final report

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Diagnostic, predictive and preventative tools for Johne's disease in sheep and cattle

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Abstract

The distribution of Johne's disease (paratuberculosis) in flocks and herds in Australia is very uneven, which creates an opportunity to reduce spread while better tools are developed to detect, control and prevent the disease. A large program of basic and applied research was conducted to provide a foundation for better tests and future control options, while ensuring that diagnostic tools remain available and are as accurate as possible. New diagnostic tools were developed and refined to replace older tools, and for early testing. A new culture medium and an enhancement to the interferon gamma test were evaluated. New genetic approaches identified resistant animals, and different breeds of sheep were tested. Genetic markers were found. Research towards a safer, more effective vaccine for sheep and cattle was conducted and an animal model developed, leading to a new more cost effective strategy for vaccine efficacy trials. Research capacity for livestock health was developed for the benefit of producers. Recommendations are made on translation of findings into livestock breeding and improving industry capacity to deal with JD in a deregulated environment.

Executive summary

Johne's disease. Johne's disease (JD) is a chronic infection of the intestinal tract and associated lymph nodes caused by *Mycobacterium avium* subsp. *paratuberculosis* (Mptb). Most infections are acquired soon after birth but progress slowly, and signs of disease are usually not seen for 1 to 3 years in sheep and for 3 to 10 years in cattle. The hidden phase is called subclinical infection. During this long incubation period tests for JD are inaccurate. Mptb is passed in faeces (termed faecal shedding) long before there are overt signs of disease, and this perpetuates the infection cycle in a herd or flock. Subclinically infected animals can be moved to other farms, thereby spreading the infection. Eventually the signs appear: weight loss, and in cattle also diarrhoea, followed by death. Initially only a few animals are affected each year, but the numbers inevitably increase. While upfront costs are less than those of other endemic diseases, internationally JD is regarded as an undesirable problem, and some countries impose control measures that can impact trade. The distribution of JD in flocks and herds in Australia is very uneven, which creates an opportunity to reduce spread, provided better tools are developed to detect, control and prevent the disease.

Scope of Project P.PSH.0576. This is a large program of basic and applied research, commissioned to provide a foundation for better tests and future control options, while ensuring that current diagnostic tools remain available and are as accurate as possible. The project was divided into three research subprograms and an animal trial program, and it contained an explicit objective to build animal health research capacity for the livestock industries.

1. Predictive and diagnostic tools - to enable producers to identify exposed and infected herds/flocks/individuals and to predict those likely to spread the disease.

2. Animal genomics - to identify resistant animals, and to determine the susceptibility of different breeds of sheep.

3. Improving vaccine technology – to conduct research towards a safer, more effective vaccine for sheep and cattle.

4. Research capacity for livestock health - to maintain and develop capacity in livestock health for the benefit of producers.

We concentrated on the disease in the period soon after exposure to Mptb (< 12 months), while animals had early subclinical infection, because we were most interested in determining early predictors of future disease outcome. This work required long term experimental infection trials in both sheep and cattle. There were remarkable discoveries and practical achievements.

Predictive and diagnostic tools

The diagnosis of JD is usually described in terms of detecting the pathogen directly, for example by culture of faeces and gut tissues, or by detecting the response of the animal to changes induced by Mptb, for example using an immunological test such as ELISA, or a microscope in the case of histopathology. Most of these tests require substantial levels of

the pathogen to be present, or substantial changes in the host, and usually this means that detection of JD is more accurate in the late stage when the disease is well established.

Early tests to predict future disease outcomes. We discovered that an early elevation in faecal Mptb DNA quantity and a lower IFNγ response identified susceptible sheep. Conversely, early low faecal Mptb DNA and higher IL-10 responses predicted immunity. In research flocks these tests had 75% accuracy. They have potential to be used to predict susceptibility to Mptb infection.

A new JD culture medium to replace BACTEC medium. We developed and validated a new culture medium for Mptb when commercial supply of BACTEC ceased. BACTEC was the only liquid culture medium able to be used in Australia for detection of both bovine JD and ovine JD. It required a radiometric assay at the test's conclusion to confirm bacterial growth. The new medium is called M7H9C and has the same sensitivity as BACTEC but is cheaper, non-radioactive and does not require regular examination during incubation. It has been adopted by all the animal health diagnostic laboratories in Australia that culture Mptb. We then developed an efficient qPCR method for confirming Mptb in liquid culture media, instead of the radiometric assay. It fits downstream of the new culture medium, is at least 1000 fold more sensitive than the standard approach and will replace this in all animal health laboratories in Australia.

Phage test for live Mptb in blood and milk. We introduced a new phage assay to detect viable Mptb in blood and milk. Attempts to use this assay for the rapid detection of Mptb directly from faeces have been unsuccessful or have demonstrated little correlation to other faecal test methods; this may be due to sample-related effects of the faeces on phage replication as well as other unknown factors. This assay is being used overseas, for example to detect Mptb in human infant milk formula and it is important that the technology is available in Australia for verification purposes. In collaboration with the University of Nottingham we showed that the assay can detect viable Mptb in blood samples and is specific. It could lead to earlier diagnosis, but this requires validation.

Implementation and validation of the IFNγPlus assay. We showed that this test can detect Mptb exposure in sheep before any other test, as early as 4-5 months after Mptb exposure and unlike standard IFNγ assays, it can be performed 2 days after sample collection, allowing sufficient time for shipment of blood samples from farm to laboratory. Sensitivity and specificity was 81%. This test may provide an opportunity for control strategies aimed at removal of young, exposed, susceptible animals before faecal shedding occurs. It can also be used to identify farms which may be contaminated with Mptb, for example trace forward investigations. There is still insufficient information about assay validation in cattle.

Other diagnostic test approaches were investigated. We trialled a lymphocyte proliferation assay to detect immunity but practical constraints were identified that precluded field validation. We showed that saliva and faeces from infected sheep contain antibodies and can be used for ELISA, but at too low a rate of positives to warrant further work. We also showed that alternative antibody ELISA formats based on IgG1 antibody isotype detection may improve commercial assays.

Novel antigens. We completed a long term discovery program for novel antigens for use in diagnostic tests and vaccines. Through international collaborations we avoided research

duplication and evaluated many candidate antigens but none had sufficient sensitivity as sole antigens in immunological tests. The global quest for better antigens for JD diagnosis will continue.

Immune signature. We studied an "Immune signature", the combination of immunological and other data with genomic data to identify variables associated with disease outcome in Mptb infection or vaccination. The statistical models used in this research need to be checked further, and other data may be able to be added to increase sample sizes. This will be reported in an Addendum to this final report as we seek to verify numerous significant associations.

Animal genomics

Transcriptomics, or the study of gene expression, allows the day to day work of the genes in the chromosomal DNA of sheep and cattle to be measured. In so doing it is possible to identify the genes that are responsible for immunity to JD. These genes work in teams or pathways, and by identifying these it should lead to new opportunities for diagnosis and vaccination, because the most likely molecular targets for tests and vaccines can be then be researched.

An aim of genetics is to find an association between important traits and genotype. Genetic variations measured as single nucleotide polymorphisms (SNPs), which are DNA deletions, mutations and other changes, can be used as selection tools in breeding programs.

Gene expression patterns in early JD. We identified gene expression consistent with a JD resistant or a susceptible outcome in both sheep and cattle. In sheep, gene expression patterns were found for a multibacillary, paucibacillary or a recovered disease outcome. Potential biomarkers for JD prediction were identified.

Breed susceptibility and gene expression. We studied JD susceptibility in Merino, Suffolk first cross Merino, Border Leicester, and Poll Dorset lambs. All these breeds were susceptible to Mptb although there were breed differences in prevalence and severity of disease. Poll Dorset and Border Leicester sheep were slower to develop clinical disease and there were variations in gene expression between breeds. Several genes were identified as markers of resistance across breeds.

Gene expression in vaccination. We discovered novel mechanisms and found gene expression changes that are potential indirect markers of efficacyGudair® for the commercial vaccine Gudair®, as a model JD vaccine. This provides a foundation for strategic research on modulation of immune function and biological targets for future vaccine development.

Genetic selection markers – single nucleotide polymorphisms. We conducted a genome-wide association study (GWAS) using a powerful tool, the Ovine Infinium® HD SNP BeadChip and we identified SNPs that may be used in the future in marker-assisted breeding approaches for JD. However, further research is recommended to verify that the SNPs are representative of populations present in Australian flocks.

Improving vaccine technology

Gudair ® vaccine is widely-used in sheep in Australia. It reduces mortality by up to 90% but some vaccinated sheep shed Mptb in faeces. This vaccine causes injection site lesions and is a workplace safety risk. The main objective was to devise a strategy for candidate vaccine evaluation in cost effective, short term trials using a defined infection model to evaluate proven correlates of protection. This would overcome impediments to new vaccine development: long term trials (years) to prove efficacy and a plethora of candidates to be evaluated. As vaccines for Johne's disease need to contain two major components, adjuvant and antigen, we conducted research on both to prove the strategy.

Immune correlates of protection associated with Gudair® vaccine. We discovered that specific lymphocyte subsets protect against Mptb-infection in vaccinated sheep, and that immune parameters other than the commonly measured IFNγ and antibody are required for vaccine efficacy. We used these measures to assess progress in a series of trials.

Reducing injection site lesions for a safer vaccine. We examined a range of adjuvants, to determine the incidence, size and persistence of injection site lesions. Novel formulations produced fewer, smaller, less persistent injection site lesions than Gudair® and the strength of the various immune responses was dependant on the adjuvant.

Candidate vaccine evaluation. We formulated novel vaccines with recombinant Mptb antigens and different adjuvants and evaluated specific immune responses in sheep. We selected four novel vaccines that promoted different immune responses in sheep, and compared these with Gudair®. The different novel vaccines provided partial protection, despite low antigen content, with fewer injection site lesions. We next evaluated the efficacy of a prototype candidate subunit antigen vaccine in sheep using the new evaluation strategy over 13 months. This trial duration was sufficient to assess vaccine efficacy, in this case ruling out a candidate antigen relatively quickly. We produced information on outcome repeatability because this knowledge is critical to have confidence in vaccine development. Small differences were seen in the responses to vaccines between two trials, and the order of protective efficacy was the same, indicating reproducible results can be achieved.

We tested a novel vaccine in calves as a demonstration of the new strategy for evaluating vaccine efficacy using the experimental infection model and immune correlates of protection in a trial of only 9 months duration. There were no injection site lesions. The novel vaccine induced a weak antibody response and a strong early IFNγ response, identified from our predictive studies as a desirable marker of protection. The novel vaccine protected against JD when assessed using faecal shedding of Mptb and histopathological lesions but like commercial vaccines did not prevent infection.

The results indicate that the predictive tools, experimental animal models and vaccine design strategies that have been developed and applied in project P.PSH.0576 provide for potentially more efficient assessment of future vaccine candidates and have strong potential to lead to the development of a safer, more effective vaccine.

Research capacity for livestock health

We built human capacity in livestock health. Four young post doctoral fellows were responsible for subprograms, ensuring a unified approach across complex disciplines. The research team trained 8 PhD students (5 have already graduated) whose skills and knowledge will be applicable as specialist immunologists, pathologists and microbiologists. All have received both field and laboratory experience. In addition, 8 domestic honours students and 2 DVM summer vacation students carried out short-term research projects. The research team is involved in extension activities with industry including committees, working groups and webinars and has many international collaborative partnerships.

An invaluable, fully traceable, biological sample archive and a legacy dataset were created. We made investments in PC2 laboratories, laboratory equipment and farm infrastructure at Camden from other funding sources. We maintained a highly skilled group of professional and technical staff capable of carrying out all diagnostic tests for Johne's disease as well as other specialised techniques that can be utilised for research on this and a variety of other livestock health issues.

The research laboratory manufactures M7H9C culture medium and distributes it to animal health laboratories across Australia for Mptb culture. It provides services as a JD diagnostic laboratory, with expert professional advice available to submitters as part of this service. The laboratory participates in quality assurance in national (ANQAP) and international (USDA NVSL) programs and will be accredited to ISO17025 under NATA in 2016.

Recommendations are made on translation of findings into livestock breeding and improving industry capacity to deal with JD in a deregulated environment.

Table of Contents

1 Background

This section explains why the research was undertaken, its significance for the industry and its main aims. This is presented in the context of previous research. Information that has arisen since 2011 from newer projects is included below (underlined), otherwise this section was as included in the 2011 MLA project agreement.

Johne's disease has an uneven distribution in Australia with a large part of the country and the majority of beef and sheep flocks apparently free of disease. Management of Johne's disease nationally, in both sheep and beef cattle, now depends on assessment of risk and acceptance of this risk by the purchaser of livestock. The specific risk is the likelihood that a flock or herd is infected when it is bought and sold. Objective information is used to quantify this risk. In the absence of diagnostic tests with a high sensitivity, certification is based on the level of assurance that can be provided through a flock or herd assessment of risk. Once a herd or flock has established a base level of risk, mitigating practices can be put in place to improve the level of assurance.

A serious problem with Johne's disease is its long incubation period, bacterial shedding by infected animals before clinical signs appear, the lack of obvious signs until late in the disease process, and the low level of accuracy of current laboratory tests when applied to individual animals with early to mid-stage disease. Thus many infected flocks and herds go unnoticed, and these often give negative results with current laboratory tests. This misdiagnosis means that animals from these herds and flocks can be sold innocently for fattening or breeding and so will spread the infection to other flocks and herds. Shedding of Mptb in faeces, which leads to environmental contamination and spread of infection to other animals, currently cannot be predicted. Furthermore, the substantial proportion of a flock or herd which does not become infected despite exposure, cannot be predicted. Key information needed for disease control and animal husbandry, such as which individuals are already infected (or will become infected), and which are not infected (or will not become infected), cannot be determined accurately until animals are slaughtered. Therefore early predictive tests are needed. A further problem is that vaccination does not prevent infection, and so vaccinated animals do become infected and can and do spread the disease, even though they may appear to be healthy. Where vaccine is used, the level of pasture contamination will be lower overall. However, "super-shedders" still occur among vaccinated sheep and cause a great deal of contamination.

For these reasons Johne's disease will continue to spread slowly in the Australian red meat industries. Producers are keen to protect Australia's relatively low prevalence and regionalisation of the disease at a time when most developed nations are seeking to reduce the level of Johne's disease infection in their national flocks and herds. At the extreme level of concern, Japan will not permit products from known Johne's disease-infected livestock to enter the human food chain, and is endeavouring to eliminate the disease from its own cattle. There has been one product recall from supermarkets in Japan due to BJD (milk). New technologies are required to tackle the problems associated with detection of disease and lack of vaccine protection.

Safety concerns relating to the existing vaccine have been raised as a significant issue for producers. There are tissue reactions in most sheep, and medical/surgical treatment is needed for humans who are accidentally injected. A serious impediment to the conduct of

trials with new vaccines is the length of the trial required, and this is a key determinant of the cost. Lengthy trials are required because animals must be studied until they are several years of age, in order to measure the degree of protection – currently there is no early test to determine whether a vaccine is working. The consequence of these factors is that the cost of a modest trial to evaluate a new vaccine formula exceeds $500,000. There are literally dozens of potential new vaccine antigens but at present there is no practical or economic method to evaluate them all. In the United States, laboratory cell cultures and inbred strains of mice are being used instead of sheep and cattle to reduce the costs of vaccine evaluation. However, the relevance of this approach has been questioned. Therefore there is a need for an indirect measure of vaccine protection that does not involve observation of large groups of sheep and cattle for 3-5 years or longer.

Prior to earlier research commissioned by MLA extensive literature searches and discussions with other research teams were held to determine whether alternative test regimes or commercial vaccines were available. In the absence of any candidates earlier R and D Projects (P.PSH.0297 and P.PSH.0311) were carried out to identify a clearer understanding of how infection establishes in animals and subsequently spreads. These have revealed exciting and previously unsuspected features about how Johne's disease develops. The researchers now know that not all animals become infected, even when there is heavy exposure – some animals appear to be resistant. Furthermore some infected animals limit the infection and others appear to recover completely from it – these animals would be valuable to retain in a herd or flock for breeding. The sheep industries are seeking to build on the knowledge obtained in P.PSH.0311 and P.PSH.0297 in the expectation that a number of significant knowledge gaps can be addressed, thereby better positioning industry to manage and control this disease and minimise any market access risk arising from a perceived public safety risk.

Most animals that have been exposed to Johne's disease can now be identified using immunology; the researchers have developed a laboratory test based on measuring the proliferation of cells from the immune system after stimulation in a specific way. With further test development which may reveal a way to separate these animals into two groups – resistant and susceptible – at an early stage, producers will be able to select seed stock that are naturally resistant to disease thereby avoiding the ongoing costs of disease control and the disease itself.

The researchers have developed new stimulants (antigens) in recent MLA Projects and these require evaluation and comparison to new stimulants from overseas, in collaboration with overseas teams. In addition, the researchers have provided the first evidence that heavy shedding of Mptb in faeces can be predicted using a faecal test at a young age, while the shedding is still light. This would enable "predictive" culling of those highly susceptible animals which are the ones most likely to be responsible for future spread of the disease. Other evidence from trials with an enhanced interferon-gamma blood test supports this idea.

