final report

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Bovine Johne's Disease: basic and applied research for improved diagnosis and prevention

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Abstract

The diagnosis of bovine 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 a direct faecal PCR test can be developed and will 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 cattle producers, and this also was achieved.

Executive Summary

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

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

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

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

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

As a result of the project it is now clear that a diagnostic test which had severe practical and technical limitations can be improved and may be of substantial benefit in the near future. This is the direct faecal PCR test which can provide results to producers within a few days instead of the current 3 months for culture. In addition there are several new research-level tests of immune function that require further development.

Direct faecal PCR test
The direct faecal PCR test will be a breakthrough for the beef cattle industry. Previously, faecal samples were collected, sent to a laboratory and 3 months would elapse before negative test results could be confirmed. For cattle sales this meant considerable forward planning and great inconvenience for the producer. Where culture was used to confirm a suspected herd infection, for example after positive or suspect ELISA test results, the long delay caused considerable additional anxiety for the producer. The new test overcomes these problems because it can provide results within a few days of receipt of samples at a

laboratory. It will cost no more than culture. Furthermore it is suitable for testing pooled faecal samples, which enables a cheap method of herd testing, either to detect infection or to show that it is not present in herds in the Market Assurance Program. The test will also be suitable for environmental testing. Additional validation of this test was recommended by the JD Research Advisory Group to provide better estimates of sensitivity and specificity. This was due to there being only a low number of samples of suitable quality from infected herds, and few culture positive samples, despite appropriate effort to obtain these. An unexpected finding of the research was a requirement for faecal samples to be stored at -80°C prior to PCR – many samples obtained from infected herds had not been stored appropriately at a commercial laboratory. A request for funding to obtain appropriate samples has been submitted to MLA; faecal samples will be obtained from a Financial non Financial Assistance Program beef herd in Tasmania which has a high prevalence of ELISA reactors. Final data will be submitted to the SubCommittee on Animal Health Laboratory Standards (SCAHLS) for approval for use of the test in the National Johne's Disease Program later in 2011.

Whole blood interferon gamma and lymphocyte proliferation tests

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

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

Gene expression studies

Gene expression studies in infected cattle in the early stages of infection revealed a remarkable pattern of regulation of genes responsible for immunological processing. This uncovered pathways that previously have not been suspected to play much of a role in the early development of Johne's disease. This new knowledge will be applied in a future project.

Mechanism of disease progression

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

the immune system of the animal functions. Further research will be conducted across these fundamental discoveries to maximise their potential.

New experimental infection model

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

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1 Background

Bovine Johne's disease (BJD) caused by *Mycobacterium avium* subsp. *paratuberculosis* (Mptb) is a significant issue for the dairy industry in south eastern Australia and a sporadic problem in beef cattle. Efforts to reduce the within-herd prevalence of BJD within the dairy industry have been quite successful using conventional technologies, and concomitant efforts to prevent spread to the beef cattle sector also appear to be well accepted by industry. A vaccine for BJD may soon be available; this is likely to be an adjunct to reduce prevalence in heavily infected dairy herds.

Internationally, BJD is considered to be a significant threat to the livestock sector. Several studies have confirmed direct economic loss, but a greater threat exists because of a perceived link with Crohn's disease in humans, 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 BJD. These will depend on accurate diagnostic tests, which are still lacking. Consequently there are large research programs on BJD in the EU/Europe, Japan, New Zealand and North America and smaller research programs in many other places. For market access insurance, Australia needs to be engaged with R&D at an international level. Currently there is very little active research on BJD in Australia. A large research program on ovine Johne's disease (OJD) under the National OJD Control Program has been completed and has led to substantial capacity in this field of research that can now be applied to 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 cattle, and they may persist in the environment for prolonged periods given the right conditions. Young calves are the most susceptible to infection, and most infections occur within the first few months of life. Bacteria ingested from the milk or 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. BJD is a very slow progressing disease with the average incubation period (time from infection to clinical signs) of 5 years (range 3-15 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 BJD 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 prior basic research projects on JD which were funded under the National Ovine Johne's Disease Control Program. Research breakthroughs in that project led to outcomes for sheep which were further evaluated and developed for cattle in this project.

Arguably the greatest knowledge gaps in BJD include:

- the means to ensure accurate early diagnosis
- how to differentiate resistant cattle from susceptible cattle
- 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
- the contribution of direct transmission from cow to calf
- ways to induce immunity without causing cross reactions in tests for TB

This project concerns the diagnosis and prevention of Johne's disease. The two topics of diagnosis and prevention are linked – if the infection can be diagnosed effectively, its spread can be prevented by identifying infected animals and intervening in a practical way before they transmit the infection. 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™ Johnes real time PCR test: "Although a new MAP 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); in this study only 143 samples, 35 of which were culture positive, were tested and compared to culture with Bayesian methods incorporating the manufacturer's estimates of sensitivity and specificity.

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, these types of methods 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, leading to new approaches for 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 characterised 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 pathogenesis 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 (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 that could be included in immunological tests to diagnose 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; Paustian et al., 2004) with in-silico epitope prediction (Leroy et al 2009) or comparative proteomics (Hughes et al., 2008; 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; Kawaji et al., 2010). 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 receptor (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, assuming that sheep were also a suitable proxy for cattle.

We developed an experimental model for paratuberculosis infection in sheep based on low dose inoculation with a pure culture of Telford 9.2 Mptb from a lyophilized seedstock (Begg et al., 2010). This model can be adapted to cattle using a cattle strain of Mptb. In

sheep it resulted 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). 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. This approach can now be applied in cattle.

