collected at 12, 14 16 and 21 months post inoculation. Surprisingly only the samples at 16 months post infection contained faecal IgA. Over half of the animals at this time point had a response greater than 50% of the positive control sample. The 16 month sample time point was when many of the animals were developing signs of the infection, there were high faecal Mptb shedding rates and presumably the environment was becoming contaminated with Mptb. Could this exposure have resulted in higher IgA responses that could not be observed at other time points? This may have been protective as many of the sheep that were not shedding and did not develop clinical disease had high IgA responses. Further studies are required to determine this.

This study has confirmed that sheep with paratuberculosis have IgA in their faecal mucus, that it can be detected and that it might offer novel diagnostic information. Other types of sample such as saliva may also contain IgA. The next steps are to re-titrate the ELISA to find out whether the samples taken at 16 months post inoculation have an abnormally high response and to test other types of samples for IgA. This work is to be undertaken in a future project.

4.3.4 Detection of Mptb in blood samples

There is considerable evidence to suggest that paratuberculosis extends beyond the gut. Dissemination has been demonstrated in clinically and subclinically affected animals with mild to severe gross pathological classification, either positive or negative serological status (Antognoli et al., 2008) and with mild to severe histopathological lesions (Dennis et al., 2008; Reddacliff et al., 2010). With this in mind, specific methods were developed to detect Mptb in blood samples because the blood stream is the most likely route by which Mptb moves from the gut to other sites in the body of infected animals. Three approaches were attempted: culture of

Mptb from blood; detection of Mptb DNA in blood using PCR, and; detection of DNA from Mptb free in the blood.

4.3.4.1 Culture of Mptb from blood samples

A new method for culture of Mptb from blood samples was developed and optimized. It is described in detail in a publication from this project (Bower et al., 2010), reproduced as Appendix 10. The method was then applied. The occurrence of bacteraemia and dissemination of Mptb to the liver and hepatic lymph node was investigated in 111 sheep. Disseminated infection was detected in 18 of the 53 sheep that were confirmed to be infected following oral exposure to Mptb while the bacterium was isolated from the blood of only 4 of these animals. Disseminated infection was detected more frequently from animals with a positive compared to a negative faecal culture result, multibacillary compared to paucibacillary lesions, and clinical compared to subclinical disease. Detection of Mptb in blood by culture was significantly associated with increased time post exposure and clinical disease, with trends for increased detection in animals with multibacillary lesions and positive faecal culture results. Prolonged incubation periods prior to growth in BACTEC were consistent with inhibition of growth or dormancy in some blood cultures. The results were also published (Bower et al., 2011) and the paper is reproduced as Appendix 11.

As Mptb was detectable by culture in the blood of only 4 of 53 infected sheep, this approach is unlikely to be useful in establishing a diagnosis of paratuberculosis in sheep, especially in preclinical stages of infection. The detection of Mptb from blood by culture may be difficult in the early stages of the disease and in paucibacillary animals as the bacteraemia may be intermittent, below the limit of detection or Mptb may be present in a non-culturable form.

4.3.4.2 Detection of Mptb DNA in blood samples using PCR

Several studies have reported finding Mptb DNA in the blood of infected sheep and cattle using nested and conventional PCR. The specificities of the blood PCR assays used in some of these studies were often not rigorously demonstrated on unexposed or uninfected animals. In addition, a number of these studies used nested PCR which is prone to contamination. If a sensitive and specific method could be established this may be useful as a diagnostic test. The numbers of organisms present in blood are unknown, but if low, present a difficulty to detect, requiring a large volume of blood to be processed in a manner which can detect very small numbers of mycobacteria. However inhibition has been noted when using larger volumes of blood. Thus an efficient method for processing blood and removing inhibitors prior to PCR is required. The aim of this study was to develop a real time, quantitative PCR assay (QPCR) that is suitable for application to buffy coat samples. This method could be used to detect Mptb in the blood of infected animals, as the culture of Mptb from blood does not appear to be very sensitive. It was hypothesised that Mptb may be detectable at lower concentrations using QPCR compared to culture by detecting both viable and non viable organisms.

A method for processing blood to detect Mptb DNA was optimised; it included processing of buffy coats, extraction of DNA and amplification using PCR. This method can detect 10 Mptb added to a buffy coat collected from 9 ml of blood or 100 Mptb added to 9 ml of whole blood. The development of this method is described in detail in Appendix 12.