Problematically, the gold standard diagnostic test for Johne's disease in sheep, BACTEC culture, is to become unavailable within 3 years when the manufacturer of the culture medium (Becton Dickinson) ceases production – an alternative needs to be found. Pooled faecal culture is the standard diagnostic test for the sheep and goat industries providing a higher level of sensitivity than ELISA or AGID tests. The lack of culture media after BACTEC is withdrawn by the manufacturer requires a response as there is no other sensitive culture

medium for Mptb. Research to replace culture with a PCR test has been successful, but culture will still be required by competent authorities for verification in new epidemiological situations and for strain typing, for instance where the disease appears to have spread to a region where it has not been previously recognised. Culture is a gold standard method, and a positive result removes perceptions about imperfect test specificity (for example with ELISA or PCR tests). The research team has access to other commercial media from the USA for trial and has conceived new approaches to manufacture media from base ingredients sourced locally.

The research team recently conducted DNA microarray experiments during the course of Johne's disease infection. This revealed that genes for markers on the surface of immune cells (MHC) are strongly and consistently associated with Johne's disease infection. Gene expression for these markers can be detected in blood samples from cattle quite soon after infection. This discovery has enormous potential to inform development of selection tools for resistance to Johne's disease. The markers are plausible candidates for marker-assisted selection because they are already known to be associated with resistance to other diseases in cattle, sheep and other species. Early work showed that resistance to some internal parasites is associated with particular markers in this part of the genome of sheep. The MHC region is known to be associated with immunity in infectious diseases.

The existence of "disease pathways" which animals may follow after infection is exciting because it offers hope that new vaccines can push animals down a more appropriate pathway towards resistance or recovery. The current vaccine appears merely to slow down progression of disease without altering its final outcome – data for this view come from MLA Projects OJD.009, OJD.033, P.PSH.0309 and P.PSH.0565. Understanding the pathways and the predictive tests for each pathway will greatly facilitate the development of new vaccines, by reducing the length of research trials.

This Project will develop tools for immediate application, specifically predictive tests that can be used for selection of resistant and susceptible livestock, and tools for prediction of protective immune responses. The work undertaken in this Project will assist in vaccine studies, and future applications will relate to selection of optimal vaccine antigens and adjuvants and field validation of these in a cost-effective manner. This project is a necessary prerequisite for critical controlled animal infection trials which are needed in the next phase of vaccine development to consolidate the results, leading to full scale field validation.

While the project will develop specific outcomes in some areas, e.g. new tests and improvements to the safety aspects of the present vaccine, other findings may represent incremental knowledge gains that can potentially be marketed (eg a realistic vaccine candidate testing model) or used by other research partners in progress towards a novel vaccine. It is acknowledged that vaccine development for diseases with a complex aetiology is slow and steps have been taken to ensure that industry has realistic expectations, particularly for marker-assisted selection and vaccine development. Marker-assisted selection for disease resistance, as in animal production research, requires complex new technology and an extended timeframe. It is likely that Johne's disease susceptibility is a complex trait. However this Project has the advantage of being able to take account of any advances in TB research, there is an excellent animal model for Johne's disease developed through Projects P.PSH.0311 and P.PSH.0297, and there is already a partially effective vaccine (Gudair®) which can be used as a starting point in experimental models. With

respect to vaccines for Johne's disease, the following are achievable goals and time-frames for this Project:

Short term 2-3 years: compare alternative adjuvants to reduce vaccine tissue reactivity and modulate Th1 immunity in sheep and cattle; measure cellular responses to new antigens in sheep and cattle; develop a greater understanding of how Gudair® vaccine works

Mid term 3-5 years: develop indirect measures of vaccine efficacy, based on measurement of immunomodulatory responses to vaccine (informed by other aspects of the Project e.g. microarray); test carefully selected antigen candidates in a new vaccine.

As soon as results suggest that a particular combination of antigen and adjuvant in a new vaccine formulation is safe and leads to significant protection from Johne's disease in experimental trials, a commercial partner would be engaged.

2 Projective objectives

2.1 Predictive and diagnostic tools

To provide new diagnostic tools to enable producers to determine more accurately whether herds/flocks are infected or uninfected, which animals in particular are exposed and infected and to predict which animals are at high risk of contaminating the environment before they do so.

2.2 Animal genomics

To develop new genetic approaches to predict accurately which herds/flocks are resistant, which particular animals are resistant and which infected animals will get sick, contaminate the environment or recover. As part of this objective, to determine the relative susceptibility to JD of different breeds, such as pure British breed and Merino sheep.

2.3 Improving vaccine technology

Conduct research towards a safer, more effective vaccine for sheep and cattle

a. To develop an indirect measure of vaccine efficacy, based on blood and faecal testing, to facilitate the development of a safer more effective vaccine

b. To improve the safety of vaccines, by reducing their tendency to cause severe tissue reactions in livestock and humans

c. To trial a prototype vaccine through experimental infection in sheep and cattle

2.4 Research capacity for livestock health

To maintain and develop capacity in livestock health for the benefit of Australian producers through the training of 4 post doctoral fellows and four postgraduate students whose skills and knowledge will be applicable and available to the broader

veterinary and agricultural research community as specialist immunologists, pathologists and microbiologists.

3 Methodology

The project required a multidisciplinary approach using state-of-the-art techniques in veterinary microbiology, immunology, pathology, molecular biology, epidemiology, bioinformatics and genomics. They were applied in four inter-related subprograms of research, each of which required methods of varying technical complexity and/or novelty, as well as farm-based flock management. A fifth subprogram built human technical capacity (Fig. 1).

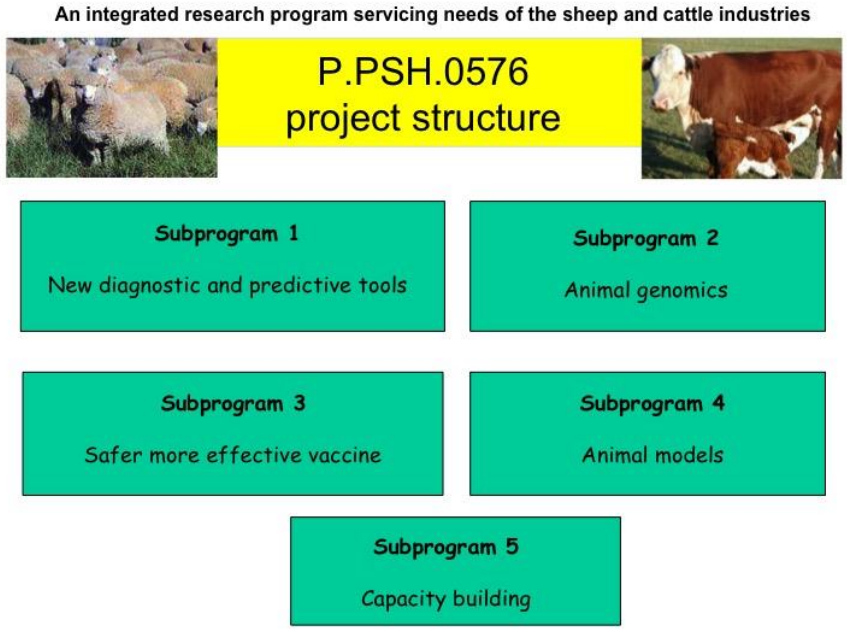


Fig. 1. The five integrated subprograms within Project P.PSH.0576

As this project involved a complex interaction between subprograms and disciplines, the general methods are provided in the context of the results for each objective in the results sections below. The methodologies are described in more detail in the appendices which are provided as a separate document linked to this report, and in reports which have already been published in the peer-reviewed scientific literature and which are listed in Section 9 at the end of this report.

4 Results and discussion

The results of this project flow from five inter-related subprograms of research (Fig. 1). Each of the research subprograms contained a body of work of great depth and complexity. In order to describe this work succinctly and to reduce the need for readers to shift back and forth between methods, results and discussion and look for relevant subheadings, the methods, results and discussion are presented below under major headings for each of the subprograms. This structure aligns closely with the objectives of the project, and it is hoped that this will make it more straightforward for readers to assess the outcomes.

4.1 Resource subprogram. Animal models (Subprogram 4)

Introduction

This subprogram addressed all project objectives by ensuring reliable access to clinical material from livestock with and without Johne's disease, throughout the project. For this reason the results from this aspect of the research work are presented first.

This project is distinguished from most other contemporary and historical studies of Johne's disease by the use of a validated animal model in sheep and cattle, by the long duration of the animal trials, the large group sizes, and by the rigour with which Johne's disease was assessed and classified for every animal. These trials were integral parts of the operational plan for this project and have been the foundation for the complex outcomes achieved and presented in this final report. Samples collected from the trial animals were directed into immunological assays, mycobacterial cultures, pathological tests and animal genomics studies. These, informed studies on the pathogenesis of Johne's disease and on diagnostics, vaccine and predictive tools. Collaborations have also stemmed from these trials and fed back into many of the above aspects, extending our capacity and maximising the output from the project.

Methods

Two experimental animal trials (P.PSH.0311.1 and P.PSH.0297.2) began in previous projects undertaken at the University of Sydney and were continued into the P.PSH.0576 project to enable long term monitoring of the animals. The necropsies, sample processing and all of the final data analysis for these trials were conducted within the current project. Trials that commenced and that were completed within the current project all have the prefix 'P.PSH.0576' and are summarised in Table 1. These trials are illustrated on a timeline in Fig. 2 to show how they were scheduled and how they provided a continuous source of research materials for the other subprograms that depended on this.

Table 1. Summary of animal trials conducted in project P.PSH.0576

Trial ID number	Trial overview	Species	Trial commenced	Duration	No. animals	Refer to page
P.PSH.0297.2	Cattle experimental infection	Cattle	Dec 2008	4.5 years	30	25, 43, 52 63, 66, 78
P.PSH.0311.1	Long duration to examine resistance/resilience	Sheep	Feb 2009	2.5 years	30	26, 47, 52 60, 62, 66 70, 72, 89, 92
P.PSH.0576.1	Breed susceptibility trial	Sheep	Feb 2012	14 months	199	66, 70, 72 83, 88, 89 92, 98
P.PSH.0576.Adj	Evaluation of novel adjuvants	Sheep	Mar 2011	6 months	90	104
P.PSH.0576.Ag	Evaluation of candidate antigens (dormancy/stress)	Sheep	Oct 2012	2 months	34	110
P.PSH.0576.2	Vaccine prototype trial	Sheep	Feb 2013	14 months	205	70, 89, 92 98, 113,117
P.PSH.0576.3	Vaccine prototype trial 2 (reproducibility)	Sheep	Feb 2014	13 months	120	118
P.PSH.0576.4	Vaccine prototype trial	Cattle	Apr 2014	9 months	30	63, 109, 120

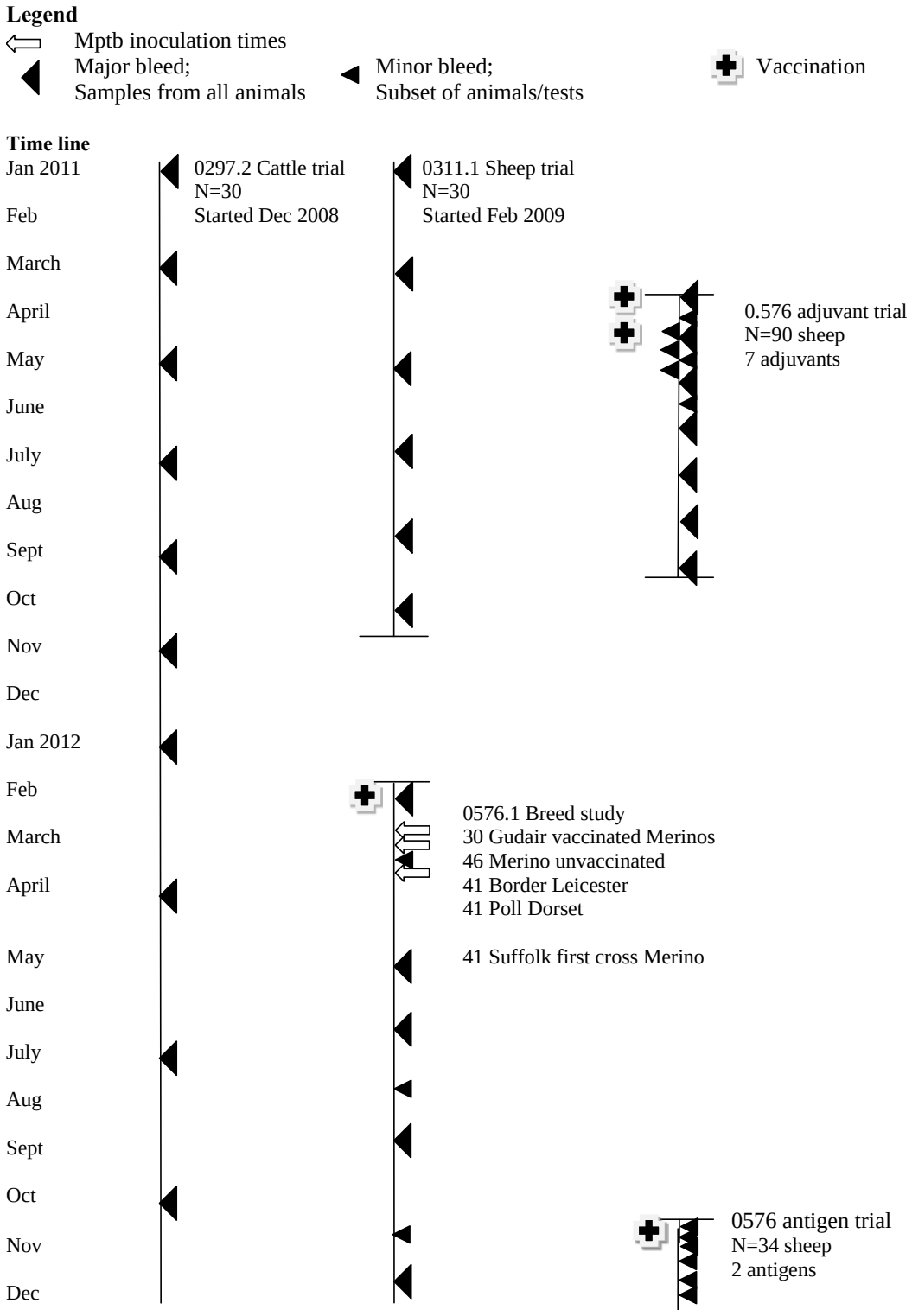


Fig. 2. Experimental infection trials conducted in sheep and cattle in project P.PSH.0576 between 2011 and 2015.

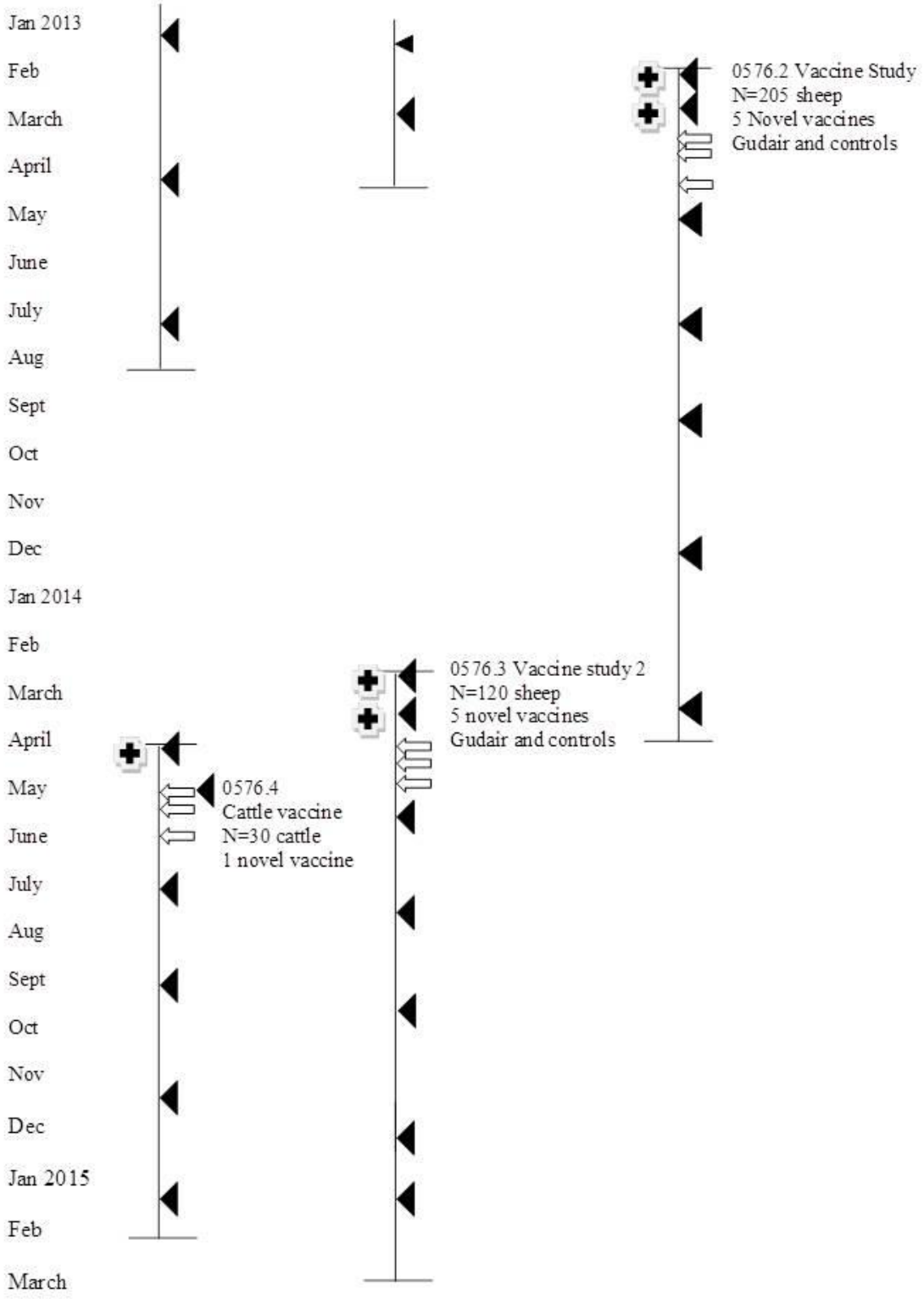


Fig. 2. Experimental infection trials conducted in sheep and cattle in project P.PSH.0576 between 2011 and 2015 (continued)

The use of all animals was approved by the University of Sydney Animal Ethics Committee.