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 MLA's satisfaction:

1. Conducted a research program over 3 years to explore current international advances in BJD diagnosis in the Australian context
2. Developed tools to understand the BJD disease process with a view to closing some of the key knowledge gaps listed above
3. Exploited Australian advances in ovine Johne's disease research for the benefit of the beef industries
4. Ensured that Australian beef producers have access to respected researchers and relevant research findings on BJD

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 herd 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 as 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 are provided in appendices to the report

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.0297

Sub project Name	Status	What it has found out	Implication of this	Outcomes it will / has addressed
Subprogram 1	**Diagnostics**			
IFN-γ technology	Active	New methods were trialled for cattle; ELISPOT was not useful while Cell-ELISA and IFN-γ ELISA appear to have some value; enhancement of cell survival by adding IL-12 and IL-7 was detrimental to test specificity	Useful. The investigation is continuing however, dead end methods have been dropped. Better antigens are required for use in IFN-γ tests in cattle.	Need for a new blood test.
Optimisation and validation of direct faecal PCR test	Active	New HT-J method developed. Further validation was recommended by JD-RAG before progressing to commercialisation.	Useful. The investigation is continuing.	Need for a new faecal test. This research has enabled rapid (within 1 week) detection of Mptb in faeces with similar sensitivity to culture.
Subprogram 2	**Basic Research on immune responses in early stages of BJD**			
Improved understanding of immune responses	Active	A novel mechanism of disruption of amino acid (tryptophan) metabolism was identified in cattle which may explain immune suppression and weight loss. Tests to measure early antibody responses, the cytokine IL-10 and cell proliferation have revealed subtle features of the immune response.	Useful. The investigation is continuing.	Need for a new blood test. Need for better understanding of the disease so that new tests and vaccines can be developed.
Novel gene expression by host and Mptb: microarray studies	Active	Immune response genes are strongly activated or suppressed during the early stages of Mptb infection.	Useful. The investigation is continuing.	Need for better understanding of the disease so that new tests and vaccines can be developed.

Subprogram 3	**Animal resources and experimental infection models**			
Animal trials	Active	Surgical biopsy confirmed that cattle have started to develop BJD in paddock-based experimental infection trials. This underpins all other subprograms by providing samples.	Useful. The investigation is continuing.	Need for better understanding of the disease so that new tests and vaccines can be developed.
Cell culture models	Active	Isolation of monocytes from bovine blood samples has been successful	Useful. The investigation is continuing.	Need for better understanding of the disease so that new tests and vaccines can be developed.

4.1 Subprogram 1 Diagnostics

4.1.1 IFN-γ technology to detect Johne's disease in cattle

Detection of IFN-γ is thought to be a good way to diagnose paratuberculosis in the early stages of infection. These assays detect IFN-γ that is produced by live blood cells specifically when these cells react to components of the mycobacterium (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.

The traditional method for detection of IFN-γ from ruminants has been to stimulate whole blood with Mptb antigens, remove the plasma and carry out an ELISA on the plasma. The existing assays on the market for detecting IFN-γ in animals with paratuberculosis are not optimal. The Bovigam® assay (Prionics, Switzerland), developed for the detection of bovine tuberculosis, may be modified by changing the component (antigen) that the blood reacts to so that it is specific for paratuberculosis. This assay, like all existing IFN-γ assays, is conducted using live blood cells and a major limitation of the on-farm application of the test has been the time required to transport blood to the laboratory. The existing assays must be set-up within 8 hours of blood collection to obtain reliable results. In addition in cattle IFN-γ ELISAs have had lower specificity in detecting animals younger than 24 months, due to non-specific IFN production by natural killer cells (Jungersen et al., 2002; Olsen et al., 2005).

In project OJD.031 it was found that novel methods for the detection of IFN-γ (ELISPOT and Cell-ELISA) were as good as if not better at the detection of Mptb infected sheep than the traditional whole blood interferon gamma ELISA. The IFN-γ responses from the ELISPOT and Cell-ELISA assays could be detected earlier after infection. The advantage of these newer assays is that they measure different aspects of the IFN-γ production; the ELISPOT measures the number of white blood cells producing IFN-γ while the Cell-ELISA measures the total amount of IFN-γ produced by the white blood cells. In contrast 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 project OJD.031 they were not commercially viable as diagnostic tests. This is due to the high cost of 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. Non-specific IFN-γ responses have been recorded in cattle less than 2 years of age (Olsen et al., 2005). It is possible that by using alternative methods of detecting IFN-γ that this non-specific response may be overcome. The aim of this research was to evaluate whether the three different IFN-γ detection methods could be modified for cattle and whether the assays could accurately diagnose infection in experimentally inoculated cattle.

The transfer of the IFN-γ technology from sheep to cattle was straight forward, as no modifications were required on the assays. The traditional whole blood interferon gamma ELISA could detect IFN-γ in the early stages of the infection but as reported previously there is an issue with Mptb-specific responses being detected from

uninfected cattle less than two years of age (Olsen et al., 2005). The Cell-ELISA assay did not have such an issue with the Mptb-specific responses from control cattle under 2 years of age and did show some useful antigen-specific responses from the inoculated cattle as early as 5 months post inoculation. Both the traditional whole blood interferon gamma ELISA and the Cell-ELISA may be useful diagnostic tools for Johne's disease, but until we have the final outcome of the experimental infection in the trial P.PSH.0297.2 it cannot be identified which test is more appropriate. The ELISPOT assay did not detect Mptb-specific IFN-γ responses in the inoculated cattle. The results indicate that the ELISPOT assay is not practical as a diagnostic aid as it does not detect potentially infected cattle. The use of the red blood cell lysis method for isolating the white blood cells may be similar to sheep in having an unknown detrimental effect on the functionality on the cells so that they cannot respond to the Mptb antigen. A switch to density gradient centrifugation for the isolation of the white blood cells may be useful as a research tool in identification of immune signatures in animals with developing Johne's disease. Detailed methods and results are presented in Appendix 1.