The blood QPCR assay in this study detected Mptb DNA in the blood of only 5 animals. These were from a diverse range of infection states and time points including unexposed, uninfected, infected and clinically infected animals. The QPCR assay detected a greater diversity of disease severity and timepoints than the culture assay. This may be due to non viable or dormant Mptb during the early stages of infection, or maybe due to a lack of specificity with unexposed and exposed-uninfected sheep testing positive. Detailed results are provided in Appendix 13.

Thus detection of Mptb from blood using PCR is unlikely to be useful in establishing a diagnosis of paratuberculosis in sheep, especially in preclinical stages of infection.

4.3.4.3 Detection of free DNA from Mptb in blood samples

The presence of cell-free DNA (CF DNA) in plasma, serum, effusions, cerebrospinal fluid (CSF) and urine has led to the discovery of potential biomarkers and diagnostic tests. Evidence is building that these fragments of DNA are derived from the breakdown of DNA released from dying microorganisms. Recently, an assay targeting the IS*6110* sequence, which is specific to *M. tuberculosis*, was positive when applied to the soluble fraction of urine from tuberculosis infected humans. The aim of this study was to determine if CF DNA was detectable in Mptb infection and if this had potential diagnostic applications. Factors necessary to develop a successful processing method for CF DNA from sheep serum were examined and the presence of detectable Mptb CF DNA from serum and plasma from sheep experimentally inoculated with Mptb was assessed. Both an established method and a method developed using serum and plasma spiked with Mptb DNA were used.

Samples were tested for the presence of Mptb CF DNA during multiple stages of the disease progression including the initial infection period as well as time points during the development of disease and the late stage of disease. Animals with clinical, subclinical, and disseminated infection were present. Mptb CF DNA was not detected in the plasma or serum of late stage paratuberculous animals or in the serum of animals with early stage and developing paratuberculosis. The lack of detection of any Mptb CF DNA indicates that it is either not present in detectable levels, is present but in fragments too small for detection due to the PCR product length (182 bp with RT-J primers), is not resilient to freeze/thawing or is present in a form that differs in its processing requirements from genomic DNA. The data from the quantification of the extract from the serum samples indicate that non specific DNA was present and was of a reasonable quality, thus it is likely that the plasma and serum samples were processed adequately. Detailed methods and results are provided in Appendix 14.

4.3.4.4 Summary

These studies confirmed, using methods that were specifically optimised, that live Mptb, dead Mptb and cell free DNA from Mptb is rarely present in the blood of most animals with paratuberculosis. When present, it is found at very low levels. However, bacteraemia does occur, mainly in advanced stages of disease and appears to be intermittent. Detection of Mptb in blood does not appear to be a useful diagnostic test. The results provide insight into the risk of contamination of tissues outside the gut with Mptb during infection, and so have public health relevance. This strengthens previous findings and is significant to the management of human exposure through meat. Specifically there is low risk of widespread contamination of tissues via blood unless there is end stage disease. Furthermore the results contrast with those in

other studies, including studies in humans with Crohn's disease, where methods have not been optimised and so may lack specificity.

4.3.5 IDO, tryptophan catabolism and Mptb infection

Virulent mycobacterial infections progress slowly with a latent period that leads to clinical disease in a proportion of cases. Mptb is an intracellular pathogen. Indoleamine 2,3-dioxygenase (IDO), an enzyme that regulates tryptophan metabolism, was originally reported to have a role in intracellular pathogen killing and has since been shown to have an important immunoregulatory role in chronic immune diseases. In this study we found a novel role for IDO in paratuberculosis, by characterising gene expression, protein localisation and functional effects. IDO mRNA levels were significantly increased in Mptb-infected monocytic cells. Both IDO gene and protein expression were significantly upregulated within the affected tissues of sheep with JD, particularly at the site of primary infection, the ileum, of animals with severe multibacilliary disease. Lesion severity was closely correlated with IDO gene expression. IDO gene expression was also increased in peripheral blood cells of Mptb-exposed sheep and cattle. IDO breaks down tryptophan and systemic increases were functional as shown by decreased plasma tryptophan levels, which correlated with the onset of clinical signs, a stage well-known to be associated with Th1 immunosuppression. IDO may be involved in down-regulating immune responses to Mptb and other virulent mycobacteria, an example whereby the pathogen may harness host immunoregulatory pathways to aid survival. These findings raise new questions about the host:mycobacteria interactions in the progression from latent to clinical disease. Complete methods and results are provided in Appendix 15.