Experimental infections in sheep with *Mycobacterium avium* subspecies *paratuberculosis* (Mptb) S strain were carried out by a method developed and validated by the University of Sydney Johne's disease research group (Begg et al. 2010) in MLA project OJD.031. The protocol depends on a pure culture of Mptb; it was originally isolated from an OJD case and was carefully purified and then archived at a low passage number with a view to future use in an animal model and in vaccine development. After reconstitution of lyophilised seed stock in liquid medium, and expansion on solid culture media, a measured dose of bacterial suspension is inoculated orally on several occasions. The resulting infection closely resembles natural OJD at individual animal and at flock level. An adaptation of this protocol that incorporated Mptb C strain was followed for trials in cattle.

Results

The main features of these trials are described in this section, but much more information can be found in the results of the other subprograms elsewhere in this report.

P.PSH.0297 Cattle trial. In December 2008 an experimental infection trial was begun in cattle similar to those run previously in sheep (Fig. 3). This trial was designed to examine the pathogenesis of disease and assist in the development of new diagnostic tools. Twenty inoculated and 10 unexposed control animals were included. The trial included a biopsy of the gut at 1.5 and 2.1 years post-inoculation on every animal, and this showed that several were infected with Mptb and had Johne's disease (JD) associated lesions. To maximise the results from the trial a decision was made to run the animals for as long as possible and therefore this trial was carried over from the P.PSH.0297 project into the P.PSH.0576 project. The animals were held for another two and a half years after the last biopsy to determine if any would recover or progress to develop clinical disease. When the trial was stopped in 2015, none of the animals had clinical signs of Johne's disease but 7 were infected (tissue culture positive). This pattern of infection is consistent with that observed in grazing beef cattle and therefore the samples derived from this study are meaningful and relevant. This is in contrast to many published studies in which very heavy doses of Mptb have been given to calves, resulting in a high prevalence of clinical cases at a very young age. It is not possible to extrapolate from such trials to evaluate diagnostic tests in practice.

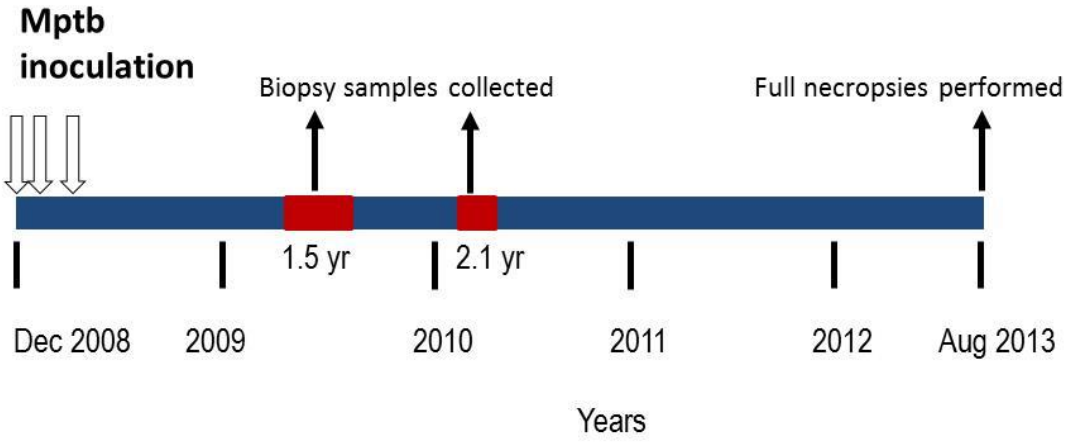


Fig. 3. Schedule of Mptb inoculation and biopsies in the P.PSH.0297.2 trial.

The experimental inoculation of the animals resulted in infection of 37% of the cattle, assessed at the end of the trial. Two animals developed histological lesions consistent with Johne's disease and that were associated with infection. One animal had stabilised the infection, as its biopsy sample at 26 months post inoculation had the same lesion severity as that seen at 56 months post inoculation. This animal, while infective as shown by faecal shedding, also had elevated Mptb-specific antibody responses at the end of the trial. This animal might have developed clinical disease if left for longer.

This is the first study in cattle to observe an animal in which intestinal lesions regressed, giving an indication that some cattle can recover from the infection. This phenomenon has previously been observed in our trials in sheep, with similarities between the two species in that regression was seen only in animals with type 3a or less severe histological lesions.

P.PSH.0311.1 Sheep Trial. This trial was also carried over from the previous project P.PSH.0311. This trial is unique as the sheep were held for up to 2.5 years post inoculation with Mptb. No-one has conducted a sheep experimental inoculation trial for this length of time as far as the researchers are aware. It was designed to look at disease progression as well as recovery/resistance, in case shorter duration trials were missing a cohort of sheep with delayed disease progression. Twenty inoculated and 10 unexposed control animals were monitored for disease development, faecal shedding, Mptb specific antibody, cytokine (IFNγ (IFNγ),interleukin (IL)10) and lymphocyte proliferation responses. As for previous trials, a cohort of animals (8 of the 20 inoculated) succumbed to clinical disease, confirmed by gross and microscopic lesions associated with JD and positive tissue culture for Mptb from intestinal tissues. The majority of the clinical cases presented 14-17 months post inoculation with the other at 21 months post inoculation. Eleven sheep were negative by tissue culture (i.e. uninfected) and histopathology at the end of the trial, with the exception of one sheep which had a minor grade 2 histological lesion; these animals were resistant. Of the animals that developed clinical disease, all had detectable persistent faecal shedding for 6-11 months prior to necropsy. Accurate classification of these sheep has enabled analyses that are presented under the other subprograms in this report.

P.PSH.0576.1 Sheep breed susceptibility trial. This experimental inoculation trial examined the infection and disease outcomes in Merino, Poll Dorset, Border Leicester and Suffolk first cross Merinos. Sheep in all of the breeds developed clinical disease, although the rate and severity was different between the breeds.

Vaccine trials. Five trials were undertaken in support of the vaccine subprogram, which aimed to define a new, efficient protocol for JD vaccine development and validation:

P.PSH.0.576.Adj to examine the effect of four different adjuvants on the immune responses and injection site lesion development;

P.PSH.0576.Ag to examine candidate antigens selected from previous dormancy /stress studies by the University of Sydney Johne's disease group;

P.PSH.0576.2 to evaluate five prototype vaccines compared to Gudair®;

P.PSH.0576.3 to test the repeatability of the outcomes obtained in vaccine trials; and

P.PSH.0576.4 to evaluate a prototype vaccine in cattle. This last trial was designed to demonstrate a strategy for vaccine evaluation and was based on data obtained and lessons learned in the previous trials. It was a short-duration vaccine trial with animals followed for only 9 months post inoculation, after which it was possible to evaluate vaccine efficacy.

Culture and strain identification of Mptb. Faecal and tissue cultures and strain identification of Mptb were undertaken using standard methods which were part of the Australian and New Zealand Standard Diagnostic Test Protocol (ANZSDP) as previously published (Whittington et al., 1998, Whittington et al., 2013, Whittington et al., 1999, Marsh et al., 1999).

Mptb faecal DNA test. The HT-J faecal DNA test was conducted using standard methods which were part of the Australian and New Zealand Standard Diagnostic Test Protocol (ANZSDP) as previously published (Plain et al., 2014).

Mptb antibody ELISA. Unless otherwise stated the IDEXX (formerly Institute Porquier) ELISA kit was used according to the manufacturer's instructions.

Histopathology. Tissues were fixed in formalin and histological processing was by standard methods at the Veterinary Pathology Diagnostic Services laboratory, University of Sydney. All sections were read by one pathologist (RW). Unless otherwise stated, lesions were graded according to a standard scoring system (Perez et al., 1996).

Histopathological sampling strategy. As the animal trials for the P.PSH.0576 study were planned it became apparent that necropsy sampling procedures would have to be modified to deal with the very large number of samples generated. This was particularly apparent for the histological analysis where 15 tissue samples were being examined using both Ziehl–Neelsen (ZN) and haematoxylin and eosin (H&E) stains to determine the lesion type. While this number of tissues was practical for trials with approximately 30 animals, it was a problem when there were 200 animals. For this reason the histological lesion results from MLA projects OJD.031 and P.PSH.0311 were re-examined to determine if a more efficient sampling method could be employed to accurately identify the lesion status of each sheep in the larger of the P.PSH.0576 trials. The standard protocol for sampling employed in past Johne's disease animal trials at the University of Sydney is shown in Fig. 4. Tissue sections were taken from the intestines and associated lymph nodes – A to F (12 tissues) of the gut plus a section from three sites not associated with the intestines, the hepatic and prescapular lymph nodes, and liver (not shown).

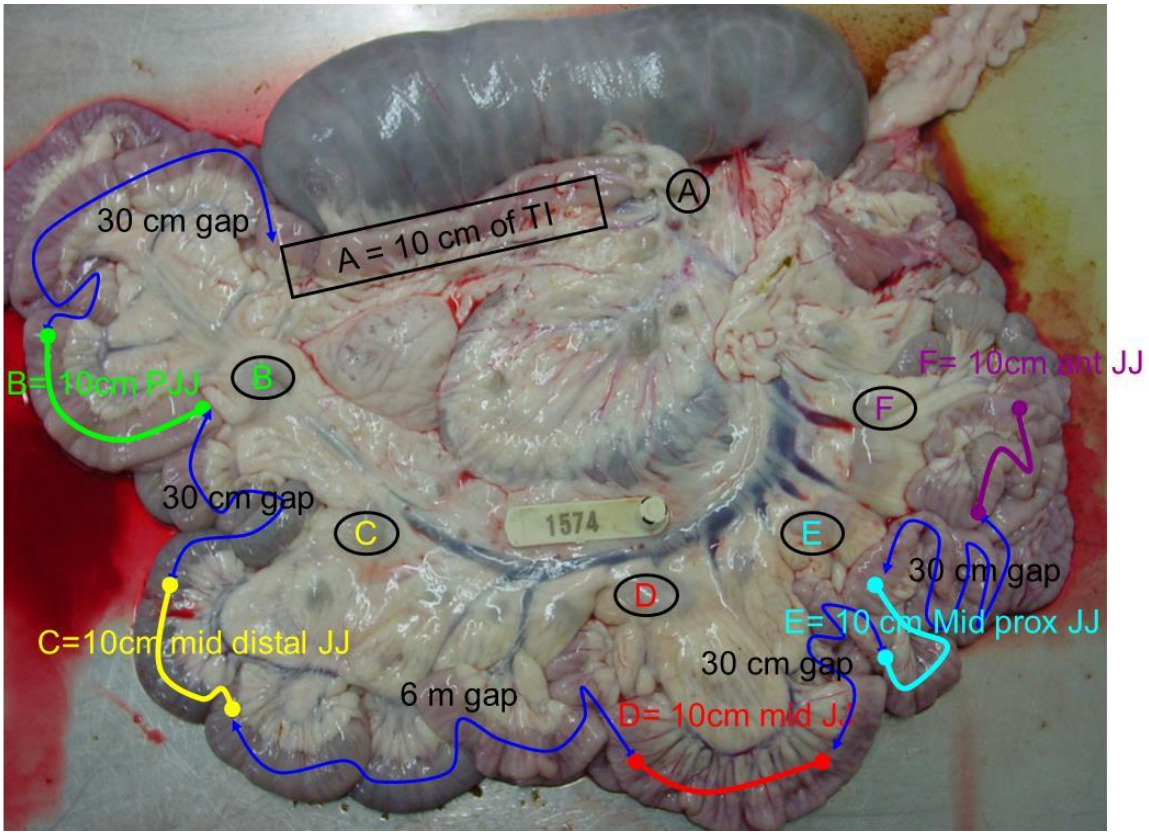


Fig. 4. Sections removed from the gut for culture and histopathlogical analysis. The sections of the ileum and jejunum removed were from the areas of the terminal ileum, in the black box, anterior ileum to posterior jejunum (Green line), mid distal jejunum (yellow line), Middle jejunum (red line), mid proximal jejunum (light blue line) and anterior jejunum (purple line). The dark blue lines indicate sections not sampled. Matching lymph nodes were taken from the site with the ovals with the letters A-F in them.

Results from 183 animals from the earlier trials were in a data base. A subset of 99 animals that had lesions of histological grade 1 or more in at least 1 tissue were selected; these were known JD affected animals. The lesion results from the intestines and associated lymph nodes were extracted to determine where the most severe lesion was in the gut and the proportion of known JD affected animals that would be detected as having a lesion in that tissue. The best combination was found to be the intestinal sections A and E with the lymph node sections B and E. This combination detected 96% of all animals with a histological lesion and 91% of the most severe lesions in the sheep. This resulted in a reduction in the number of samples to be examined from approximately 3000 to 800 for an animal trial with 200 sheep. This sampling protocol was adopted in trials P.PSH.0576.1, P.PSH.0576.2 and P.PSH.0576.3.

Classification of disease outcome. In order to conduct analyses of disease signatures in this project it was necessary to develop and adopt case definitions for various categories of outcome following exposure to or infection with Mptb. In brief, three variables were considered in the analysis of the dataset.

1. Mptb exposure status - exposed or not exposed

2. Disease status defined by a histopathology lesion score - multibacillary (score 3b), or paucibacillary (score 1, 2, 3a, 3a-b, 3a-c, 3d). This was based on published criteria (Perez et al., 1996) with modifications to account for predominantly submucosal lesions (score 3d), transitional lesions (3a-b, 3a-c) and non-specific focal granulomatous lesions containing debris (score 0.5), as well as the number of acid fast bacilli observed.

3. Finally, within the Mptb exposed subset the animals were classified as resistant or susceptible.

Resistant animals were defined as those within an experimental infection trial known to have received an infectious dose of Mptb but in which the infection did not establish, did not progress, remained in a dormant state or from which the animal recovered. When examined at necropsy, the infection could be detected using standard tests such as culture of tissues and histopathology. Necropsy examination must have been conducted when the animal was old enough to have had identifiable histopathological lesions, that is, more than 6 months of age. In addition the following criteria needed to be met: the animal was <6 months of age at the time of exposure and was directly exposed to Mptb by oral inoculation on more than one occasion. A spectrum of disease was observed in the cohort of animals that were inoculated at the same times with the same doses, showing that the inoculum was infective and the host/environmental conditions were conducive to disease expression. There was evidence of exposure of the animal to Mptb in the form of a positive IFNγ, and/or lymphocyte proliferation assay and/or antibody ELISA test result and/or faecal shedding of Mptb occurred. If faecal shedding of Mptb occurred, it was intermittent and then ceased, and through longitudinal sampling, a period of non-shedding lasting > 6 months was observed. Sheep would usually be older than 6 months of age when shedding ceased (i.e. this assessment cannot be made in sheep <12 months old). Mptb DNA quantities in faeces assessed by the HT-J test were low. Cultures on tissues collected from multiple sites were negative and histopathological lesion grades were Perez score <2.

Susceptible animals were defined as those within an experimental infection trial known to have received an infectious dose of Mptb in which the infection did establish and progress. When examined at necropsy, the infection was detected using standard tests such as culture of tissues and histopathology. The relevant provisos as stated above must have been met however, in the case of faecal shedding this may have been intermittent but there must have been evidence of multiple shedding events over longitudinal sampling. Tissues were culture positive and histopathological lesion grades were Perez score $\geq$2.

4.2 Subprogram 1. Predictive and diagnostic tools

This subprogram addressed the following project objective:

To provide new diagnostic tools to enable producers to determine more accurately whether herds/flocks are infected or uninfected, which animals in particular are exposed and infected and to predict which animals are at high risk of contaminating the environment before they do so.

The operational plan agreed with MLA for this subprogram included research on the following aspects:

1. Identification of immune profiles associated with disease status through statistical analysis of data from animal infection trials
2. Development and validation of a new culture medium for Mptb to replace commercial BACTEC medium which was being discontinued by the manufacturer, and developing a new strategy for confirmation of Mptb in culture medium using PCR
3. Introducing a new mycobacteriophage assay from Ireland to Australia, testing this as a live-dead assay using samples from the animal trials, and confirming applications for the test
4. Extending, implementing and validating a lymphocyte transformation assay by trialling new antigens, testing the effects of storage and transport conditions and conducting a field trial
5. Implementing and validating the IFNγ release assay (IFNγ^{PLUS})
6. Evaluating antibody isotype assays using novel samples including saliva and faeces
7. Developing an immune signature of disease resistance and vaccine efficacy using multiplex cytokine assays
8. Incorporating genomics data into the immune signature

4.3 Subprogram 1.1. Immune profiles associated with disease status

Introduction

Disease outcomes following exposure to Mptb are not uniform; not all exposed individuals become infected and, amongst those that do, factors such as the rate of disease progression and disease pathology are variable. Some animals recover from infection, while some remain carriers. Some of the subclinically infected carriers become clinical cases when the infection progresses and lesions in the gut become severe (Fig. 5).