4.1.2 Enhancement of interferon-gamma (IFN-γ) technologies using IL-12 and IL-7

The aim of this study was to investigate and develop techniques that may improve sensitivity and permit a longer period between blood collection and culture for the existing whole blood IFN-γ ELISA protocol. This may be translated to other cytokine detection assays, for example the interleukin-10 ELISA. Addition of IL-12 to IFN-γ stimulation cultures has been previously shown to increase the survival of the white blood cells and IFN-γ production in these assays (Jungersen et al., 2005). This protocol involved the addition of IL-12 to whole blood cultures in order to 'rescue' the ability of Th1 cells to produce IFN-γ in response to Mptb antigen. Jungerson *et al* found that the time period from collection of blood to set-up of the culture in the laboratory could be prolonged out to 24 hrs, which reduced the logistical issues with the assay. There was a problem identified in that the addition of IL-12 could give non-specific positive results in cattle. This has also been found in studies on younger cattle that were prone to non-specific false positive responses in IFN-γ assays for paratuberculosis, possibly related to IFN-γ release by natural killer cells (Jungersen et al., 2005; Jungersen et al., 2002; Olsen et al., 2005).

The role of IL-7 as a survival factor for certain white blood cells (T cells) was first identified in studies of cell cultures (Vella et al., 1997). IL-7 has also been shown to maintain cell size, promote glucose and protein metabolism to avert 'death by neglect' (Rathmell et al., 2001) and upregulate anti-apoptotic protein expression, Bcl-2 (Masse et al., 2007). Therefore, we proposed that IL-7 may be beneficial as an additive in IFN-γ stimulation cultures due to its role as a cell survival factor and as it and has been shown to maintain T cell responses in culture in other types of live cell assays (eg. mixed lymphocyte cultures). IL-7 does not by itself induce resting T cells to proliferate, but stimulates isolated or 'neglected' T cells to maintain function and metabolic rate similar to freshly isolated cells and promotes their subsequent ability to respond in culture.

In this study, we used our in-house IFN-γ ELISA protocol and attempted to prolong the time between collection of blood and set-up of the culture. The experiments were adapted from IL-12 potentiation studies. Initially, we assessed the response of control cattle and cattle which had been immunised with Mptb-antigen to create positive

control samples. The optimised method was used to potentiate responses for blood samples from experimentally infected cattle. The goal was to have a simple, cost efficient method that allowed for a reasonable transport time of blood from the farm to the laboratory and retained or improved sensitivity of the assay. Detailed methods and results are presented in Appendix 2.

Overall, IL-12 and IL12/IL-7 addition to cattle blood cultures led to high media background levels and loss of discrimination of Mptb-specific responses between control and vaccinated/inoculated cattle. This was particularly evident for cattle in the experimental infection trial. A further issue was identified in that storage of cattle blood at room temperature led to non-specific release of IFN-γ and this was heightened by the addition of cytokines**.**

Factors that may have contributed to the loss of specificity include the age of the cattle tested. It is known that young cattle can have non-specific IFN-γ responses (Jungersen et al., 2002; Olsen et al., 2005). The cattle in the experimental infection trial were <9 month of age when tested. It is possible that as the trial progressed and the animals aged, the specificity would improve. This will need to be further investigated, and can be in an ongoing trial. Other factors that may have adversely affected specificity include the storage temperature and the Mptb antigen. Cattle blood may be particularly sensitive to storage temperature and require storage at 4^{o}C. A more specific antigen rather than the Mptb antigen used in these studies (316v, which is whole pressed Mptb) may give better specificity. This work will be conducted in a future project.

4.1.3 Direct faecal PCR tests for Johne's disease

The development of new and improved diagnostic tests for both OJD and BJD has remained at the forefront of Johne's disease research since the mid 1990s at both the University of Sydney and the Elizabeth Macarthur Agricultural Institute (EMAI, Industry and Investment NSW, formally NSW Agriculture). This research resulted in the development of the pooled faecal culture (PFC) test (Whittington et al., 2000; Whittington et al., 1999), currently the best available test for OJD, and other polymerase chain reaction (PCR) based assays including a direct-PCR (DPCR) assay for the detection of Mptb in faeces (Marsh and Whittington, 2001). The initial direct PCR test developed in the late 1990s as part of the MLA funded project (TR.060) showed promise but lacked the sensitivity of faecal culture, a prerequisite if it was to be offered as a routine test for the diagnosis of Johne's disease.

An MLA funded follow up project (AHW.079) was undertaken at EMAI to elevate the sensitivity of the original EMAI DPCR test to be equal or greater than that of faecal culture while maintaining the technical practicalities of the test and low cost. Unfortunately, we were not successful in achieving the desired outcome. Although we were able to establish that the poor performance of the new test was directly attributable to the Mptb isolation and DNA extraction procedure, time and financial constraints prevented us from developing the test further.

Fortunately, just before the conclusion of the AHW.079 project a new DPCR test that incorporated real time PCR technology became available. This new test was developed and trialled initially in Japan at the Japanese National Institute of Animal Health combined with a Japanese faecal extraction technique (JohnePrep™, Shimadzu Corporation). At the time the test was not commercially available in

Australia but was being evaluated on faecal samples from both cattle and sheep at the Faculty of Veterinary Science at the University of Sydney by Satoko Kawaji, a PhD student under the supervision of Professor Richard Whittington. The new DPCR test (known as RT-J) (Kawaji et al., 2007) had 86% sensitivity compared with faecal culture on faecal samples previously examined at EMAI.