4.3.6 Toll-like receptor (TLR)6 and TLR1 differentiation, and TLR9 and TLR10 gene expression studies of ovine Johne's disease

Diagnosis of sheep with subclinical Johne's disease is difficult and limited by the poor knowledge of pathogenesis of the infection. The role of toll-like receptors (TLR) in the pathogenesis of JD has been previously identified at the level of gene expression. Gene expression studies using reverse transcriptase polymerase chain reaction (RT-PCR) are widely used and powerful, but the results obtained from such studies are dependent on the specificity of the assay. We developed assays designed to detect TLR6, TLR9 and TLR10 in sheep. Discrimination between TLR1 and TLR6 at the level of gene expression was challenging due to extensive tracts of homology and identity within the two sequences. Both TLR1 and TLR6 can form heterodimers with TLR2 in order to bind the ligands of microbial pathogens. The expression of TLR6 and TLR9 was increased in the ileum and jejunum of sheep infected with Mptb. TLR9 was significantly reduced in peripheral blood cells of multibacilliary sheep while there was a trend towards TLR6 upregulation in peripheral blood cells in response to Mptb exposure. A likely role for TLR6/TLR2 heterodimers and TLR9 in the pathogenesis of JD was identified. TLR6 and TLR9 may be potential markers of exposure and could aid in the development of a gene signature for sheep resistant to Mptb infection. Complete methods and results are provided in Appendix 16. The discoveries will be explored further in a future project.

4.3.7 Immunological assays based on cell proliferation

The cell mediated immune response is important in eliminating intracellular pathogens such as Mptb. One feature of this immune response is the ability to recall previous exposure to a pathogen. T cells that have encountered Mptb once will react strongly on subsequent encounters to Mptb antigens and this can be detected by the

proliferation assay. The proliferation assay developed for the OJD.031 project demonstrated that Mptb exposure in sheep could be detected as early as 4 months after inoculation in experimentally challenged animals (de Silva et al., 2010). This was the earliest time point tested post inoculation. Therefore in the current project this response was monitored at earlier time points in sheep. In addition, in the current study, only the animals that showed signs of clinical disease were removed from the trial. Mptb-exposed sheep that have shown no signs of clinical disease continue to be monitored.

The Mptb antigen-specific proliferative response was tested in experimentally inoculated lambs from before they were exposed as lambs to when they were over two years of age. The response in non-exposed controls remained low throughout this period while it increased and remains elevated in inoculated sheep. The Mptb antigen-specific response varied with disease status; sheep with multibacillary lesions had a low proliferative response compared to sheep with no clinical disease at this early stage. Interestingly, from within the group of animals removed from the trial due to a decrease in body weight, the proliferative response of those which had paucibacillary lesions started to increase at least two months prior to the onset of clinical signs. While the Mptb antigen-specific response of currently non-diseased inoculated sheep remains elevated these animals are yet to show signs of clinical disease. The proliferative response appears to vary with final disease outcome and may be helpful when assembling disease signatures for Johne's disease.

5 Discussion/conclusion

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 first principles, including those derived from study of human tuberculosis in the early 1900s. Practical applications of this include culture, histopathology, whole blood interferon gamma assay, ELISA and intradermal skin tests for JD, 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. It was designed to use basic research and combine it with strategic elements to both 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.

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

- interferon gamma tests can be improved and with new antigens could be made practical 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 tryptophan 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.

Two existing diagnostic tests which had severe practical and technical limitations can be improved and may be of substantial benefit. These include a direct faecal PCR test and an interferon gamma blood test.