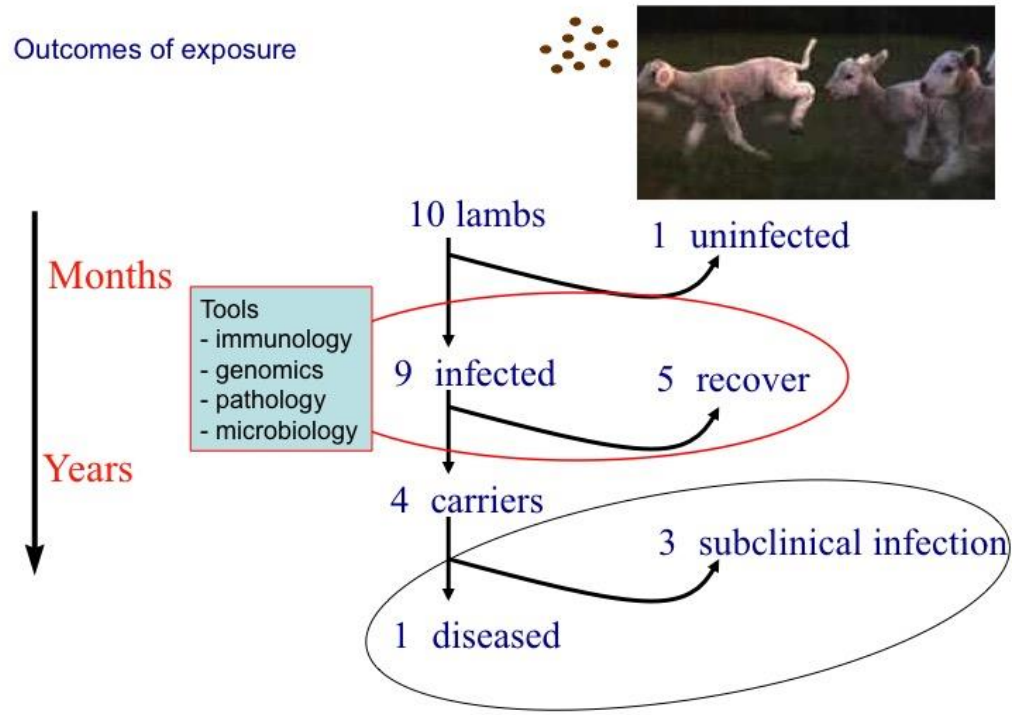


Fig. 5. Pathways leading to different outcomes in individual sheep after exposure to Mptb, and the tools that can be used to investigate these. These pathways are based on observations from experimental infection trials conducted in MLA projects OJD.031 and P.PSH.0311. There is evidence that the same pathways exist in cattle, but there are fewer observations.

In ruminants the usual route of exposure to Mptb is oral, via ingestion of contaminated milk or faecal matter. Once Mptb enters the intestinal wall, initial contact is with phagocytic cells such as macrophages. At this stage, these cells of the innate immune system may be able to destroy the pathogen. Alternatively, Mptb may actively evade intracellular killing mechanisms and take residence within these cells. As a result, a more complex host response is required. The accepted view has been that the initial response predominantly involves the antigen-specific release of IFNγ by T lymphocytes and as disease progresses this response is replaced by an antibody response. IFNγ activates bystander macrophages and facilitates intracellular killing of Mptb. While the IFNγ response is important in the cell-mediated control of intracellular pathogens like Mptb it is not always a predictor of disease outcome. To counteract the host's immune response, Mptb can also actively induce certain cytokines to suppress and evade immune cells; IL-10 and tumour growth factor (TGF)β are two such cytokines. IL-10 can also reduce the ability of macrophages to kill intracellular Mptb. The presence of specific antibodies produced by the humoral arm of the adaptive immune system is widely used as an indicator of disease although the exact mechanism by which it acts against an intracellular organism is not clear. As disease progresses (with the expression of clinical disease) in some animals there is a general suppression of the immune system and this is thought to be due to an increase in the secretion of immunosuppressive cytokines such as IL-10. However, we have previously shown that an early IL-10 response also occurs after exposure to Mptb.

Infected sheep can shed huge amounts of Mptb in their faeces – as high as 10^8 mycobacterial bacilli per gram of faeces – and thus contaminate pasture and act as a major source of infection for susceptible animals. Therefore, control of Johne's disease is usually based on culling these highly infectious animals or by using management practices to avoid or minimise their contact with susceptible animals. Although vaccines can be used as a preventative measure, they are not fully protective in sheep as some continue to shed Mptb after vaccination. For any of the control measures to be fully effective, there is need of a sensitive and specific diagnostic test, or a suite of tests, to identify infectious animals – not when they are already shedding huge amounts of bacilli – but at a younger age when they are still in early stages of infection. Achieving this goal would enable removal or separation of such animals from a flock prior to their being able to spread the disease. Such a test should also prevent removal of animals that, although infected, are unlikely to become highly infectious but instead are likely to clear infection.

In this study, we monitored several potential indicators of infection and immune responses in experimentally challenged sheep from time of exposure to up to 1 year post inoculation (p.i.) and evaluated if indicators from an early age can predict pathological and clinical status of animals 1 year post-infection. The important switch points shown in Fig. 5 between exposure, infection and recovery, and between sub-clinical infection and progression to severe disease were the focus of the research.

Methods

Fifty-seven Merino lambs (3-4 months of age) were drafted into control and exposed groups using systematic sampling and either left unexposed (n=20) or orally exposed to Mptb S strain (n=38). Blood/serum and faecal samples were collected at 4, 8 and 12 months p.i. and tissue samples were collected at 12 months p.i. when the animals were sacrificed. For the IFNγ, IL-10 and lymphocyte proliferation assays whole blood or cells were cultured with medium alone (RPMI 1640/10% foetal calf serum/Penicillin/Streptomycin/β-mercaptoethanol) or with added Mptb antigen (316v, 10 µg/mL). Faecal pellets were collected for detection of viable Mptb by culture or Mptb DNA by PCR. Tissue sections from the terminal ileum, ileocaecal lymph node, ileum (mid and anterior) and jejunum (mid, mid-proximal and anterior) and their associated lymph nodes, prescapular lymph node and liver were collected for culture (stored at -80°C until processed) and histopathology. Three main parameters were considered when categorising disease outcomes: tissue culture (infected or uninfected), faecal shedding (infectious or non-infectious) and histological lesion type (Table 2).

Table 2. Disease outcome of experimental exposure of sheep to Mptb

Outcome variables	Categories	Frequency	Percent	No. of culture positive tissue sections per animal	Lesion type	No. of tissue sections with lesions per animal	Extra-intestinal spread of Mptb
Tissue culture							
(Infected)	Negative	12	31.58				
	Positive	26	68.42	2-6	3a, 3c, 3b	3-6	
Faecal culture							
(Infectious)	Negative	21	56.76	0-4			
	Positive	16	43.24	3-6	3a, 3c, 3b	5-6	
Multibacillary							
	No	27	71.05	0-4			
	Yes	11	28.95	4-6	3b	6	Detected in hepatic tissue
Resistant/Recovered							
	No	26	68.42				
	Yes	12	31.58	0	None, except for two with 3a lesion with no AFB	0-1	None
Increasing disease severity-I							
	Recovered/resistant	12	31.58				
	Paucibacillary lesions*	15	39.47	2-4	3a, 3c	3-6	
	Multibacillary lesions	11	28.95				
Increasing disease severity-II							
	Recovered/resistant	12	34.29				
	Paucibacillary lesions	12	34.29				
	Multibacillary lesions	11	31.43				

Increasing disease severity-II excludes infected animals with no lesions which were included in the Paucibacillary group within the Increasing disease severity-I classification. Faecal culture data were excluded for one animal with insufficient data points

Faecal Mptb DNA, serum antibodies, antigen-specific PBMC IFNγ, IL-10 and proliferation index measurements made at 4, 8 and 12 months p.i. were used as explanatory variables in statistical analyses to investigate their associations with the outcome variables. Univariable logistic regression analyses were conducted to evaluate the association of explanatory variables with all outcomes – binomial logistic regression for the binary outcome variables (infected, infectious, multibacillary and resistant/recovered) and ordinal logistic for the ordinal outcome variables (increasing disease severity-I and II). Multivariable logistic regression analyses were then conducted to evaluate the association of explanatory variables after adjusting for each other. Receiver Operating Characteristic (ROC) curves were created based on the binomial multivariable logistic regression models to determine cut-off values at which the variables will have the maximum sum for sensitivity and specificity to discriminate infected and non-infected sheep if used as diagnostic tests. Sensitivity and specificity

achieved by the variable at the determined cut-off values and Positive Predictive Value (PPV) and Negative Predictive Value (NPV) were also calculated.

Results

Non-exposed control sheep were included in the trial to ensure that the parameters measured were different to the Mptb-exposed sheep. The control group were all uninfected based on negative tissue culture and histopathology at the trial endpoint and were consistently negative for faecal Mptb DNA, faecal culture and antibody ELISA results and did not respond in the IFNγ assay (S/P < 0.05) at any time point tested throughout the trial period. These results indicate that the control group had no environmental exposure to Mptb during the study period. The trial was terminated at 12 months p.i. when weight loss greater than 10% of body weight was observed in some sheep within the exposed group. Faecal shedding of Mptb was not detected in any of the Mptb-exposed uninfected sheep at any of the time points sampled during the study. About 30% of Mptb-exposed sheep either resisted or recovered from infection. In these, the absence of histological lesions or viable Mptb in any of the 6 intestinal tissue sections tested and the absence of faecal shedding throughout the trial indicate an absence of infection. It is certain that Mptb exposure occurred as the lambs were dosed orally three times and all had an antigen-specific IFNγ response. None of the resistant/recovered group was infectious while all of the multibacillary group were infectious during the trial period. Of the 12 sheep with paucibacillary lesions 7 were non-infectious and 5 were infectious.

Overall, serum anti-Mptb antibodies and faecal Mptb DNA increased with time while IFNγ and lymphocyte proliferation peaked at 8 months p.i. Six logistic regression models were built for as many outcome variables. Single variables representing faecal Mptb DNA (log 10) at 4, 8 and 12 months had significant associations with most outcomes. As faecal Mptb DNA content increased, the odds of a sheep to be tissue culture positive, faecal culture positive or being multibacillary increased and odds to be disease-free (resistant/recovered) decreased. The results of final multivariable logistic regression models are presented in Fig. 6 as odds ratios and 95% confidence intervals. Faecal DNA at 4 months p.i. was significant in all of the models and had a similar direction of association except for animals being resistant, i.e. a higher value of faecal DNA at 4 months p.i. increased the likelihood of an animal to be infected (tissue culture positive), infectious (faecal culture positive) and multibacillary at the termination of the trial and reduced the likelihood to be resistant/recovered (free from disease at 12 months p.i.).

If a lamb had a higher specific IFNγ response at 4 months p.i., it was less likely to be infectious (faecal culture positive), less likely to become multibacillary and less likely to have severe disease (Fig 6). If a lamb had a lower Mptb-specific IFNγ response at 4 months p.i. it was more likely to have positive faecal culture (infectious), multibacillary disease (severe pathology) and increasing disease severity (Fig. 6).

Lambs with a higher specific IL-10 response at 4 months p.i. were less likely to be infected (tissue culture positive), more likely to be disease-free (resistant/recovered) and less likely to have severe disease at 12 months p.i. (Fig. 6).

The only other significant variable was the lymphocyte proliferation index at 12 months p.i. and this suggested that animals with a higher proliferation index were less likely to develop multibacillary disease.

Based on ROC curves, we selected cut-off values in order to achieve maximum combined sensitivity and specificity. The cut-off values for a 'positive' result were: >3.39 fg for faecal Mptb DNA, <0.38 for IFNγ S/P and <0.28 for IL-10 S/P. Using these cut-off values, we calculated sensitivity and specificity of diagnosing various disease outcomes using different tests and combinations of tests (Table 3). Combinations of two diagnostic tests were assessed in parallel (i.e. a positive result in either test was considered positive) and in series (i.e. both tests needed to give a positive result to be considered positive). Based on these results, the potential for early identification of the likelihood of a sheep becoming Mptb infected, infectious or progressing to multibacillary disease was greatest when a positive result was recorded in either the faecal DNA test or the IFNγ assay i.e. when faecal Mptb DNA was high or when IFNγ was low. For this combination, sensitivity ranged from 58-75% and specificity ranged from 67-83%. The positive and negative predictive values (PPV and NPV) for this combination of tests under conditions of this experimental trial ranged from 47-88%.

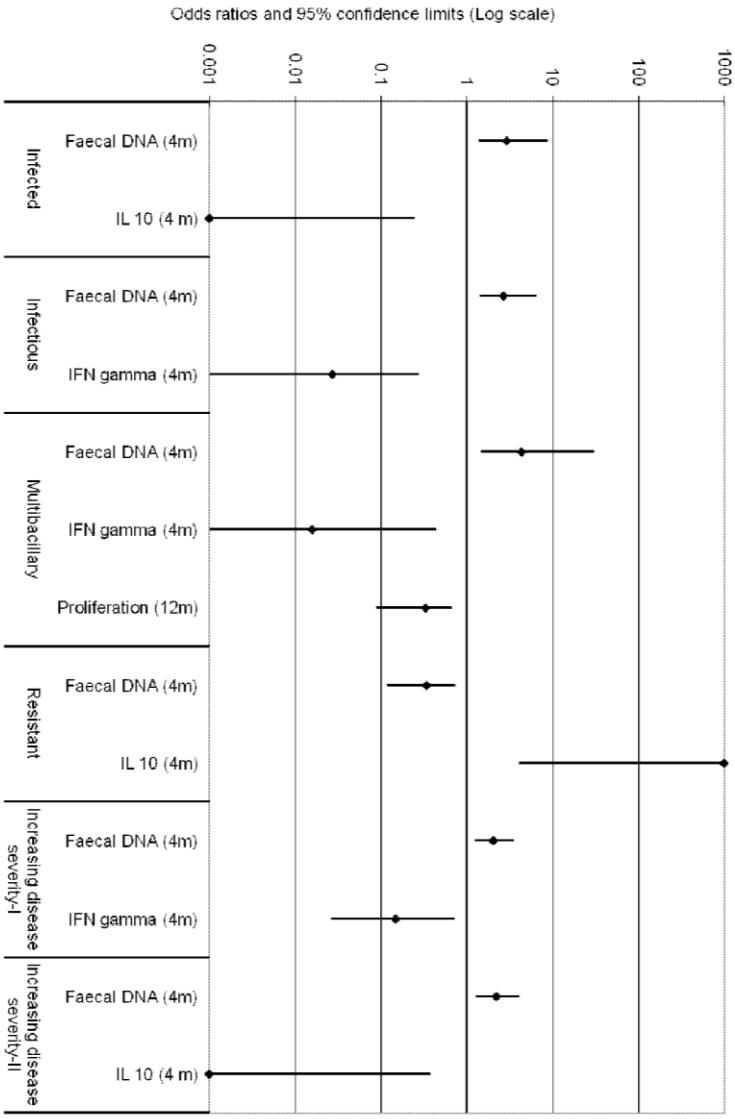


Fig. 6. Odds ratio and confidence limits for association of predictor variables with the six outcome variables. The bars indicate confidence intervals around odds ratios. Only the significant associations are shown. An odds ratio > 1 indicates that an elevated value of the explanatory variable increases the odds of the outcome. The time post inoculation that the test was carried out is indicated in months (m). Infected = tissue culture positive at 12 months p.i.; Infectious = faecal culture positive at least one sampling time point; Resistant = uninfected and non-infectious at 12 months p.i.

Table 3. Sensitivity and specificity values for diagnostic tests done at 4 months post inoculation for various outcomes at 12 months post inoculation.

	Infected[1] at 12 months p.i.		**Infectious[2]**		**Severe pathology[3] at 12 months p.i.**		**Resistant[4] at 12 months p.i.**	
	Se	Sp	Se	Sp	Se	Sp	Se	Sp
Faecal DNA	34.6 (0.093)	91.7 (0.079)	43.8 (0.124)	90.5 (0.064)	45.5 (0.150)	81.5 (0.074)	8.3 (0.079)	65.4 (0.093)
IFNγ	30.8 (0.091)	91.7 (0.079)	43.8 (0.124)	90.5 (0.064)	45.5 (0.150)	85.2 (0.068)	8.3 (0.079)	69.2 (0.091)
IL-10	92.0 (0.054)	33.3 (0.136)	93.8 (0.061)	20.0 (0.089)	90.9 (0.087)	19.2 (0.077)	66.7 (0.136)	8.0 (0.054)
Faecal DNA and IFNγ	7.7 (0.052)	100.0 (0.00)	12.5 (0.083)	100.0 (0.00)	18.2 (0.117)	100.0 (0.00)	0.0 (0.00)	92.3 (0.052)
Faecal DNA or IFNγ	57.7 (0.097)	83.3 (0.11)	75.0 (0.108)	81.0 (0.086)	72.7 (0.134)	66.7 (0.091)	16.7 (0.108)	42.3 (0.097)
Faecal DNA and IL-10	32 (0.093)	100 (0.00)	43.75 (0.124)	95 (0.049)	45.45 (0.150)	88.46 (0.062)	0 (0.00)	68 (0.093)
Faecal DNA or IL-10	96.0 (0.039)	25.0 (0.13)	93.8 (0.061)	15.0 (0.079)	90.9 (0.087)	11.5 (0.063)	75.0 (0.125)	4.0 (0.039)

Standard errors are shown in parentheses; Cut-off values: Faecal DNA 3.39 fg, IFNγ S/P <0.38, IL-10 S/P <0.28
[1]Tissue culture positive, [2]Faecal culture positive at any one sampling, [3]Multibacillary disease pathology, [4]Tissue culture negative

Discussion

In this study we have successfully identified indicators from a young age that reflect future disease outcome, albeit under experimental infection conditions. Most mycobacterial diseases are inherently long-term in nature and subclinically infected individuals can appear unaffected for many years. Therefore it was surprising to find that at only a few months after exposure to Mptb samples collected from sheep contain a wealth of information regarding the eventual disease outcome.