Overall the preliminary results from the RT-J test were very promising and indicated that a more thorough examination of the test was required. Optimal results appear to be achieved when the new faecal extraction method (JohnePrep™, Shimadzu Corporation) was used in conjunction with real-time PCR. Unfortunately, the RT-J test was procedurally difficult and prone to cross contamination in the hands of inexperienced operators. The test required further development to be suitable for routine use and further evaluation under Australian conditions.

Collaboration between the Faculty of Veterinary Science at the University of Sydney and EMAI (I&I, NSW) was undertaken to further develop the new direct faecal PCR test (RT-J) for Johne's Disease based on the procedure described in a recent publication by Satoko Kawaji (Kawaji et al., 2007). Steps in this method were redesigned to make them more practical and robust, without loss of sensitivity, leading to two new test formats called DF-J and HT-J. Because the RT-J test appeared to be the best available worldwide based on literature review, and because HT-J was the better of the two new versions of RT-J, an intensive investigation was undertaken to develop the HT-J test. This work is described in detail in Appendix 3.

Validation of the HT-J test commenced with 1202 faecal samples representing 22 infected properties from New South Wales (9), Tasmania (4), and Victoria (9) and at least 8 unexposed properties from Queensland (7) and Western Australia. As samples from unexposed properties in Western Australia were collected at an abattoir without matching NLIS data the exact number of properties represented is uncertain. When requested, the laboratory reports for these samples were issued to submitters within a 7 day timeframe, which confirmed 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. The HT-J test was conducted at two laboratories and the results were interpreted in parallel. A summary of the results are provided in Table 2.

Table 2. A summary of results in the culture and HT-J faecal PCR tests. The data are the number of positive results with the total number evaluated in each test given in parentheses. There were a total of 1202 faecal samples, 35 of which were not tested in the HT-J test due to limited sample volume.

Category of sample	Culture	HT-J PCR test
Not exposed to JD	0 (471)	0 (465)
Infected farms	13 (513)	83 (494)
Infected farms – private lab sample storage	101 (218)	87 (208)

Only 13 culture positive samples with acceptable cold chain history (i.e. stored and shipped at an acceptable temperature) were available in this study. This was despite the concerted efforts to obtain suitable samples from beef herds enrolled in the Cattle Council of Australia Financial Non Financial Assistance Program. Sample collection from these herds commenced in November 2009 and concluded in September 2010. Unfortunately the majority of samples yielded negative culture results, providing

relatively few useful samples for estimating test sensitivity. To supplement these samples when their negative culture results were known (at least 3 months after collection), samples from two dairy herds in Victoria were obtained by arrangement with Pfizer Animal Health. The samples had been stored at a private laboratory following culture and it was hoped that a large number would be suitable for PCR analysis. However, the only submissions where there were fewer HT-J positive samples than culture positive samples involved samples from the two dairy herds that were stored at the private laboratory at -20°C, and subjected to several freeze thaw cycles. We suspect the discrepancies between the culture and HT-J results are most likely attributable to the storage temperature. We showed experimentally that the HT-J test requires samples that are freshly collected, or stored at -80°C. Storage at -20°C is detrimental. Therefore the results from these samples were excluded from the main analysis.

The specificity of the HT-J test appears to be very high. None of the 465 samples from cattle from regions where BJD is not known to be present were positive (Table 2). These included 248 samples from 7 different herds in Queensland and 223 from various herds in Western Australia (6 samples were not tested due to limited sample volume). Therefore specificity was 100% (95% confidence limits 99.2 to 100).

The sensitivity of the HT-J test cannot be determined accurately at this time because there were insufficient culture positive samples of suitable quality. However, some general conclusion can be made. More samples from infected herds were HT-J positive than culture positive, and the number of culture positive samples from the two Victorian herds that had been stored poorly was only about 10% greater than the number of HT-J positive samples. Therefore it is probable that the sensitivity of the HT-J test is as high as that of culture. HT-J positive results in excess of culture positive results in infected herds could also be explained by false positive results (low analytical specificity) but given that none occurred in unexposed herds this seems unlikely. However, an important issue is pass-through shedding. The high analytical sensitivity of the HT-j test and its ability to detect non-viable Mptb means that individual animal specificity in infected environments may be poor due to detection of pass-through organisms (i.e. cattle may consume Mptb which pass through the gut into the faeces without infecting the animal). Sensitivity will be difficult to define in absolute terms because we do not know the true infection status for any of the cattle. Gold standard histopathology and tissue culture results were not available from any of the animals.

In order to properly determine the sensitivity of the HT-J test relative to culture a greater number of samples from culture positive cattle are needed. The Johne's Disease Research Advisory Group recommended in March 2011 that additional samples from infected herds be examined to improve estimates of test sensitivity. A suitable herd has now been located in Tasmania through the Financial Non Financial Assistance Program and the owners have agreed to allow access to their cattle. The beef herd has an unusually high seroprevalence in ELISA for BJD. MLA has been approached to fund sample collection, culture and PCR testing of an additional 200 samples from ELISA positive cattle. Completion of this work will enable final analysis of the data and estimation of sensitivity with more confidence.

The HT-J test provides results on a continuous scale, making it possible to set different positive negative cut-offs. The optimum cut-off has not been determined and this also will be done when the additional samples have been collected and tested.

The selection of cut-off affects both sensitivity and specificity and may need to be set differently at different laboratories because different PCR equipment is used at each laboratory.