Direct faecal PCR test
Culture of sheep faeces for OJD became possible only in the late 1990s as a result of research in NSW laboratories. However, the prolonged wait for results from this test is a recurring problem for industry. Faecal samples are collected, sent to a laboratory and 3 months elapse before negative test results can be confirmed. For sheep sales this means considerable forward planning and great inconvenience for the producer. Where culture is used to confirm a suspected infection, the delay causes additional anxiety for the producer. The new faecal PCR test overcomes these problems because it can provide results within a few days of receipt of samples at a laboratory. It is estimated that it will cost no more than culture, and we have some good evidence that it 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 has high flock-level specificity and is slightly more sensitive than culture. A further advantage is that the new test is suitable for testing pooled faecal samples, which enables a cheap method of flock testing. Pooled faecal PCR will be able to be used 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. This will involve the testing of a larger number of samples in the second part of 2011, as part of a new project. The researchers are not aware of any laboratory technical or study design issues which will adversely affect the performance of the test during its further validation, because the method has been standardised and the samples tested thus far are likely to be representative of other field samples. After completion of further validation, the 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. It is anticipated that a submission on this test will be made to SCAHLS in late 2011, and the test should be available commercially in animal health diagnostic laboratories in 2012, subject to SCAHLS processes. The direct faecal PCR test will be recognised as a breakthrough for the sheep industry.

Interferon gamma blood test
Interferon gamma detection assays offer the potential to detect infected animals at a very early stage of the disease, unlike an antibody ELISA or direct detection of *Mycobacterium paratuberculosis* (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. A whole blood interferon gamma assay which was developed to prototype stage in a previous project was 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. Therefore this new test will not be available, except in research trials, for several years. The main work to be done is to identify an antigen (component part of Mptb) that is specific for this organism. Such an antigen is sought also for inclusion in ELISA assays which detect antibodies against Mptb. There is an international research effort to discover the key specific antigen. The international collaborations of the Australian researchers should ensure that any useful antigens which are discovered and publicly disclosed will be able to be evaluated in Australia.

Some tests were unsuccessful
There were a number of potential new tests which were unsuccessful for diagnosis. Two involved detection of interferon gamma – the Cell-ELISA and the ELISPOT assay. Culture of blood, detection of Mptb in blood by PCR, and detection of free Mptb DNA in blood also were deemed to be unsuitable for diagnostic purposes following their application to sheep with OJD.

Other key discoveries that may benefit future diagnosis
In the basic research program, computer-based methods were used to mine the DNA sequence of the causative bacterium Mptb to identify new components to include in future diagnostic tests with the objective of greater accuracy than current tests. This work is part of an antigen discovery program and will feed into the research to be conducted on the interferon gamma blood test mentioned above.

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. A great deal more research is required to confirm this and to determine whether it is a reliable predictor. It is possible that this test when used in conjunction with others such as the interferon gamma assay may be used to detect exposure to Mptb and then to decide which sheep have become infected and which are likely to progress in the disease. Practical outcomes are unlikey for a number of years due to the complexity of this research. One of the key requirements to conduct such research is a reliable way to create infection under controlled conditions. Considerable progress was made in this regard.

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

Public health
During this project it was shown that Mptb does not circulate very often in blood at detectable levels. Three parallel testing approaches were used to show this, so the results are robust. This has positive implications for public health because it means that meat and other products from infected sheep are unlikely to be regularly contaminated from the bloodstream. Furthermore, contamination is most likely to occur in animals with advanced disease. These can be readily detected and not enter the food chain.

Mechanisms of disease progression and weight loss in OJD
A novel 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.

Future research
Outcomes from this project that need to be taken forward into a new research project include immunological discoveries about cell proliferation and the whole blood interferon gamma assay. These should be combined with data from the sheep genome, specifically, an assessment of the types of genes which are switched on and switched off during infection. By combining results from multiple tests it is hoped that patterns will emerge that can be used to predict outcomes of infection at an early age, or predict responses to vaccines. Vaccine development will be facilitated by the controlled infection model, and the discovery of antigens in Mptb which may be involved in its virulence and that can be evaluated using the infection model. The timeframe for development of a new commercial vaccine is likely to be very long, but as a result of this project the methods to do so are now much more reliable.