Although all exposed sheep have an IFNγ response, the strength of this early IFNγ response reflects future disease outcome. Sheep that had a weaker early IFNγ response were the ones that were more likely to be infectious (i.e. shed Mptb in their faeces), more likely to be truly infected (i.e. have viable Mptb in intestinal tissues) and more likely to have severe multibacillary disease pathology. IFNγ is the main cytokine which regulates the function of macrophages, activating these cells to produce cytotoxic free radicals which are an essential antimicrobial defence mechanism. This cytokine also enhances expression of MHC molecules which enable interaction with lymphocytes and as a result facilitates cell-mediated immune mechanisms. Thus the stronger IFNγ response in sheep that are able to remain free from disease is perhaps a reflection of efficient control or elimination of the pathogen by the host.

An IL-10 response at 4 months p.i. was also associated with increased likelihood of disease resistance and decreased likelihood of infection. Two cytokines with very different actions, IFNγ and IL-10, were associated with protection. In *Mycobacterium tuberculosis* (Mtb)-infected human macrophages, pre-treatment with IFNγ facilitates expression of Mtb antigens on the cell surface while pre-treatment with IL-10 results in retention of Mtb antigens within endosomal compartments (Bobadilla et al., 2012). Hence while IFNγ is associated with a favourable host response, IL-10 allows pathogen persistence. With such seemingly diametrically opposing actions at the cellular level, how do we reconcile the paradoxical effect of both IFNγ and IL-10 being protective at a whole animal level? Our results are

supported by findings in experimentally infected calves: at 15 months p.i. a lower IL-10 response in peripheral blood cells is associated with a greater extent of intestinal tissue infiltration by Mptb (Subharat et al., 2012). While IL-10 has potent immunosuppressive effects it also enhances survival and differentiation of B cells (Mocellin et al., 2004). Perhaps, at a tissue level the immunosuppressive properties of IL-10 are important to enable a tightly controlled immune response to minimise tissue destruction. Sheep that are resistant to Mptb infection have higher numbers of B cells in lymph nodes draining sites of infection (Begg and Griffin, 2005). We have also previously shown that a combined IFNγ and antibody response is a common response in Mptb-exposed sheep (Begg et al., 2011). In brief, sheep that are resistant to Mptb infection have higher numbers of B cells in lymph nodes draining sites of infection and a combined IFNγ and antibody response is a common response in Mptb-exposed sheep. While the benefits of an antibody response to an intracellular pathogen are not entirely certain, it is possible that this antibody response is a reflection of the effects of an early IL-10 response.

The positive predictive value indicates the proportion of test positive animals that are truly diseased while the negative predictive value indicates the proportion of test negative animals that do not have the disease. Although the positive predictive value of these tests is quite high there were limited numbers within some subgroups. In addition, in practice, these values are affected by disease prevalence. Therefore, these results in an experimental infection model only provide guidelines as to which tests are likely to be beneficial when attempting to use early host responses to predict eventual disease outcomes.

Sheep that progress to multibacillary disease pose the greatest threat to the rest of their flock and other flocks as they are most likely to become infectious and shed greater numbers of Mptb in their faeces and contaminate the environment. Based on the tests used in this study, faecal Mptb DNA and the peripheral blood IFNγ response have the potential for predicting which animals will eventually succumb to multibacillary disease. Selective removal of such animals early would be valuable in limiting the spread of paratuberculosis.

This work has been published in a peer-reviewed scientific journal (de Silva et al., 2013).

4.4 Subprogram 1.2. Part 1. Development and validation of a new culture medium for Mptb to replace commercial BACTEC medium

Introduction

Liquid culture of Mptb from clinical samples is the most sensitive *ante-mortem* diagnostic test for Johne's disease. Modified BACTEC 12B is the only commercial liquid culture medium that has been validated and shown to support the growth of the two major strain types of Mptb, S and C. Mptb S strain does not grow reliably in the commercial culture media that are marketed for Mptb detection. Consequently the culture of Mptb in all animal health laboratories in Australia and New Zealand and in some laboratories in other countries has been based on modified BACTEC 12B medium. In 2012 Becton Dickinson discontinued the supply of BACTEC 12B medium, an antibiotic additive (PANTA-PLUS) containing polyoxyethylene stearate (POES) and support for the associated BACTEC 460 instrumentation, as this radiometric technology had become redundant in human applications such as *M. tuberculosis* diagnosis, where it was mainly used.

The aim of this study, which was conducted over 2 years using tissue and faecal samples from both sheep and cattle, was to develop and validate a liquid culture medium that would emulate the essential composition of modified BACTEC 12B medium and would support the growth of both major strain types of Mptb from domestic ruminants.

Methods

Modified BACTEC 12B medium (Becton Dickinson, BD) was prepared according to the ANZSDP and contained egg yolk, PANTA-PLUS and mycobactin J. Following a literature review, and based on prior experience, a new medium was developed and tested in pilot studies. The newly develop medium, M7H9C medium was prepared by combining 1.86 g Middlebrook 7H9 broth base (BD), 0.4 g casitone (BD) and 385 mL water to form the base, which was autoclaved at 121°C for 20 minutes. To the base the following was added: 100 mL egg yolk (obtained aseptically from fresh commercial eggs sold for human consumption), 20 mL PANTA-PLUS (BD) reconstituted in sterile water, 10 mL 50µg/mL mycobactin J stock solution (Allied Monitor), 70 mL sterile water and 16 mL ADC (0.136g NaCl, 0.8 g bovine albumin fraction V, 0.32g dextrose, 4.8 mg catalase) (BD). The final pH does not require adjustment and was 6.3 to 6.4. After mixing, the medium was dispensed (6 mL per tube) into 30 mL polycarbonate tubes, each 25 mm diameter by 80 mm high.

Two reference strains of Mptb were used, Telford 9.2 S strain, and CM00/416 C strain. Mptb was cultivated in BACTEC 12B medium and subcultured to modified 7H10 agar slopes as described above. The lawn cultures were harvested in sterile phosphate buffered saline with 0.1%v/v Tween 20 to produce suspensions. Aliquots of 50 µl from three replicate serial 10 fold dilutions of the suspensions were inoculated into BACTEC 12B and M7H9C media. After 12 weeks incubation PCR evaluation of each culture tube was conducted, regardless of whether a growth index was detected in the BACTEC 12B medium. The most probable number (MPN) of viable Mptb in each suspension was determined using end point titration. The limit of detection was determined for each medium and Mptb strain, defined as the highest dilution where all three replicates grew Mptb, based on growth at 12 weeks. Counts within 1 log were regarded as sufficiently similar to enable pooling of data for some calculations.

Analytical sensitivity was determined using faeces from healthy animals that were spiked with Mptb. Serial dilutions of each Mptb strain were prepared as above and spiked into faeces (pre-diluted 1:1 with sterile water to form a slurry) to achieve concentrations ranging from 10^8 to 10^1 Mptb/g based on prior microscopic counts of the bacterial suspension determined using a Helber counting chamber. After adding Mptb suspension the faecal slurry was mixed thoroughly. Non-spiked controls were included. One aliquot from each dilution was cultured in BACTEC 12B and M7H9C media.

Faecal and tissue samples from ruminants were cultured in M7H9C medium and in BACTEC 12B medium (Table 4). There were 175 faecal samples from sheep from 9 different flocks in New South Wales, and 496 faecal samples from cattle from 9 different herds in New South Wales, Victoria and Tasmania. There were fewer tissue samples available: 37 from sheep and 76 from cattle. Tissues comprised intestine, mesenteric lymph node and liver. The strain of Mptb that was present in clinical samples was determined using IS*1311* PCR and REA. Each sample was processed to create an inoculum using ANZSDP methods. Briefly, faeces were prepared using a double incubation centrifugation method, and were decontaminated

in hexadecylpyridinium chloride (HPC) then in vancomycin, amphotericin B and nalidixic acid (VAN), while tissues were homogenized using a stomacher apparatus and the supernatant was used as inoculum following decontamination in HPC. A single inoculum was prepared from each sample, mixed, and divided in two for inoculation of the two media. Only one tube of each medium was used per sample. Mptb was cultured in BACTEC 12B medium as previously described. Each culture was incubated for 12 weeks. The growth index (GI) was measured weekly and samples of the broth were removed from the bottles for PCR analysis when GI reached 999. For M7H9C medium, a sample of broth was removed after 12 weeks incubation. For part of the study, samples were taken from M7H9C media on more than one occasion. The first sample (T1) was usually taken within a week of the time that the GI in the parallel BACTEC 12B culture reached 999 while the second sample (T2) was usually collected after 12 weeks incubation. For both media, 200 µl of the broth was collected after 12 weeks incubation and Mptb bacteria were detected using IS*900* PCR with restriction endonuclease analysis of the PCR product.

Table 4. Samples used to evaluate M7H9C medium in comparison to modified BACTEC 12B medium

Species of origin	Type of sample	Strain of Mptb present in sample	No. of samples tested	No. culture positive	
				BACTEC 12B	M7H9C
Sheep	Faeces	S	175	50	50
	Intestinal tissue	S	12	8	8
	Lymph node	S	20	10	10
	Liver	S	5	2	2
Cattle	Faeces	C	496	135	125
	Intestinal tissue	C	38	2	3
	Lymph node	C	38	2	3
Total			784	209	201

BACTEC 12B medium when supplemented with PANTA-PLUS solution as recommended contains an aqueous reconstituting fluid that itself contains POES. Two other PANTA products are available from BD (BBL MGIT PANTA and BACTEC PANTA/F), but neither contains POES. To test the requirement of Mptb for POES in *in vitro* culture, aliquots of 50 µl from three replicate serial tenfold dilutions of suspensions of S and C strain Mptb were inoculated into BACTEC 12B and M7H9C media, as for an MPN, with and without POES and growth was monitored for 12 weeks.

Results

Serial dilutions of pure culture Mptb suspensions were cultured in triplicate as for a most probable number (MPN) determination to compare growth in the two media. MPN estimates were within 1 log for both strains and media: the limit of detection was approximately 7 viable bacilli per inoculum. The analytical sensitivity for Mptb spiked into faeces for both media for S strain was approximately 1.1×10^3 and for C strain was 2.4×10^1 viable bacilli per gram faeces. As only one replicate of each dilution was used, these results should not be considered to be true limits of detection. POES was not essential for growth of Mptb.

Faecal samples from sheep and cattle were cultured in BACTEC 12B and M7H9C media (Tables 3 and 5). No cultures were lost due to visible contamination. The samples contained a spectrum of concentrations of viable Mptb. Of the 671 faecal samples, 185 were positive in BACTEC medium compared to 175 in M7H9C medium (Table 5). However, there was no significant difference in the detection rate between the two media and the level of agreement

between them was excellent. Findings were similar when the data were stratified by host species (Table 5).

Table 5. Cross tabulation of the results of cultures of clinical samples from sheep and cattle in M7H9C and modified BACTEC 12B media. Data are the number of samples.

	BACTEC 12B		Total
	Positive	Negative	
Sheep and cattle faeces			
M7H9C positive	164	11	175
M7H9C negative	21	475	496
Total	185	486	671
McNemar's Chi square 2.53, P>0.05; Kappa 0.88, 0.84-0.92			
Sheep faeces			
M7H9C positive	46	4	50
M7H9C negative	4	121	125
Total	50	125	175
McNemar's Chi square 0.13, P>0.05; Kappa 0.89, 0.81-0.96			
Cattle faeces			
M7H9C positive	118	7	125
M7H9C negative	17	354	371
Total	135	361	496
McNemar's Chi square 3.38, P>0.05; Kappa 0.88, 0.83-0.92			
Sheep and cattle tissues			
M7H9C positive	24	2	26
M7H9C negative	0	87	87
Total	24	89	113
McNemar's Chi square 0.50, P>0.05; Kappa 0.95, 0.88-1.0			
Sheep tissues			
M7H9C positive	20	0	20
M7H9C negative	0	17	17
Total	20	17	37
No discrepant results			
Cattle tissues			
M7H9C positive	4	2	6
M7H9C negative	0	70	70
Total	4	72	76
McNemar's Chi square 0.5, P>0.05; Kappa 0.79, 0.5-1.0			

Tissue samples were cultured in both media without visible contamination (Tables 4 and 5). Twenty four of 113 were positive in both media, while 2 (cattle tissues) were positive in M7H9C medium but negative in BACTEC medium. There was no significant difference in detection rate between the two media and the agreement between the two tests was excellent (Table 5).

Overall, there were 784 cultures undertaken, of which 21 were positive in BACTEC and negative in M7H9C medium, and 13 negative in BACTEC but positive in M7H9C medium (McNemar's Chi sq. 1.44, p>0.05; Kappa 0.89, 0.85-0.93 95% CL) (data pooled from Table 5). Based on this analysis the performance of the two media was not significantly different.

Of the relatively small percentage (<5) of samples which were positive in one medium and negative in the other, most contained few viable Mptb. Under such circumstances not every 1 g subsample of faeces will contain a viable organism, and this fact alone can account for

discrepant results between two tests. Seventeen of the 21 BACTEC 12B positive/M7H9C negative faecal samples were from cattle (Table 5).

Discussion

The culture of Mptb will remain a definitive test for confirmation of Johne's disease despite the development of DNA detection methods. Ideally liquid culture media should be used for cultivation of Mptb from clinical samples because of greater analytical sensitivity compared to solid media. The diversity of Mptb strains globally, and the tendency for some types to grow poorly *in vitro*, necessitates the use of media capable of supporting the growth of all strains of Mptb. The recently discontinued BD product BACTEC 12B was the only commercial liquid medium that provided some certainty in culture of both S and C strains.

The M7H9C medium described in this study gave comparable results to BACTEC 12B. The agreement between the two media in culture outcomes for 784 clinical samples, more than 25% of which were culture positive, was excellent and there was no statistically significant difference in sensitivity.

M7H9C medium does not include a growth indicator and it is necessary to conduct secondary tests on every culture to determine whether Mptb is present. However, even with the BACTEC commercial system, the growth readout is generic and secondary tests are required if growth is detected. Secondary tests include examination of a smear of broth stained using a Ziehl Neelsen method, antigen capture ELISA, subculture to solid media, or PCR. Smear examination lacks sensitivity, is not specific for Mptb and is very time consuming for the operator. Subculture to solid media with and without mycobactin J to show mycobactin dependency is a definitive taxonomic test for Mptb. However, because solid media have lower analytical sensitivity than liquid media for both C and S strains of Mptb, subculture from broth to solid media has a false negative culture rate of about 30%, and requires another 6 weeks of incubation. Antigen-capture ELISA to detected secreted products in growing cultures is useful for confirming the presence of *M. avium* complex but requires a specific PCR to confirm Mptb. Therefore in this study we chose to use IS*900* PCR with confirmatory restriction endonuclease analysis.

Some Mptb cultures begin to grow within a week or two and can be detectable by PCR, whereas others may not grow for 6 weeks or more. This is dependent on the number of Mptb in the sample, which in turn is dependent on the stage of disease in the animal. Unfortunately samples with very low numbers of Mptb, or Mptb in a dormant state, may require prolonged incubation. In Australia a minimum incubation of 10 weeks has been recommended for bovine and caprine pooled faecal culture and 12 weeks is routinely used for sheep pooled faecal culture. In this study a few cultures were extended for 17 weeks incubation to confirm that there was no detrimental effect of prolonged incubation on PCR detection in the culture. Recently, incubations as short as 6 weeks have been recommended for liquid culture of Mptb, but based on available evidence, such a protocol would lead to substantial rates of false negative culture test outcomes unless there were above average numbers of Mptb in the clinical samples. Such samples are most likely to be from cases in a relatively advanced stage of disease. We recommend a standard incubation of 12 weeks.

A second question is whether or not samples should be removed from the broth cultures for examination within the standard incubation period. While many positive cultures could be

detected early, for example at 4 weeks or 6 weeks, it would add to the cost of confirmation if all cultures were tested at intermediate times as well as at the end of the standard incubation period.

The detection of Mptb in a sample is indicative of Mptb infection in the herd or flock because the organism is considered to be an obligate parasite. Therefore for herd-level diagnosis it is not important to determine whether the organisms are viable or non-viable. However, there may be a need to confirm viability to confirm a diagnosis at individual animal level. Mptb viability can be inferred when using M7H9C medium by performing PCR immediately after inoculation and again after 12 weeks incubation to show an increase in Mptb DNA concentration.

The ideal culture medium supports the growth of all strains of Mptb, has high analytical sensitivity, a low contamination rate, a short incubation period, enables easy identification of Mptb*,* is low cost, entails a low risk to laboratory staff and the environment. It is unlikely that any medium can fulfill all these criteria. In the present study the contamination rate was negligible, but it is recognized that contamination is highly clustered with origin of samples and is unpredictable. The advantages of M7H9C medium are that it directly replaces BACTEC 12B medium, and its use does not require any change to existing culture protocols with respect to sample preparation, decontamination or inoculation. There is reduced risk to the operator and the environment because the medium is not radioactive. During incubation no weekly monitoring of cultures is required apart from a routine check after a week to look for gross contamination, something that most laboratories would do with any media. With the BACTEC 460 system it was recommended that all cultures be checked every week for development of a growth index, which involved technician and machine time. The main disadvantage of M7H9C medium is that it lacks a growth read out and a PCR must be conducted on every culture. While this may appear to be a disincentive many faecal cultures contain irrelevant microorganisms which will produce a growth signal in systems like BACTEC 460 and BACTEC MGIT 960. Each of these cultures would require follow-up, usually by PCR.