Finally, data on the consistency of results between the two laboratories will be conducted after completion of testing of the additional samples and establishing independent positive-negative cut-points for each laboratory. This will establish the robustness of the test.

4.2 Subprogram 2 Basic research on immunity in early stages of BJD

4.2.1 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, characterizing gene expression, protein localization 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 4.

4.2.2 Improved understanding of antibody responses in cattle

Knowledge of antibody responses in cattle in the early stages of Johne's disease infection is somewhat limited. For a long time it was thought that very little antibody was present in the early stages of infection, although more recent studies have discovered that there are antibodies present (Bannantine et al., 2008; Waters et al., 2003). These studies focused on the type of antigen used in the ELISA assay to detect the early antibody responses in cattle. Other approaches that may aid the understanding of the antibody response are the type of antibody isotype that is measured; isotypes such as IgG1 have been suggested to be better diagnostic indicators of infection than IgG or total antibodies, as detected by most commercial ELISA assays (Griffin et al., 2005). The implementation in Australia of the IgG1 assay developed in New Zealand by Griffin et al (2005) has been hampered by intellectual property issues, although as discussed later this may not be a constraint at this time.

The aim of this study was to examine the early antibody responses using the animals in experimental inoculation trials. A commercial ELISA (Institute Pourquier, France) was used. The antibody responses from cattle in both experimental inoculation trials

were negative. Trial P.PSH.0297 is on-going and further results will be obtained through a future research project. That there have been no detectable Mptb specific IgG antibody responses as measured by the Pourquier ELISA is somewhat surprising as other studies have discovered early antibody responses in experimentally inoculated cattle using other specific recombinant antigens. However, the other studies differed from our own in that there was a very artificial experimental inoculation method used (Bannantine et al., 2008; Waters et al., 2003). By utilising an intra-tonsillar inoculation method there may have been bias in the immune response as this occurred when this method was used in sheep (Begg et al., 2005) where an earlier antibody and IFN-γ response were induced compared to an oral infection. Examination of new recombinant antigens that have been identified may detect earlier responses in these animals. This will be conducted in a future study.

4.2.3 Immunological assays based on the cytokine IL-10

The immune response in Johne's disease is traditionally gauged in terms of antigen-specific IFNγ production, cell proliferation or antibody production. IFNγ and cell proliferation are markers of the cell-mediated immune response considered 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 (Buza et al., 2004; Lybeck et al., 2009). IL-10 is known for its role in maintaining a controlled immune response. Results from the earlier OJD.031 project demonstrated that in sheep the IL-10 response in peripheral blood cells starts within a few months after exposure to Mptb in experimentally challenged animals (de Silva et al., 2011). Prior to the present P.PSH.0297 study, the IL-10 response had not been investigated in cattle throughout the course of development of Johne's disease, i.e. from pre-infection to clinical disease. In addition, in this study the cellular source of IL-10 in Johne's disease was assessed for the first time. Such knowledge is needed for the planning of future diagnostic strategies. Blood samples were collected from calves prior to Mptb challenge and then every 4 weeks until 7 months post challenge and then every 8 weeks. Blood samples continue to be collected from these animals. IL-10 was detected using an in-house ELISA.

An antigen-specific response was detected from 15 weeks after inoculation. However, controls animals and Mptb-inoculated cattle both respond similarly to Mptb antigen as well as to the *M. avium* antigen (PPDA) (Figure 3A and 3B). Unlike in sheep, there does not appear to be an elevated early IL-10 response in cattle and therefore monitoring this parameter will not be advantageous in the detection of bovine Johne's disease.

An intracellular staining protocol was established to determine the phenotype of IL-10 producing cells. In six Mptb-exposed cattle assessed at 17 months post challenge there was no distinct pattern of cell types responsible for the secretion of IL-10 in response to recall of Mptb. In some animals CD8 T cells and B cells were the main cells producing IL-10 in response to the Mptb antigen. Interestingly in those animals where the CD8 and B cell IL-10 response was low, the main IL-10 responding cells were γδ T cells. The phenotype of IL-10 producing cells was only determined at one time point. Assessing the phenotype of IL-10-producing cells at a later time point, as the cattle start showing signs of clinical disease, may inform the Johne's disease signature. It is hypothesised that patterns of immunological responses may be predictive of future disease outcomes. They will be investigated in a future project.

4.2.4 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). Prior to this project P.PSH.0297, the antigen-specific proliferative response has not been monitored in cattle throughout the course of Johne's disease, from pre-infection to clinical disease. Blood samples were collected prior to Mptb challenge and then every 4 weeks until 7 months post challenge and then every 8 weeks. Blood samples continue to be collected from these animals. Faecal samples were also collected at the same time points. The Proliferation Assay was carried out as described (de Silva et al., 2010). Mptb-specific proliferation was seen in inoculated animals at 33-97 weeks post inoculation but not in non-exposed controls. When a level of 10% Mptb antigen-specific proliferation was chosen as the cut-off for a positive reponse, 50-90% of inoculated cattle were positive for the test at 33-97 weeks post inoculation while all non-exposed controls gave a negative response at all time points. In the inoculated cattle, the proliferative response decreased at later time points.

It is not possible to catergorise experimentally infected cattle in trial P.PSH.0297.2 into disease status as yet, but immune response analysis has revealed differences between animals. For example in two animals: faecal shedding has been detected continually for the last 7 samplings (14 months) in one while faecal shedding was detected only at one time point soon after experimental exposure in the other, and the immune response profiles of the two cattle were different. Therefore it is likely that immune response profiles for different disease outcomes in Johne's disease could be developed by combining these parameters with others such as gene expression profiles.
Full results for this study are provided in Appendix 7.