6 Bibliography

Alinovi, C.A., Ward, M.P., Lin, T.L., Moore, G.E., Wu, C.C., 2009, Real-time PCR, compared to liquid and solid culture media and ELISA, for the detection of Mycobacterium avium ssp. paratuberculosis. Veterinary Microbiology 136, 177-179.
Antognoli, M.C., Garry, F.B., Hirst, H.L., Lombard, J.E., Dennis, M.M., Gould, D.H., Salman, M.D., 2008, Characterization of *Mycobacterium avium* subspecies *paratuberculosis* disseminated infection in dairy cattle and its association with antemortem test results. Vet Microbiol 127, 300-308.
Bannantine, J.P., Baechler, E., Zhang, Q., Li, L., Kapur, V., 2002, Genome scale comparison of *Mycobacterium avium* subsp. *paratuberculosis* with *Mycobacterium avium* subsp. *avium* reveals potential diagnostic sequences. Journal of Clinical Microbiology. 40, 1303-1310.
Bannantine, J.P., Hansen, J.K., Paustian, M.L., Amonsin, A., Li, L.L., Stabel, J.R., Kapur, V., 2004, Expression and immunogenicity of proteins encoded by sequences specific to *Mycobacterium avium* subsp. *paratuberculosis*. Journal of Clinical Microbiology 42, 106-114.
Bannantine, J.P., Paustian, M.L., Waters, W.R., Stabel, J.R., Palmer, M.V., Li, L., Kapur, V., 2008a, Profiling bovine antibody responses to Mycobacterium avium subsp. paratuberculosis infection by using protein arrays. Infect Immun 76, 739-749.

Bannantine, J.P., Rosu, V., Zanetti, S., Rocca, S., Ahmed, N., Sechi, L.A., 2008b, Antigenic profiles of recombinant proteins from Mycobacterium avium subsp. paratuberculosis in sheep with Johne's disease. Veterinary Immunology and Immunopathology 122, 116-125.
Begg, D.J., de Silva, K., Bosward, K., Di Fiore, L., Taylor, D.L., Jungersen, G., Whittington, R.J., 2009, Enzyme-linked immunospot: an alternative method for the detection of interferon gamma in Johne's disease. J Vet Diagn Invest 21, 187-196.
Begg, D.J., de Silva, K., Carter, N., Plain, K.M., Purdie, A., Whittington, R.J., 2011, Does a Th1 over Th2 dominancy really exist in the early stages of *Mycobacterium avium* subspecies *paratuberculosis* infections? Immunobiology in press.
Begg, D.J., de Silva, K., Di Fiore, L., Taylor, D.L., Bower, K., Zhong, L., Kawaji, S., Emery, D., Whittington, R.J., 2010, Experimental infection model for Johne's disease using a lyophilised, pure culture, seedstock of *Mycobacterium avium* subspecies *paratuberculosis*. Vet Microbiol 141, 301-311.
Bower, K., Begg, D.J., Whittington, R.J., 2010, Optimisation of culture of Mycobacterium avium subspecies paratuberculosis from blood samples. J Microbiol Methods 80, 93-99.
Bower, K.L., Begg, D.J., Whittington, R.J., 2011, Culture of Mycobacterium avium subspecies paratuberculosis (MAP) from blood and extra-intestinal tissues in experimentally infected sheep. Veterinary Microbiology 147, 127-132.
Buckley, J.F., Cashman, W.J. 2009. Limitations of single tests in the diagnosis of MAP, pp. 282-a-.
Buza, J.J., Hikono, H., Mori, Y., Nagata, R., Hirayama, S., Aodon, g., Bari, A.M., Shu, Y., Tsuji, N.M., Momotani, E., 2004, Neutralization of interleukin-10 significantly enhances gamma interferon expression in peripheral blood by stimulation with Johnin purified protein derivative and by infection with *Mycobacterium avium* subsp. *paratuberculosis* in experimentally infected cattle with paratuberculosis. Infect Immun 72, 2425-2428.
Cho, D., Shin, S.J., Talaat, A.M., Collins, M.T., 2007, Cloning, expression, purification and serodiagnostic evaluation of fourteen Mycobacterium paratuberculosis proteins. Protein Expr Purif 53, 411-420.
de Silva, K., Begg, D., Carter, N., Taylor, D., Di Fiore, L., Whittington, R., 2010, The early lymphocyte proliferation response in sheep exposed to *Mycobacterium avium* subsp. *paratuberculosis* compared to infection status. Immunobiology 215, 12-25.
de Silva, K., Begg, D., Whittington, R., 2011, The interleukin 10 response in ovine Johne's disease. Veterinary Immunology and Immunopathology 139, 10-16.
Dennis, M.M., Antognoli, M.C., Garry, F., Hirst, H., Lombard, J.E., Gould, D.H., Salman, M.D., 2008, Association of severity of enteric granulomatous inflammation with disseminated *Mycobacterium avium* subspecies *paratuberculosis* infection and antemortem test results for paratuberculosis in dairy cows. Veterinary Microbiology 131, 154-163.
Dennis, M.M., Reddacliff, L.A., Whittington, R.J., 2011, Longitudinal study of clinicopathological features of Johne's disease in sheep naturally exposed to *Mycobacterium avium* subspecies *paratuberculosis*. Veterinary Pathology 48, 565-575.
Eda, S., Bannantine, J.P., Waters, W.R., Mori, Y., Whitlock, R.H., Scott, M.C., Speer, C.A., 2006, A highly sensitive and subspecies-specific surface antigen enzyme- linked immunosorbent assay for diagnosis of Johne's disease. Clin Vaccine Immunol 13, 837-844.
Griffin, J.F., Spittle, E., Rodgers, C.R., Liggett, S., Cooper, M., Bakker, D., Bannantine, J.P., 2005, Immunoglobulin G1 enzyme-linked immunosorbent assay