The cost of M7H9C medium including labour and materials is about half the purchase price of modified BACTEC 12B medium based on data in Australia in January 2013. M7H9C medium is also considerably cheaper per unit than modified Middlebrook 7H10 agar or 7H11 agar, which are the only other media known to support the growth of the two common strains of Mptb. There are hidden costs inherent in the use of solid media, such as the common practice of using multiple slopes per sample compared to one tube for liquid culture. The labour and materials costs of culture set-up are identical for M7H9C and BACTEC 12B media. During incubation BACTEC 12B requires greater labour inputs to read growth, and at the completion of incubation M7H9C may require greater inputs to confirm the presence of Mptb*,* but the extent to which this increases the cost of the procedure relative to BACTEC 12B is highly dependent on the proportion of BACTEC 12B cultures with a growth signal. This depends on the prevalence of Mptb and on the rate of isolation of irrelevant microbes that cause a growth signal.

The new M7H9C broth medium has been adopted for routine culture of Mptb in animal health diagnostic laboratories throughout Australia, and it is included in the Australian and New Zealand Standard Diagnostic Protocol for Paratuberculosis (www.scahls.org.au). The Johne's disease laboratory at The University of Sydney manufactures M7H9C medium and

supplies it upon request to these laboratories, in support of the Australian livestock industries.

This work has been published in a peer-reviewed scientific journal (Whittington et al., 2013).

4.5 Subprogram 1.2. Part 2. Development of an efficient high throughput method for confirmation of Mptb in M7H9C liquid culture medium

Introduction

As all M7H9C cultures must be examined by PCR to detect growth of Mptb, an efficient, high throughput method was needed. The original validation of M7H9C media performance used a simple DNA isolation method involving ethanol precipitation. This method effectively removes PCR inhibitors present in the culture medium, such as egg yolk constituents but is time consuming and costly. The aims of this study were to develop and validate a sensitive, specific and efficient method for detection of the growth of Mptb in liquid broth cultures containing egg-yolk to meet the need for cheaper high throughput testing.

Methods

A panel of BACTEC 12B liquid culture samples was selected in order to compare results from the different DNA extraction methods (Table 6). There were 54 cattle and 70 sheep faecal culture samples and 20 sheep tissue culture samples. Both culture positive and negative samples were included in similar numbers for each species and sample type in a retrospective study.

Table 6. Samples included in the feasibility study to compare ethanol precipitation/conventional PCR and optimised magnetic bead DNA isolation/qPCR methods for Mptb in liquid culture.

Species of origin	Sample type	Strain of Mptb present	Sample numbers and original culture results		Results[a]	
			Positive (wks to 999[b])	Negative	EtOH precip.	Mag bead
Cattle	Faeces	C	-	30	2[c]	3[c]
Cattle	Faeces	C	4 (4 weeks)	-	4	4
			10 (5 weeks)	-	10	10
			5 (6 weeks)	-	5	5
			3 (7 weeks)	-	3	3
			2 (8 weeks)	-	2	2
Sheep	Faeces	S	-	30	All negative	All negative
Sheep	Tissues	S	-	10	All negative	All negative
Sheep	Faeces	S	10 (4 weeks)	-	10	10
			10 (5 weeks)	-	10	10
			10 (6 weeks)	-	10	10
			10 (7 weeks)	-	10	10
Sheep	Tissues	S	10	-	10	10

a. EtOH precip.: ethanol precipitation and IS*900* qPCR method; Mag bead: optimised magnetic bead DNA isolation and IS*900* qPCR method.
b. Weeks of growth in BACTEC 12B liquid culture media to reach a growth index of 999.
c. These samples had GI of 999 but the original PCR results were negative. Two were positive on secondary testing with both methods and an additional sample was detected by magnetic bead isolation/qPCR. The remainder of the samples in this group was negative using both methods.

DNA was extracted using the commonly used ethanol precipitation method, as previously described. Briefly, 500 µl absolute ethanol was added to 200 µl of culture medium, left to

stand for 2 min then vortexed briefly and centrifuged at 28 × *g* for 2 min to allow the partially flocculated egg yolk to accumulate at the base and sides of the tube, in order to remove inhibitory egg components. The supernatant was transferred to a clean 1.5 ml centrifuge tube and centrifuged at 18,000 × *g* for 5 min. The resulting pellet was washed twice in 200 µl of sterile PBS, resuspended in 50 µl of sterile distilled water and incubated at 100°C for 20 min; 5 µl was added to each PCR.

A new method based on bead beating was developed and optimised. Briefly, 200 µl of the liquid culture medium was added to 200 µl Buffer RLT (Qiagen), then transferred to a 2 ml conical base screw-capped tube containing 0.3 g of Zirconia/Silica beads and disrupted using a Fast Prep-24 bead-beater (MP Biomedicals) at 6.5 m/sec for 60 sec, twice. The tubes were centrifuged at 16000 x *g* for 2 min and 100 µl of the homogenate was added to the deep 96-well (S-Block) plate. DNA was isolated using the BioSprint® 96 One-For-All Vet kit (Qiagen) and a MagMAX-96 automated magnetic processor, following the protocol for tissue homogenates using Buffer RLT, according to the manufacturer's instructions.

Results

A pilot study was conducted to determine whether a bead-beating/magnetic bead DNA isolation method was likely to detect Mptb in liquid culture. This showed that zirconia/silica beads in the bead-beating step appeared to be superior to ceramic beads, and both were far superior to samples processed without a bead-beating step. Two buffers supplied with the BioSprint® 96 One-For-All Vet magnetic bead nucleic acid isolation kit were tested and Buffer RLT gave superior sensitivity.

The analytical sensitivity was assessed on replicate samples of M7H9C culture media (n=4/treatment and dilution) spiked with known quantities of Mptb. No significant difference in the detection of the two Mptb strains was found. The limit of detection was 10^4 to 10^5 fold better for the newly optimised magnetic bead method compared to the original ethanol precipitation method (10^2-10^3 compared to 10^7 Mptb organisms/ml, respectively) (P<0.001).

Cultures from both cattle faecal and sheep faecal samples as well as tissue samples were included in a validation study to compare the optimised magnetic bead DNA isolation method with the ethanol precipitation method (Table 5). Samples chosen had been previously grown in radiometric liquid media (BACTEC 12B) and categorised as negative (n=70) or positive (n=74) based on the original culture results using the standard ethanol precipitation/IS*900* conventional PCR method. Individual culture samples were divided into two equal aliquots and processed in parallel using the two DNA isolation methods, with detection of Mptb DNA using the IS*900* qPCR on both extracts. There was a high level of agreement in the positive and negative results obtained using the two methodologies (Table 7) (McNemar's Chi squared P=1.0). Two cattle samples with late growth indices (12 weeks) that were negative in the original PCR had Mptb DNA detectable using both the ethanol precipitation and optimised magnetic bead DNA isolation methods coupled with qPCR and an additional sample was positive with only the magnetic bead isolation method (Table 7).

Table 7. Two by two contingency table of frequencies for detection of Mptb in the feasibility study, comparing the ethanol precipitation/conventional PCR method and the optimised magnetic bead DNA isolation/qPCR method.

		EtOH/Conv. IS*900* PCR		
		Positive	*Negative*	TOTAL
Mag bead/ IS*900* qPCR	*Positive*	76	1	77
	Negative	0	67	67
	Total	76	68	144

Discussion

The new method is many-fold more sensitive than the previously used detection method; the increased sensitivity is likely to be attributable to the inclusion of a bead-beating step to disrupt the cell membrane as well as the advantages offered by using magnetic bead DNA isolation technology and a qPCR detection assay. The optimised magnetic bead method removes inhibitors in egg yolk that can affect the detection of Mptb in liquid culture by PCR.

This work has been published in a peer-reviewed scientific journal (Plain et al., 2014).

4.6 Subprogram 1.3. Introducing a new mycobacteriophage assay to Australia, testing this as a live-dead assay using samples from the animal trials, and confirming applications for the test

Introduction

International efforts to manage Johne's disease have been hampered by the lack of rapid, specific detection tests for live Mptb. Culture is the gold standard test, however culture methods require long incubation periods and decontamination protocols that can decrease sensitivity. An ideal detection test for Mptb would be rapid, specific and provide live/dead differentiation.

Phage assays are rapid live-dead assays that have been applied for the detection of *M.tuberculosis* in human tuberculosis patient samples, such as sputum. The methodology has been adapted in other countries for the detection of viable Mptb in blood and milk. The phage assay utillises a mycobacteriophage (D29), which is a virus that specifically infects mycobacteria by attaching to the bacterial cell wall via specific receptors. Once bound, the phage injects its genome into the host cell and uses the cells machinery to produce nucleic acids and phage proteins. After assembly, the bacterial cell is lysed to release multiple bacteriophages to infect other cells. Samples are incubated with the mycobacteriophage to allow any mycobacteria in the sample to be infected. Excess phage is eliminated and the sample is then cultured on a lawn of sensor cells (*Mycobacterium smegmatis*) that indicate the presence of mycobacteria from the original sample by the formation of a plaque. If there are no mycobacteria in the original sample (or negative control), there should be no phage amplification and therefore no plaques at the end of the assay. The specificity of the method for Mptb is dependent on combining the phage-based assay with PCR amplification of IS*900*.

The blood phage assay developed by researchers at the University of Nottingham has been shown to successfully detect Mptb in blood with a limit of detection of 10 pfu/ml. A bacteraemic phase in paratuberculosis in some sheep with severe infection has been

previously demonstrated by culture of blood samples from exposed animals at the University of Sydney. It can be assumed that the presence of this organism in the blood of the animal indicates that it has crossed the gut and disseminated to other tissues such as milk and skeletal muscle.

The aim of this study was for the phage assay developed by Dr Cath Rees and Dr Ben Swift, University of Nottingham to be introduced to Australia and optimised for local conditions. The phage assay would then be trialled on animal samples and applications for the test assessed.

Methods

The phage assay and experimental controls were carried out according to published methods and introduced to The University of Sydney by Dr Ben Swift, University of Nottingham through a collaborative visit to Sydney. Mptb cells were isolated from blood prior to detection by the phage assay using magnetic bead separation. The magnetic beads were coated with two peptides that specifically bind to Mptb. Briefly, each sample (1ml total volume) was placed onto a magnetic rack for 10 min, the supernatant was removed and the beads were resuspended and the magnetic separation process repeated. The samples were resuspended prior to testing using the phage detection assay. As an alternative sample preparation method, peripheral blood mononuclear cells (PBMC) were isolated from cattle blood using Ficoll-Paque. Briefly, 2 ml of whole heparinised blood was mixed with 2 ml of PBS. This was carefully layered on 3 ml of Ficoll-Paque Plus. The samples were centrifuged then the upper layer (plasma) of the sample was removed. The buffy coat layer was carefully removed ensuring the red blood cells were not disturbed. The PBMCs were washed with 6 ml of PBS by centrifugation, the supernatant was removed and the pellet was resuspended in 1 ml of FPTB (Fast Plaque TB) Medium Plus for the phage detection assay. To detect mycobacteria in this assay, samples were mixed with D29 bacteriophage in FPTB Medium Plus and incubated for 1 h to allow the phage to infect mycobacteria present in the sample. Phage that had not infected host cells were inactivated using a virucide (ferrous ammonium sulphate; 10 mM) and then the samples were mixed with *M. smegmatis* cells before plating with soft 7H10 agar (0.75 % final agar concentration). Two negative controls (1 ml of FPTB Medium Plus without sample added) were included in each experiment; one prior to the samples and one at the end. The positive control was prepared by serially diluting the *M. smegmatis* sensor cells and adding as a sample to the D29 bacteriophage to obtain positive plaques. The number of Mptb cells detected in the original sample was determined by counting the number of plaques formed (data reported as $pfu.ml^{-1}$) in the lawn of *M. smegmatis* cells. Enumeration of Mptb cells in the inocula was achieved by diluting samples until countable numbers of plaques are obtained. DNA from up to five plaques formed on each plate was extracted using a gel DNA extraction kit. To confirm the plaques formed were due to the detection of Mptb in the original sample, a IS*900* PCR assay was used.

Cattle from experimental infection trial P.PSH.0297.2 were tested. In this trial, 30 calves (aged 2-4 months) were age matched then randomly allocated into a group of 20 to be experimentally exposed to Mptb (Numbers 11-30, Table 9.1) along with a group of 10 age-matched unexposed control animals (Numbers 1-10, Table 9.1). Blood samples were collected at 4 years and 8 months post-exposure from all animals for the phage assay.

Sheep blood samples from trial P.PSH.0576.2 were tested at 14 months post-exposure, which was at the conclusion of the trial, to determine the ability of the phage assay to detect S strains of Mptb. Twenty-eight sheep were tested, eight that had been experimentally inoculated and 20 unexposed controls.

Results

The phage assay was used to test blood samples from cattle in trial P.PSH.0297.2 at 4.6 years post-exposure. None of the cattle had clinical signs of Johne's disease. Shedding of Mptb in the faeces of some of the 20 exposed cattle was detected by faecal culture intermittently following exposure. None of the control animals were positive for Mptb in the phage assay or any of the other methods used. Seven out of the nineteen (37%) Mptb-exposed animals were positive in the phage assay, indicating that viable Mptb cells were detected in the blood samples. Only very low numbers of plaques (2-5) were produced in the Mptb-positive samples.

The phage assay was also applied to sheep samples. It is known that viable Mptb cells can be cultured from the blood of infected sheep, but it was not known whether sheep strains of Mptb could be captured using PMMS or whether they are as efficiently infected using the bacteriophage (D29). The results show that out of the twenty-eight sheep samples, 5 were positive for Mptb by the phage assay, 3 from the exposed and 2 from the unexposed animals. All eight of the exposed animals were positive for Mptb by faecal HT-J PCR and five of the samples (excluding the vaccinated samples; # 6-10) were positive for Mptb by serum ELISA (Table 9.3). Two of the control animals gave positive results for Mptb which was unexpected and did not agree with the results of the other diagnostic tests.

As the number of Mptb cells detected using phage was very low in both sheep and cattle samples, the efficiency of the magnetic bead isolation step was investigated. The limit of detection of Mptb cells was poor (7.3×10^2 pfu ml^{-1}), explaining the low levels of Mptb detected in the blood by the phage assay.

Since the magnetic bead separation step requires expensive reagents and optimisation of magnetic bead coating, experiments were conducted to determine whether Mptb cells could be detected from whole blood without the need to conduct a PMMS step. There was no significant difference in the number of Mptb cells detected, but the detection of Mptb in PBMCs was more reproducible than isolating Mptb cells using the PMMS method on whole blood samples. The appearance of plaques is illustrated in Fig. 7.

The PBMC isolation method was applied to test blood samples from cattle trial P.PSH.0576.4. DNA was extracted from a random mixed sample of plaques from every plate and tested using IS*900* qPCR; all samples from the unexposed cattle were negative and one positive sample was found within the exposed cattle. However, the large number of plaques that had to be screened by PCR to rule out Mptb in the control cattle made the method impractical.

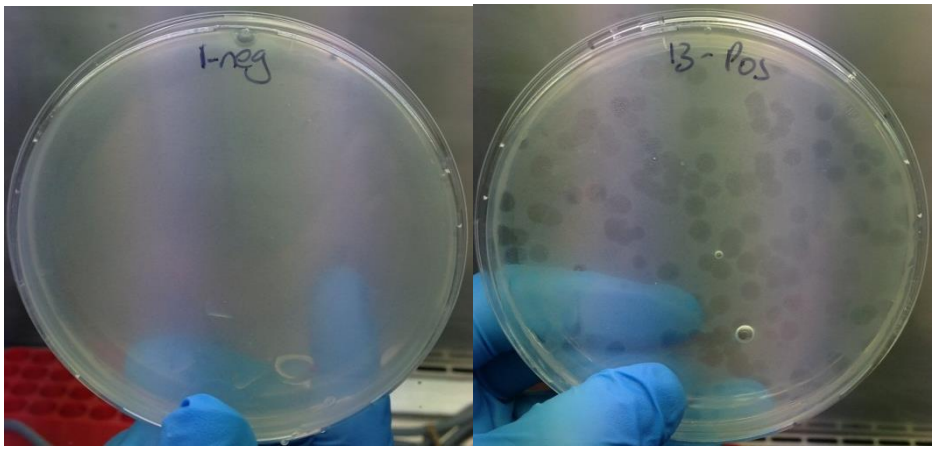


Fig. 7. Representative plaque results for the PBMC isolation phage detection method for cattle from trial P.PSH.0576.4. The clear spots represent proliferation of phage in and lysis of *M. smegmatis* bacteria which are growing as a lawn culture on the agar plates. IS900 PCR must be conducted on DNA obtained from each plaque to determine whether it contains Mptb.

Discussion

The results in this study show that experimentally infected animals with variable blood ELISA status can have viable Mptb in their blood that can be detected in a phage assay. The assay was specific; although plaques were detected in two unexposed control cattle, no positive Mptb DNA was detected in the plaques, so the test result was "negative". Approximately one third of Mptb-exposed cattle had viable Mptb in their blood that was detectable by the phage assay. Although two animals were shedding Mptb in their faeces, no Mptb was detected in the blood of these individuals. The results suggest that the presence of viable Mptb cells in the blood may be intermittent and non-aligned with faecal shedding, which can also be intermittent. Longitudinal studies in a larger herd with greater levels of disease would be required to confirm the pattern of bacteraemia detected by the phage assay.