4.2.5 Gene expression in bovine Johne's disease

The primary objective of this study was to evaluate gene expression profiles in cattle exposed to known amounts of Mptb and compare these to control cattle at three very early post exposure time points (9, 13 and 21 weeks post exposure). The samples were drawn from the experimental Johne's infection trial P.PSH.0297.2, consisting of a mixed population of Holstein cattle. A total of four Mptb exposed and 4 control cattle were chosen from each post exposure time point on the basis of observed interferon gamma expression and the 24 resulting samples were processed to microarray. Extensive data analysis including ontological analysis revealed a number of differences in gene expression between the Mptb exposed and control animals at the individual time points. In particular a subset of genes associated with the MHC were found to be consistently altered suggesting significant changes to the antigen processing and presentation pathways in Mptb exposed animals in comparison to the control cohort. Ontological analysis revealed a stark variation in the expression of MHC genes along the known antigen presentation pathways which suggest that Mptb exposure potentially results in the host immune response switching to a CD8+ biased antigen presentation profile. This association requires further in-depth analysis since it exposes a hitherto unconfirmed association between Mptb exposure

and MHC gene modulation. Previous studies of *M. tuberculosis* have identified a similar association however within the Mptb research field this larger functional association is novel.

IFNγ enhances cellular expression of both MHC Class I and MHC Class II molecules. Although the Mptb-challenged cattle in this study were selected for their enhanced IFNγ response at 9 weeks post exposure, differential expression of Class I and Class II molecules was found. Previous studies have shown that mycobacteria can downregulate MHC Class II expression on antigen presenting cells, therefore we hypothesise that in these Mptb-exposed cattle the tendency towards decreased MHC Class II expression, despite an elevated IFNγ response, is due to active effects of Mptb to avoid exposure to host defence mechanisms. The consistent upregulation of MHC Class I molecules in the initial few months after exposure to Mptb will allow interaction with cytotoxic CD8+ T cells. Thus we hypothesise that the CD8+ T cell response may be more important than previously thought in the elimination of Mptb.

Based upon these initial findings further research will be performed to determine allele specificity of MHC components within the trial animal cohort as well as analysis of other regulatory genes associated with MHC function. Microarray and ontological analysis revealed a number of differences in gene expression between the Mptb exposed and control animals at the individual time points. Detailed methods and results are provided in Appendix 8.

4.3 Subprogram 3 Animal resources and experimental infection models

4.3.1 Development of an experimental infection model for bovine Johne's disease

The aims of this study were to create a similar experimental inoculation protocol as was developed for sheep (Begg et al., 2010). The first trial was a short term pilot study to see if a defined cattle strain of Mptb would result in infection; the second trial was a long term study to evaluate infection outcomes in cattle.

Thirty nine Holstein cattle aged 2-4 months were used in 2 trials. In the first trial, P.PSH.0297.1, five calves were experimentally inoculated orally with a dose of 4.3 x 10^8 viable cells followed one week later by a second dose of 9.3 x 10^6 viable cells. One month after the first inoculation a third dose was given of 4.3 x 10^7 viable cells. Two months after the first inoculation two of the animals were euthanased and necropsied; the remaining 3 animals were culled at 3 months post initial inoculation. None of the calves had detectable Mptb specific antibody responses throughout the trial while the IFN-γ responses had a high background initially then decreased. There were no histological lesions but culture of the tissues from the cattle confirmed that 4 of the 5 inoculated animals were infected with Mptb. One of the animals was also faecal culture positive. This experiment confirmed the validity of the infection methodology, enabling a large trial to begin.

In the second trial, P.PSH.0297.2, thirty calves aged 2-4 months were age matched then randomly allocated into a group of 20 to be inoculated and a group of 10 controls. Control animals were housed separately from the inoculated animals, in paddocks where no Mptb infected livestock cattle had been housed in the past. The 20 animals to be inoculated were dosed using the same schedule as the pilot trial with doses of 2.3 x 10^8, 9.3 x 10^7 and 1.5 x 10^8 viable cells. To monitor the development of intestinal lesions in these cattle, intestinal and ileal lymph node

biopsies were carried out at 16 to 18 months post inoculation and again 8 months later.

Faecal culture showed a spike of shedding of Mptb at 5 months post inoculation with almost half of the cattle shedding (Figure 7.3.1.1). One animal has remained a consistent shedder while another has shed intermittently from 13 months post infection. There is a substantial implication from these findings – calves may be able to transmit JD to other calves.

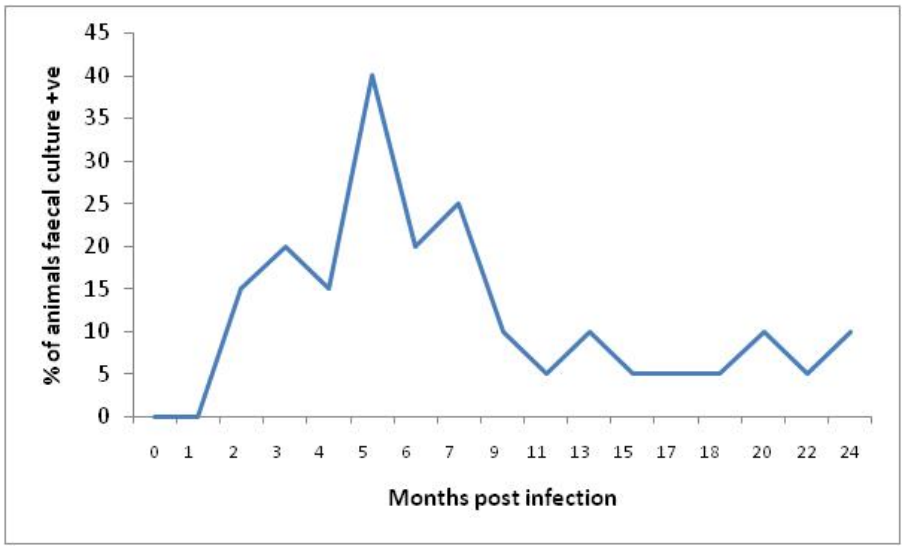

Figure 7.3.1.1. The Mptb faecal shedding rate of the cattle in trial P.PSH.0297.2