for diagnosis of Johne's Disease in red deer (Cervus elaphus). Clinical and Diagnostic Laboratory Immunology 12, 1401-1409.
Gumber, S., Eamens, G., Whittington, R.J., 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, 91-101.
Gumber, S., Taylor, D.L., Marsh, I.B., Whittington, R.J., 2009a, Growth pattern and partial proteome of *Mycobacterium avium* subsp. *paratuberculosis* during the stress response to hypoxia and nutrient starvation. Vet Microbiol 133, 344-357.
Gumber, S., Taylor, D.L., Whittington, R.J., 2009b, Evaluation of the immunogenicity of recombinant stress-associated proteins during Mycobacterium avium subsp. paratuberculosis infection: Implications for pathogenesis and diagnosis. Vet Microbiol 137, 290-296.
Gumber, S., Whittington, R.J., 2009, Analysis of the growth pattern, survival and proteome of *Mycobacterium avium* subsp *paratuberculosis* following exposure to heat. Veterinary Microbiology 136, 82-90.
Hughes, V., Bannantine, J.P., Denham, S., Smith, S., Garcia-Sanchez, A., Sales, J., Paustian, M.L., McLean, K., Stevenson, K., 2008, Proteome-determined *Mycobacterium avium* subspecies *paratubersulosis* proteins: immunogenicity in ovine paratuberculosis. Clinical and Vaccine Immunology 15, 1824-1833.
Hughes, V., Smith, S., Garcia-Sanchez, A., Sales, J., Stevenson, K., 2007, Proteomic comparison of *Mycobacterium avium* subspecies *paratuberculosis* grown in vitro and isolated from clinical cases of ovine paratuberculosis. Microbiol 153, 196-206.
Jungersen, G., Grell, S.N., Clemensen, A., Roust, T., Huda, A., Howard, C.J., 2005, Interleukin-12 potentiation of the interferon-gamma test rescues day-old blood samples for diagnosis of paratuberculosis PPD specific cellular mediated immune response., In: Manning, E.J., Nielsen, S.S. (Eds.) Proceedings of the Eighth International Colloquium on Parratuberculosis, Copenhagen, Denmark, August 14-17., pp. 501-506.
Kawaji, S., 2009, Pathogenesis and Diagnosis of Johne's Disease with Emphasis on Subclinical Infection: PhD thesis.
Kawaji, S., Zhong, L., Whittington, R.J., 2010, Partial proteome of *Mycobacterium avium* subsp. *paratuberculosis* under oxidative and nitrosative stress. Veterinary Microbiology 145, 252-264.
Koets, A.P., Rutten, V.P.M.G., de Boer, M., Bakker, D., Valentin-Weigand, P., van Eden, W., 2001, Differential changes in heat shock protein-, lipoarabinomannan-, and purified protein derivative-specific immunoglobulin G1 and G2 isotype responses during bovine Mycobacterium avium subsp paratuberculosis infection. Infection and Immunity 69, 1492-1498.
Leroy, B., Roupie, V., Noel-Georis, I., Rosseels, V., Walravens, K., Govaerts, M., Huygen, K., Wattiez, R., 2007, Antigen discovery: a postgenomic approach to paratuberculosis diagnosis. Proteomics 7, 1164-1176.
McConnel, C.S., Churchill, R.C., Richard, M.M., Corkhill, C.M., Reddacliff, L.A., Whittington, R.J., 2004, Surgical method for biopsy of terminal ileum and mesenteric lymph node of sheep for detection of *Mycobacterium avium* subsp *paratuberculosis*. Australian Veterinary Journal 82, 149-151.
Orpin, P., Sibley, R., 2009, Approaches to Johne's disease. Vet. Rec. 164, 311-a-.
Paustian, M.L., Amonsin, A., Kapur, V., Bannantine, J.P., 2004, Characterization of Novel Coding Sequences Specific to *Mycobacterium avium* subsp. *paratuberculosis*: Implications for DIagnosis of Johne's Disease. Journal of Clinical Microbiology 42, 2675-2681.