The magnetic bead separation step of the phage method requires expensive reagents, but is not needed and the phage assay can be carried out directly from the sample. However, this may lead to large numbers of plaques that have to be screened by PCR. Thus the PMMS method step should be retained when testing samples in Australia.

There is a risk in the use of the phage assay in proximity to laboratories where Mptb culture is undertaken, due to the potential for cross-contamination of cultures with bacteriophage, leading to false negative culture results.

This study showed that viable Mptb may be present in the blood of Mptb-exposed cattle prior to the onset of clinical signs. The ability to detect viable Mptb in the blood of subclinically infected animals may lead to increased sensitivity of diagnosis in the early stages of infection, but this requires validation. A longitudinal study testing the blood, milk and faeces of animals would enable a better understanding of paratuberculosis disease progression, determine when systemic infection becomes established in animals, and how this influences the serum and milk ELISAs, and faecal culture or PCR results. In sheep, the results were consistent with prior studies in which a small proportion of Mptb exposed animals had bacteraemia, but positive results from two control sheep suggest that assay specificity may need to be addressed carefully.

4.7 Subprogram 1.4. Extending, implementing and validating a lymphocyte proliferation assay by trialling new antigens, testing the effects of storage and transport conditions and conducting a field trial

Introduction

Lymphocytes are mononuclear white blood cells that circulate between the blood and lymphatic systems. Their main role is defending an individual against infection. Lymphocytes can be categorised based on function and identified by 'cluster of differentiation' (CD) molecules expressed on their cell surface. Briefly, T lymphocytes can be classified into many subtypes including $CD4^+$, $CD8^+$and γδ T cells. Presentation of antigens by professional antigen presenting cells such as monocytes/macrophages and dendritic cells signal the presence of an infection. Interactions between lymphocytes and these antigen presenting cells initiate a cascade of events directed towards eliminating the pathogen. This includes the secretion of cytokines such as IFNγ and lymphocyte proliferation. Proliferation of antigen-specific lymphocytes, and generation of lymphocytes which bear long lasting 'memory' of the encounter with the pathogen, are hallmarks of the adaptive immune response to pathogens. The lymphocyte proliferation assay detects immune memory.

Previous contact with a specific antigen (either via exposure to the pathogen or vaccination) is measured by the proliferative response of blood lymphocytes in vitro which provides a general overview of the capacity of an individual's immune system to respond to infection. Historically, lymphocyte proliferation has been measured by radioactive methods. The assay used here avoids the use of radioactive materials and incorporates a fluorescent dye to track proliferation via flow cytometry. An additional advantage of this method is the ability to study proliferation of specific subtypes of lymphocytes.

Methods

Blood was collected from the jugular vein into lithium heparin-coated tubes. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation and were labelled with the fluorescent tracking dye CFSE (carboxyfluorescein diacetate succinimidyl ester) (5 mM). The cells were then plated into a 96-well plate and cultured in medium alone, 10 µg/mL Mptb antigen or 5 µg/mL pokeweed mitogen (PWM) as the positive control for 5 days. At the end of the culture period, samples were acquired on a flow cytometer to determine total cell proliferation.

The lymphocyte proliferation assay was applied to samples collected in many of the experimental infection trials listed in Table 1.

To obtain more field-relevant data, the lymphocyte transformation assay was assessed to determine if stored blood samples could be used under conditions similar to the $IFN\gamma^{PLUS}$ assay (see section 4.8). This would allow for transport of blood samples from farms to diagnostic laboratories. Blood was collected from the jugular vein into lithium heparin-coated tubes. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation on the day of blood collection (Day 0) or after 1 and 2 days of storage. On each day of PBMC isolation, cells were labelled with CFSE. PBMC cultures were setup with either culture medium alone or with pokeweed mitogen (PWM). The following supplements were added to both in an attempt to restore cell function: IL-12 or IL-7 or both IL-12 and IL-7.

One set was left free of supplements. Each condition was carried out in triplicate. Cells were assessed by flow cytometry after 5 days of culture.

New antigens were tested in the lymphocyte proliferation assay, as well as in the IFNγ release assay and in enzyme-linked immunosorbent assays (ELISA). The discovery of antigenic targets that have the potential for use in diagnostic tests and vaccine development has been an ongoing aim of the MLA Johne's disease (JD) research program at the University of Sydney. We have engaged with international collaborators who have identified Mptb candidate antigens to avoid duplication of these research efforts. Novel approaches have been applied for the identification of potential candidate antigens of Mptb. Table 8 summarises the work conducted in this area. Antigens related to Mptb stress proteins were developed at the University of Sydney by PhD students aligned with this and prior MLA projects (OJD.031, P.PSH.0297, P.PSH.0311).

Table 8. Summary of studies conducted at the University of Sydney on novel Mptb antigens identified and/or assessed for immunogenicity in lymphocyte proliferation, serum antibody and/or IFNγ assays.

Lead investigator/ Collaborator	Antigen identified/cloned	Immunogenicity assessed	References
Dr Sanjeev Gumber Dr Satoko Kawaji	Mptb stress-regulated proteins	Yes	(Gumber et al., 2009a, Gumber et al., 2009b, Gumber and Whittington, 2009, Kawaji et al., 2012a, Kawaji et al., 2012b, Kawaji et al., 2010) S. Kawaji PhD thesis 2009 (Chapter 5).
Dr Ratna Gurung	*In silico* identified B and T cell epitopes of Mptb	Yes	(Gurung et al., 2012a, Gurung et al., 2012b)
Dr Ratna Gurung	Recombinant proteins[c] and synthetic peptides[c] of Mptb stress regulated proteins	Yes	(Gurung et al., 2013, Gurung et al., 2014c)
Dr Ratna Gurung and Dr Doug Begg/ Dr John Bannantine (USDA)	ORF[a] of Mptb/stress-regulated proteins	Yes	(Gurung et al., 2014b, Gurung et al., 2013)
Dr Shyamala Thirunnavukkarasu / A.Prof Torsten Eckstein (Colorado State University)	Lipid antigen specific for Mptb	Yes	(Thirunavukkarasu et al., 2013)
Dr Ratna Gurung and Dr Auriol Purdie	Mptb stressome meta-analysis of published studies	Pending experimental analysis[b]	
Dr Ratna Gurung and Dr Doug Begg/Dr Horachio Bach (University of British Columbia, Canada)	Ptpa antigen[d], an Mptb secreted protein critical for survival within macrophages	Yes[c]	(Gurung et al., 2014a)

a. ORF: Open reading frames
b. A comprehensive list of Mptb proteins was identified that may have diagnostic significance, pending experimental assessment.
c. Serum antibody reactivity was assessed with experimental work ongoing regarding IFNγ responses (Dr Doug Begg).
d. Protein tyrophosphatase A

Results

The lymphocyte proliferation assay is a marker for exposure to Mptb in sheep. An example of results from a longitudinal study are provided in Fig. 8. Sheep in the P.PSH.0311.1 trial were held for 2.5 years post Mptb exposure. This trial was conducted on sheep that were located immediately adjacent to our laboratory, and sample transit times were less than 4 hours between collection and processing.

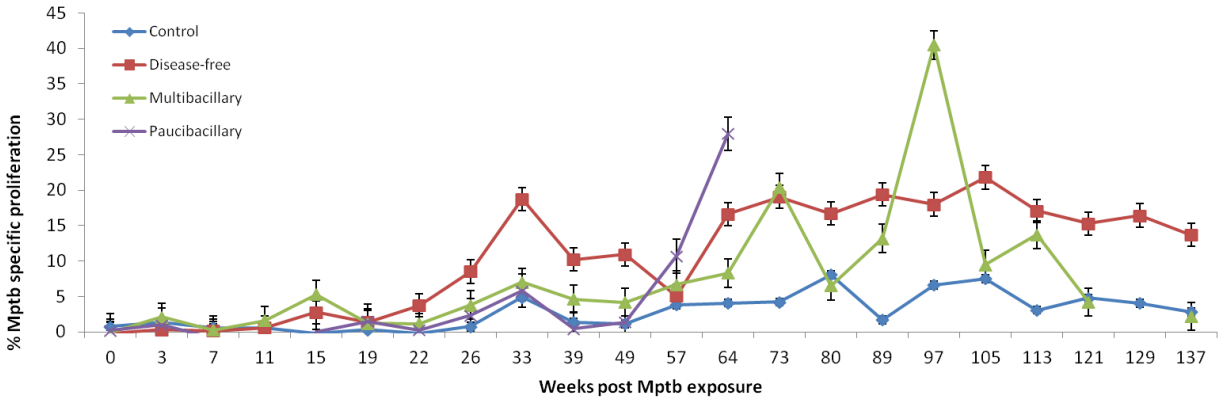


Fig. 8. P.PSH.0311.1 trial Mptb specific lymphocyte proliferation results based on final disease outcome. Data are mean +/- se. The disease free group were exposed to Mptb but not diseased at the end of the trial. Only one animal remained in the multibacillary category at greater than 97 weeks post Mptb exposure.

In practice, blood samples often have to be transported over long distances and times between farms and laboratories, and they can deteriorate as a result. Therefore methods to preserve or enhance blood sample viability were investigated. In the absence of supplement addition (No cytokines in Fig. 9) lymphocyte proliferation decreased as time after blood collection increased. The proliferative response could not be revived by the addition of supplements (cytokines IL-7 and IL-12) at the time of assay set up (Fig. 9). This result is very different from the successful outcomes observed in the IFNγ assay, which also requires live cells (see next section of this report). Further studies were undertaken to improve the proliferation assay when using stored blood. Blood tubes stored at room temperature were compared with tubes stored at $4^{\circ}C$. Reducing storage temperature or duration of the culture period did not restore the proliferative response. This was shown to be due to the deterioration of the white blood cells during storage.

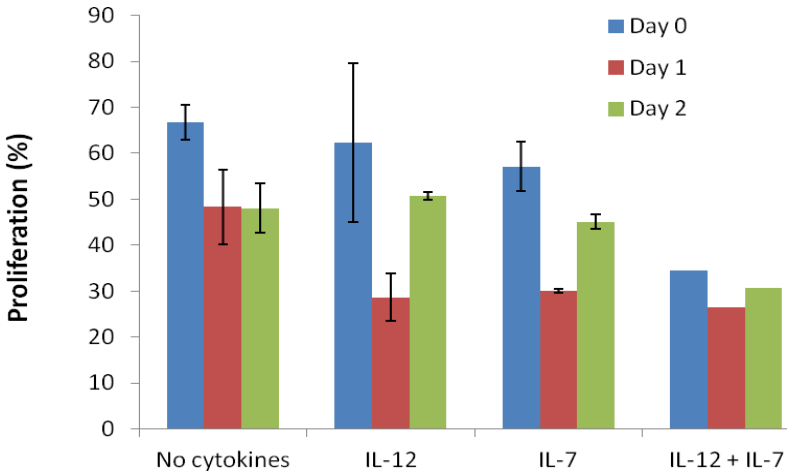


Fig. 9. Effect of cytokines on storage of blood tubes prior to set up of the Proliferation Assay. The assay was carried out using peripheral blood mononuclear cells from two sheep in the presence or absence of additional cytokines (IL-12 and/or IL-7). The assay was setup within 2 hours on the day of blood collection or blood tubes were stored at room temperature for up to 2 days prior to isolation of cells. Cells were cultured for 6 days. Mean ± standard deviation for pokeweed mitogen-stimulated proliferation – proliferation in medium alone is shown.

While use of novel antigens did not appear warranted in any practical field applications of the lymphocyte proliferation assay, they were applied in other assays in this project and so are reported here in proximity to Table 8 where they are summarised. Studies in previous projects undertaken by Dr Sanjeev Gumber and Dr Satoko Kawaji involved the identification and cloning of a range of Mptb proteins that were expressed under varying stress conditions, based on the hypothesis that stress and/or dormancy-associated pathways may be triggered during disease pathogenesis. Mptb is known to enter a dormant phase when exposed to certain conditions, such as the external environment on pasture or in soil. Stress conditions that are similar to those that trigger dormancy were applied in vitro and the 'stressome', a set of proteins differentially expressed under stress conditions, of Mptb was characterised (Gumber et al., 2009a, Gumber et al., 2009b, Gumber and Whittington, 2009, Kawaji et al., 2010). Dormancy associated genes and proteins identified *in vitro* to be regulated by Mptb under stress may be similarly regulated *in vivo* during infection and it was hypothesised that these may be good targets for immune assays. A few of the identified stress-regulated proteins were found to be immunogenic in a previous project, though none were found to be viable alternatives to the currently used antigens (Gumber et al., 2009b, Kawaji et al., 2012a, Kawaji et al., 2012b).

Dr Ratna Gurung extended this work in the current project as part of his PhD studies. An alternate approach was undertaken to the identification of potential candidate antigenic targets, targeting proteins that are likely to be recognised by B and T cells in the host immune system. This involved selection of 25 Mptb genes that had been reported to be upregulated in response to stress and assessment of these using a bioinformatic tool for the prediction of T and B cell epitopes and cloning of these proteins, prior to assessment of their immunogenicity (Gurung et al., 2012a, Gurung et al., 2012b).

A meta-analysis of the published literature relating to the Mptb stressome was conducted mining available data archives to identify potential future targets for research. An approach to the production of candidate antigens that was considered to be more cost-effective than cloning was assessed, examining the effectiveness of synthesised short fragments (peptides) of Mptb stress-regulated proteins that had been identified (Gurung et al., 2014c).

A collaboration with Dr John Bannantine (USDA), who had been involved in a major consortium in the USA (Johne's Disease Integrated Program) to clone all open reading frames of Mptb, was also established (Gurung et al., 2013, Gurung et al., 2014b). This allowed screening of additional antigenic candidates that had been cloned as part of this consortium, to avoid replication of effort. A similar collaboration was established with Dr Horatio Bach (University of British Columbia) in order to examine Mptb PtPa (protein tyrophosphatase A) antigen (Gurung et al., 2014a).

A collaboration with A.Prof Torsten Eckstein (Colorado State University) was established to examine the novel Mptb-specific lipopeptide antigen Para-LP-01. Many cell wall-associated lipid components of Mycobacterium avium complex have been found to be antigenic; most of these are glycopeptidolipids (GPL) containing sugar moieties and are highly immunogenic in nature however Mptb differs from other members of the *Mycobacterium avium* complex in that it lacks GPL and has a unique lipopentapeptide. The study assessed the ability of synthetic Para-LP-01 to invoke specific serum antibody and IFNγ responses in sheep exposed to Mptb with responses compared to those elicited by the crude whole-cell derived Mptb 316v antigen (Thirunayukkarasu et al., 2013).

Discussion

The lymphocyte proliferation assay is able to detect exposure to Mptb, but it is possible to undertake the assay only when freshly collected blood can be used. Antigen-stimulated proliferation decreases when blood has been stored. Further studies assessing the lymphocyte proliferation assay under field conditions were not undertaken as the developmental work concluded that it was not suitable for use on blood samples that had been stored or transported between farm and laboratory within the usual timeframes. However, the lymphocyte proliferation assay remains an effective research tool. .

All of the novel antigens described in Table 8 remain research tools, none so far showing sufficient promise on their own, due to lack of sensitivity as sole antigens in immunological tests. Some will be mentioned in other sections of this report.

4.8 Subprogram 1.5. Implementing and validating the IFNγ release assay (IFNγ^{PLUS})

Introduction

Many studies have shown that IFNγ is a key cytokine involved in protective immune responses and is essential to enable animals and humans to survive mycobacterial infections. The Mptb antigen specific IFNγ response has been used throughout this project as a research tool, as a key immune parameter in the experimental infection model and in prototype vaccine trials.

The specific IFNγ response offers the potential to detect Mptb exposed aninmals. This may provide an opportunity for control strategies aimed at removal of young exposed animals, or for tracing purposes.

A protocol to potentiate IFNγ cellular responses in cultures of sheep blood was developed and optimised in the previous projects (P.PSH.0311 and P.PSH.0297). This involves supplementation of blood samples with the cytokines (interleukins IL) IL-12 and IL-7 at the time the culture is set-up in the laboratory. This protocol, termed the IFNγ^{Plus} assay, had sensitivity comparable to the standard assay set-up within 8 hours of blood collection from naturally infected sheep. The advantage of this assay in comparison with the standard IFNγ assay that needs to be performed within 8 hours of blood collection is that it can be set-up after 2 days, allowing a more realistic time frame for shipment of blood samples from the farm to the laboratory.

In the current project, this assay was analysed further. The IFNγ assay platform was confirmed as a test for exposure to Mptb in both cattle and sheep, rather than a test for active infection. A field trial was conducted to validate the transport protocol from farm to laboratory. The sensitivity and specificity of the test was assessed. The ultimate aim of the research was to test the applicability of this assay in its current format and enable immediate implementation. Further enhancements may be achieved if a promising new Mptb antigen were to be identified in the antigen discovery subprogram of this project or by other researchers in the field. The antigen ESAT-6, which is not relevant to JD, has been included in human TB applications of interferon-gamma tests. No antigen like ESAT-6 has been discovered so far in Mptb despite global research efforts.