No animals have had an antibody response that would indicate an infection at any period up to 26 months post inoculation. In contrast to the antibody ELISA results, mean Mptb antigen-specific whole blood IFN-γ ELISA levels increased steadily until 13 months post inoculation at which time the responses began to slowly decrease (Figure 7.3.1.2). As seen in the pilot study there were some background responses in the control animals especially during the first year post inoculation.

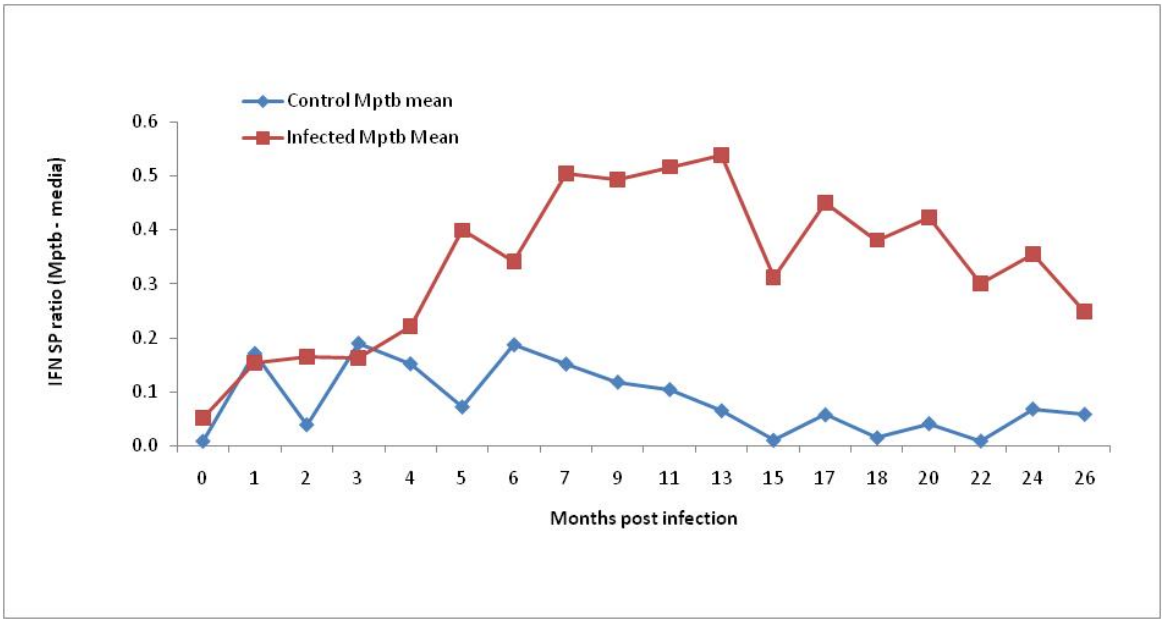

Figure 7.3.1.2. Mean whole blood IFN-γ ELISA responses from the inoculated and control cattle in trial P.PSH.0297.2

There was no histological or tissue culture evidence of infection from the biopsy results at 17-18 months post inoculation. However, the second round of biopsies at 26 months post infection indicated 2 of the animals had microscopic lesions associated with Johne's disease in sections of ileum.

Experimental inoculations of cattle with Mptb have resulted in variable outcomes with infection rates from 13- 100%. Since a trial conducted in 1989 (Lepper et al., 1989) very few clinical cases have been recorded from experimentally inoculated cattle, and this may be at least partly due to the short duration of the trials relative to greater than 2 years required between inoculation of Mptb and clinical disease development in natural infection in cattle. Holding animals for long periods of time can be prohibitively expensive especially if the cattle must be held indoors. It is unknown what dose of Mptb is required to lead to a clinical disease outcome in cattle as much of the previous research was not based on accurate methods of enumerating the viable bacteria. Most recent experimental inoculation trials in cattle have used very low numbers (~4) of cattle (Allen et al., 2011; Stabel et al., 2009), which risks the ability of being able to observe the potential wide spectrum of responses seen between individual animals in naturally infected herds. Furthermore most researchers have used the detection of sub-clinical infection as an end point in vaccine and pathogenesis studies (Stabel et al., 2009; Sweeney et al., 2009); a potential limitation with this approach is that it is unknown whether any animals would have progressed to clinical disease, or recovered from infection, and therefore whether any animals in the trial have undergone a pathogenesis reflective of natural infection.

This experimental infection trial is one of the largest and longest to be conducted in cattle over the past 30 years anywhere in the world. It has utilised a number of recommended protocols (Begg and Whittington, 2008) such as accurate enumeration of the bacteria, low inoculation doses of a low passage strain of Mptb, the use of liquid culture (the most sensitive method to detect Mptb) and frequent blood and faecal samplings to monitor the infection. Most importantly, this trial has given the

research group the ability to monitor these animals for a long period of time post inoculation. The inoculation doses used in this trial are 10 to 1000 lower than other recently conducted infection models in cattle (Buddle et al., 2010; Stabel et al., 2009) which should ensure that the outcomes are realistic and relevant to natural infection on farms.