Reddacliff, L.A., Marsh, I.B., Fell, S.A., Austin, S.L., Whittington, R.J., 2010, Isolation of *Mycobacterium avium* subspecies *paratuberculosis* from muscle and peripheral lymph nodes using acid-pepsin digest prior to BACTEC culture. Veterinary Microbiology 145, 122-128.
Robbe-Austerman, S., Gardner, I.A., Thomsen, B.V., Morrical, D.G., Martin, B.M., Palmer, M.V., Thoene, C.O., Ewing, C., 2006, Sensitivity and specificity of the agar-gel-immunodiffusion test, ELISA and the skin test for detection of paratuberculosis in United States Midwest sheep populations. Veterinary Research 37, 553-564.
Ruiz-Larrañaga, O., Garrido, J.M., Iriondo, M., Manzano, C., Molina, E., Koets, A.P., Rutten, V.P.M.G., Juste, R.A., Estonba, A., 2010a, Genetic association between bovine NOD2 polymorphisms and infection by Mycobacterium avium subsp. paratuberculosis in Holstein-Friesian cattle. Animal Genetics 41, 652-655.
Ruiz-Larrañaga, O., Garrido, J.M., Manzano, C., Iriondo, M., Molina, E., Gil, A., Koets, A.P., Rutten, V.P.M.G., Juste, R.A., Estonba, A., 2010b, Identification of single nucleotide polymorphisms in the bovine solute carrier family 11 member 1 (SLC11A1) gene and their association with infection by Mycobacterium avium subspecies paratuberculosis. Journal of Dairy Science 93, 1713-1721.
Santema, W., Overdijk, M., Barends, J., Krijgsveld, J., Rutten, V., Koets, A., 2009, Searching for proteins of Mycobacterium avium subspecies paratuberculosis with diagnostic potential by comparative qualitative proteomic analysis of mycobacterial tuberculins. Veterinary Microbiology 138, 191-196.
Taylor, D.L., Zhong, L., Begg, D.J., de Silva, K., Whittington, R.J., 2008, Toll-like receptor genes are differentially expressed at the sites of infection during the progression of Johne's disease in outbred sheep. Veterinary Immunology and Immunopathology 124, 132-151.
Waters, W.R., Miller, J.M., Palmer, M.V., Stabel, J.R., Jones, D.E., Koistinen, K.A., Steadham, E.M., Hamilton, M.J., Davis, W.C., Bannantine, J.P., 2003, Early induction of humoral and cellular immune responses during experimental *Mycobacterium avium* subsp. *paratuberculosis* infection of calves. Inf Immun 71, 5130-5138.

Acknowledgements

The authors would like to thank the staff of the Farm Animal and Veterinary Public Health Laboratory group Mrs Anna Waldron, Ms Adelyn Bolithon, Ms Sophie Hoft, Mrs Rebecca Maurer, Mr Jessie McIvor, Mrs Ann-Michele Whittington, Mrs Pabitra Dhungyel, Mr Ankit Srivastava, Ms Nicole Carter, Dr Deborah Taylor, Mrs Reena Mehta, Mr Craig Kristo, Mr Nobel Toribio, Mr Lee White and Ms Marion Saddington. Thanks are also due to Ms Elaine Chew, VPDS Histopathology Laboratory, Dr Peter Thomson and Dr Navneet Dhand.