The results from the main trial are of major significance and it will continue in a future project. Full methods and results are provided in Appendix 9.

4.3.2 Isolation of bovine monocytes for *in vitro* studies

Pure isolated bovine monocytes can be used for research into the responses of ruminant macrophages to Mptb to gain greater understanding of pathogenic processes occurring within infected animals. Macrophages are key cells in the pathogenesis of Mptb, being both the target cell of the infection and the cells involved in pathogen recognition and initiation of immune responses. Mptb and other virulent mycobacteria inhibit or modify many of the antibacterial processes of macrophages in order to survive intracellularly (Weiss and Souza, 2008; Whittington et al., 2011). However, there is still much that is not known regarding the way Mptb organisms interact with macrophages. Alteration in macrophage function can have a major impact on the establishment of infection and initiation and regulation of the immune response, such as the cytokines produced by macrophages (interleukin (IL)-12, IL-10, tumor necrosis factor alpha), cell surface molecules they express (CD14, toll-like receptors, CD40, MHC class II molecules) and intracellular signalling pathways (nuclear factor kappa beta (NFκβ), mitogen-activated protein kinase (MAPK) p38) (Sanchez et al., 2006; Weiss and Souza, 2008). Changes in macrophage phenotype and function can be studied using *in vitro* infection models or by examining macrophages from animals that are infected with Mptb. In order to perform these experiments, we must first be able to isolate monocytes/macrophages in sufficient numbers and purity. Previous work by this group on primary ovine macrophages identified yield and purity as the major obstacles in performing functional and phenotypic studies.

The MACS® separation system is designed to separate cell populations from mixed biological samples, such as peripheral blood mononuclear cells (PBMC) or lymph node tissue suspensions. The MACS® magnetic bead particles are nanosized particles that cannot be seen microscopically; they can only be visualised on a transmission electron micrograph. An antibody (Ab) to the macrophage cell surface molecule is attached to the magnetic bead. Cell populations are enriched by binding of labelled (Ab + magnetic bead) cells to a column that is placed in a strong magnetic field. Unlabelled cells pass through the column while labelled cells are retained and can be recovered from the column after removal from the magnetic field. Both fractions, the positively selected fraction retained on the column and the negatively selected fraction that passes through the column, can be used for downstream experiments.

This work describes the development of an isolation technique for bovine monocytes that gives high purity and yield. Healthy Holstein cross cattle were maintained at the University of Sydney research farm. Blood was collected into 10ml lithium heparinised vacuum blood collection tubes, the buffy coat of white blood cells was removed, purified over Ficoll paque, resuspended and enumerated. The cells of interest were magnetically labelled by targeting a specific molecule on the cell

surface, passed though a column placed within a strong magnetic field and the positively labelled monocytes were retained while other cells passed through. The monocytes were then eluted from the column once it was removed from the magnetic field. Phenotypic analysis of positively selected, negatively selected and unfractionated PBMCs was performed using a panel of antibodies and respective isotype controls for the different surface markers indicative of each cell subtype. Monocytes isolated from normal bovine blood using the MACS® protocol were highly purified, with a yield of approximately 4% of the starting PBMC cell numbers. In an initial stimulation experiment, it was confirmed that the isolated monocytes were capable of producing nitric oxide upon stimulation with interferon-gamma/LPS. This gave an indication that the cells were functional after isolation.

These cells will be used in further studies of *in vitro* Mptb infection to examine the effect on macrophage function in a future project. Studies on cattle experimentally infected with Mptb will also be conducted to determine if there are changes in the monocyte populations from these control and exposed animals. Full methods and results are provided in Appendix 10.

5 Discussion/conclusion

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

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

As a result of the project it is now clear that a diagnostic test which had severe practical and technical limitations can be improved and may be of substantial benefit in the near future. This is the direct faecal PCR test which can provide results to producers within a few days instead of the current 3 months for culture. The direct faecal PCR test is a breakthrough for the beef cattle industry. Previously, faecal samples were collected, sent to a laboratory and 3 months would elapse before negative test results could be confirmed. For cattle sales this meant considerable forward planning and great inconvenience for the producer. Where culture was used to confirm a suspected herd infection, for example after positive or suspect ELISA test results, the long delay caused considerable additional anxiety for the producer. The new test overcomes these problems because it can provide results within a few days of receipt of samples at a laboratory. It will cost no more than culture, and may be of similar accuracy. Furthermore, following validation of pooling rates, it may be suitable for testing pooled faecal samples, which would enable a cheap method of herd testing, either to detect infection or to show that it is not present in herds in the Market Assurance Program. The test will also be suitable for environmental testing. Additional validation of this test 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.

In addition to the direct faecal test there are several new research-level tests of immune function that require further development. A whole blood interferon gamma assay has been 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. Two blood additives were trialled to extend the life of the blood samples to 48 hours. This would make it possible to ship samples from most places in Australia to a laboratory in time to conduct the test. The additive may have worked, but there was concomitant loss of specificity. Additional research is now required to find a way to make the test more specific, and this will be done in project P.PSH.0576, with an aim to validate the new procedure within the life of that project. Interferon gamma detection assays offer the potential to detect more infected animals at an earlier stage of the disease compared to an antibody ELISA or direct detection of Mptb in the faeces. This may provide opportunity for control strategies aimed at removal of young infected animals before they start shedding bacteria into the environment.

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

Gene expression studies in inoculated cattle in the early stages of infection revealed a remarkable pattern of regulation of genes responsible for immunological processing. This uncovered pathways that previously have not been suspected to play much of a role in the early development of Johne's disease. This new knowledge will be applied in a future project.

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

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

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8 The Appendices are not available publically as they contain confidential information