Methods

To study IFNγ^{PLUS} responses in infected or vaccinated sheep, blood samples were collected from: 30 ewes that had been vaccinated previously with Gudair® vaccine and had a history of Johne's disease from an OJD affected farm near Bathurst, NSW, Australia (trial P.PSH.0311.A1), and; experimentally challenged sheep and cattle were from trials outlined in Table 1. To assess the specificity of the IFNγ Plus assay, a sheep property near Walcha in the New England region of NSW with a history of being JD-free (MN3) was selected (P.PSH.0576.A); blood and faecal samples from 150 Merino sheep were collected for testing; the disease-free status was supported by 100% negative serum antibody ELISA and HT-J faecal PCR results.

Blood was collected into lithium heparin vacuum blood tubes. The standard IFNγ stimulation protocol was performed on the day of blood collection. Replicate blood tubes were collected and stored unopened at room temperature in the dark for 2 days prior to set-up of the culture for the IFNγ^{Plus} assay.

For the standard IFNγ stimulation assay, 0.5 mL blood was placed into wells of a 48 well plate and stimulated with 0.5 mL of Mptb 316v antigen at 10 µg/mL in culture media (10% foetal calf serum made up in RPMI 1640 supplemented with L-glutamine and penicillin/streptomycin). The unstimulated control consisted of blood with 0.5 mL of culture medium alone. The positive control had 0.5 mL of media with pokeweed mitogen (PWM) added (5 µg/mL). After 48 hours of culture at 37°C in air supplemented with 5% CO2, the plasma supernatant was collected and stored at -20°C .

For the IFNγ^{Plus} assay, stimulation cultures were set-up after storage of blood samples for 2 days. The antigens and concentrations (Mptb 316v, PWM and medium alone control) were as described above. These cultures were performed with the addition of recombinant human (rh) IL-12 p70, rhIL7 or a combination of these cytokines to potentiate the cells. The cytokines (IL-12, IL-7) were added at the same time that stimulation cultures were established. Plasma supernatants from these stimulated cultures were harvested after 48 hours and stored as per the standard assay.

For the standard and IFNγ^{Plus} assays, ELISA plates were coated with 50 µL of 1.5 µg/mL mouse monoclonal anti- IFNγ antibody (IFN 6.19, generous gift of Dr G. Jungerson) and incubated overnight at 4°C. Frozen plasma supernatant samples were thawed and brought to room temperature. The plates were machine washed 5 times (Tecan, Austria) using wash buffer (PBS with 0.05% Tween 20) prior to adding 50 µL of PBS to appropriate wells followed by 50 µL of the plasma supernatant sample. The plates were incubated at room temperature for 1 hour and then washed 5 times using wash buffer. The secondary antibody (mouse monoclonal anti- IFNγ antibody biotin-conjugated) (50 µL) was added at a concentration of 0.5 µg/mL diluted in PBS. The plates were incubated at room temperature for 1 hour, then washed 5 times using wash buffer and 50 µL of horseradish peroxidase streptavidin was added (0.01 µg/mL in PBS). The plates were incubated at room temperature for 1 hour and machine washed 5 times using wash buffer. TMB (3,3´,5,5´-tetramethylbenzidine) substrate (100 µL) was added to each well as required and the plate was incubated in the dark for 30 min. The reaction was then stopped with 100 µL of 2 M sulphuric acid. The optical density (OD) was measured in an ELISA reader at 450 nm. Results were expressed as signal of the test sample as a proportion of the positive control, corrected for the negative control (S/P) according to the formula: S/P = 100 × (OD450 value of the sample - OD450 value of the negative control)/(OD450 value of the positive control - OD450 value of the negative control). Specific S/P was determined by subtracting the appropriate media control S/P from the Mptb antigen or PWM S/P result.

Results

Comparison of the IFNγ^{Plus} assay with the standard assay. Analysis of data from the IFNγ^{Plus} assay, including IL-12 and IL-7, was conducted with the assistance of Dr Navneet Dhand. For the on-farm trial (P.PSH.0311.A1), conducted on 30 vaccinated sheep, blood was collected and then transported to Camden and stored at room temperature overnight. Addition of IL-12/IL-7 significantly enhanced Mptb-specific IFNγ responses ($P<0.001$) but had no effect on unstimulated controls which remained at background levels.

For the results derived from experimental infection trial P.PSH.0311.1, REML statistical analysis was performed. The mean responses to Mptb antigen of all experimentally inoculated sheep throughout the trial are shown in Fig. 10. This showed a trend in the peak of specific IFNγ responses between 4 to 8 months post-inoculation using both the standard and IFNγ^{Plus} tests, however the responses in the IFNγ^{Plus} assay peaked earlier than those in the standard assay. At early time points (4-6 months), this was significant ($P<0.001$) with the inoculated sheep showing higher Mptb antigen specific IFNγ responses in the IFNγ^{Plus} assay compared to the standard assay. Addition of the combination of IL-12 and IL-7 led to significantly enhanced ($P<0.001$) responses compared to IL-12 alone at all time points ≥4 months post-inoculation (Fig. 10). At 8 and 12 months post-inoculation the standard assay had higher responses than the modified assay. The sudden decline in mean IFNγ responses

at late time points post inoculation (12-14 months) corresponded to the time that clinical disease became apparent and six animals were removed from the trial.

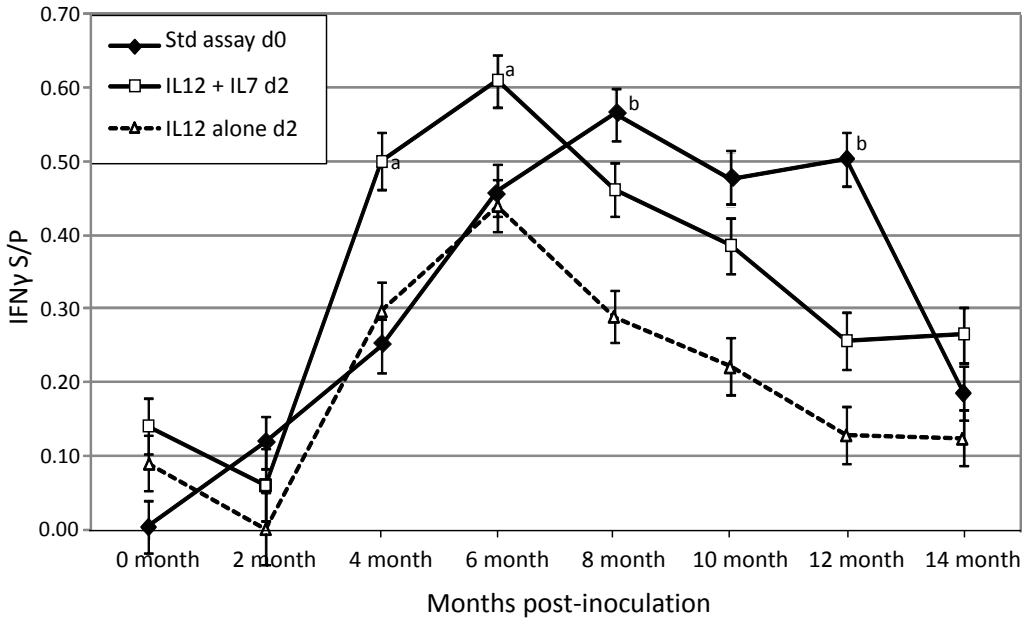


Fig. 10. IFNγ responses of experimentally inoculated sheep from Trial P.PSH.0311.1. Data are predicted mean ± SEM derived from REML statistical analysis. Significant differences: a, increased IFNγ responses ($p<0.001$) of inoculated sheep in the IL-12 + IL-7 modified assay at day 2 compared to the standard assay at day 0; b, increased IFNγ responses ($P<0.001$) of inoculated sheep in the standard assay (Day 0) compared to the modified assay at day 2.

Detection of sheep and cattle exposed to Mptb. The ability of the IFNγ test to differentiate between unexposed and Mptb exposed sheep and cattle was assessed under experimental conditions where the exact Mptb exposure history of each animal was known. When the results for sheep in trial P.PSH.0311.1 were stratified by infection status at the end of the trial and histological lesion grade, it became clear that the IFNγ immune response is a reflection of exposure to Mptb rather than active infection with Mptb, as sheep that had resisted or recovered from the exposure had elevated responses along with the affected sheep (Fig. 11).

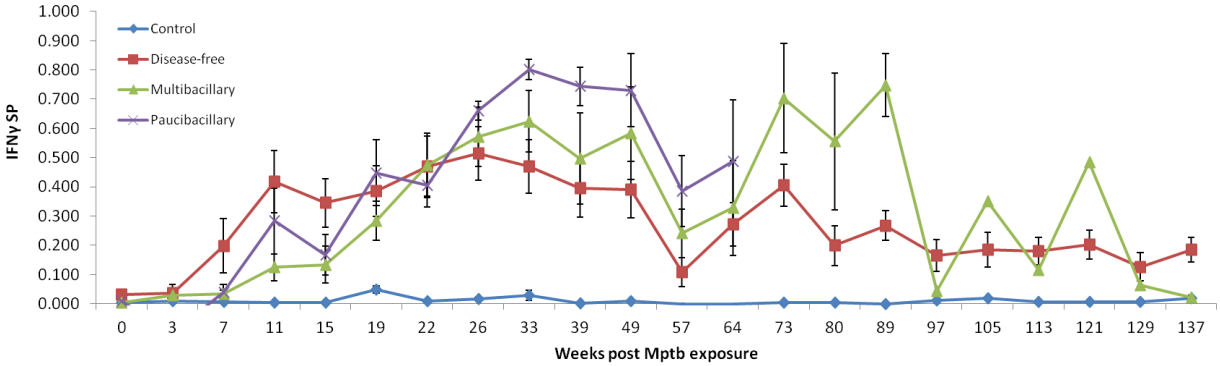
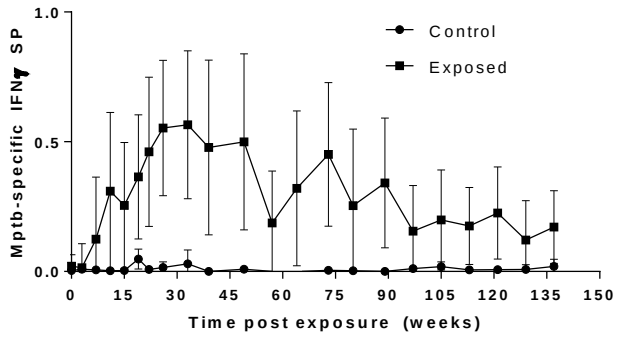


Fig. 11. P.PSH.0311.1 trial Mptb specific IFNγ immune responses based on final disease outcome. Data are mean +/- se. Only one animal remained in the multibacillary category from 97 weeks post Mptb exposure

There was a clear difference in the Mptb specific IFNγ response in both sheep and cattle exposed to Mptb as early as 4-5 months post exposure compared to the non-exposed controls (Fig. 12). In sheep, this was similar in all breeds studied. Statistical analyses confirmed that these were significant differences.

A.



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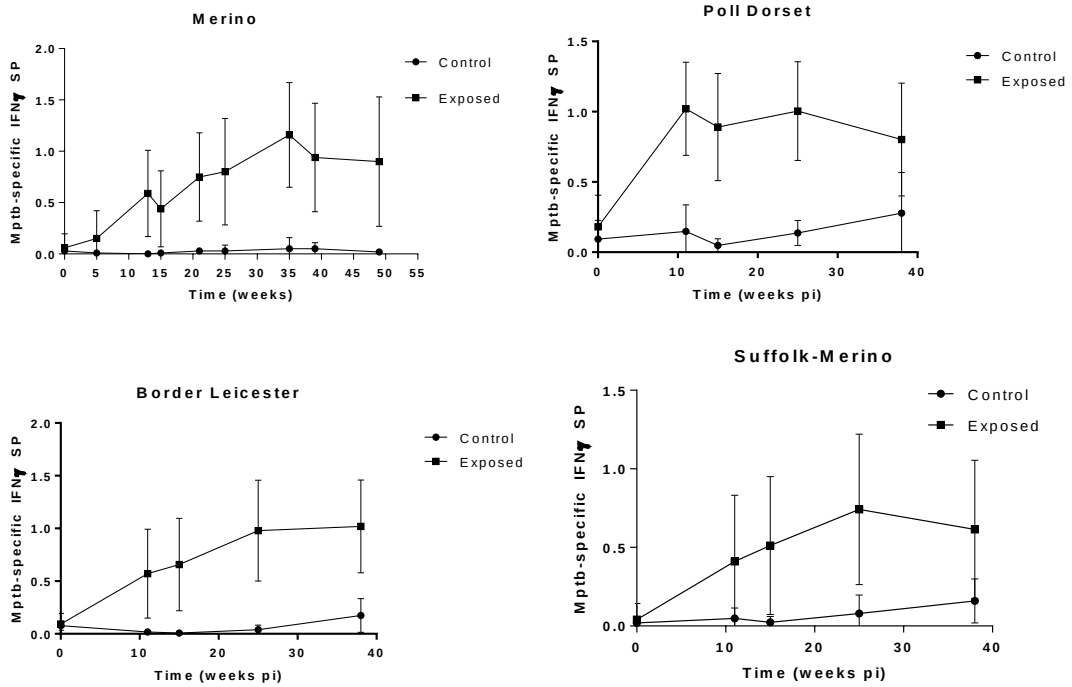

C.

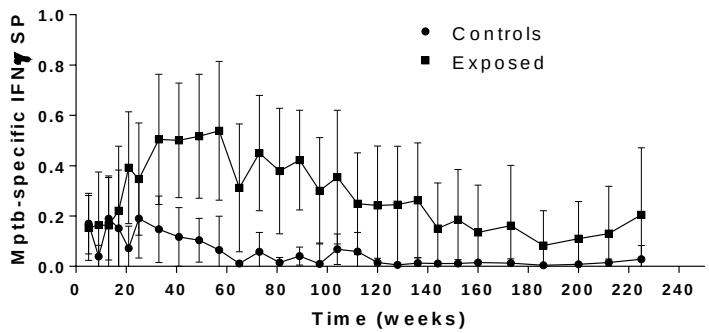

Fig. 12. IFNγ response in Merino and other sheep breeds in A. Trial P.PSH.0311.1 and B. Trial P.PSH.0576.1, and C. in cattle in trial P.PSH.0297.2.

For sheep, the IFNγ response at 16-22 weeks post Mptb exposure was analysed for all non-vaccinated, experimentally exposed sheep in trials P.PSH.0311.1, P.PSH.0576.1, P.PSH.0576.2 and P.PSH.0576.3 (n=39 controls and 93 Mptb exposed). Using a cut-point of 0.105 for Mptb-specific IFNγ SP, the test had 100% specificity and 89.5% sensitivity for detecting sheep that have been exposed to Mptb. Similar analysis for cattle from trials P.PSH.0297.2 and P.PSH.0576.4 at 15-21 weeks post Mptb exposure with a cut-point of 0.18 gave 71.4% specificity and 80% sensitivity.

It was important to obtain additional data from non-exposed sheep from another farm to estimate specificity of this test. Therefore samples were collected from sheep from an MN3 farm with a strong history of being JD-free (P.PSH.0576.A). The OD values for testing of these samples in the IFNγ^{Plus} assay are shown in Fig. 13. The blood samples were tested two-days after collection following the transportation and test protocols developed in the previous project (P.PSH.0311). The results show firstly that the transportation protocol for blood was successful as the majority of samples were strongly positive for the PWM positive control; two samples were outliers due to low OD values and these were excluded from further analysis.

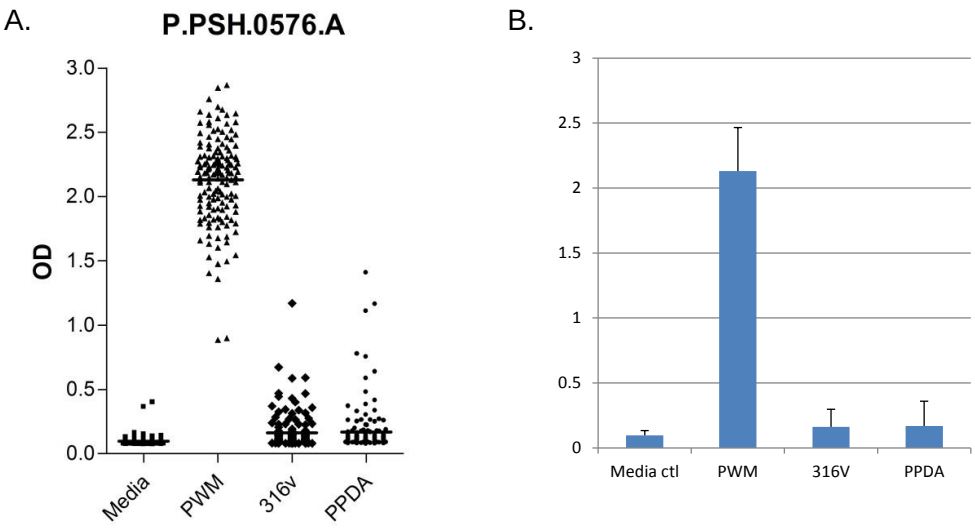

Fig. 13. OD values derived from IFNγ^{Plus} assay of sheep in trial P.PSH.0576.A. All samples were tested 2 days after collection. A. Responses of all individual animals. B. Average responses (mean ± SD).

The majority of the 148 samples had low OD values (<0.4) to the Mptb 316v antigen. Nine of the 148 samples gave an OD≥0.4 to this antigen. These samples tended to also have high responses to PPDA (purified protein derived from M. avium subsp avium) but responses to the media control were at background levels. This cross-reactivity to PPDA is a common finding for positives in Mptb-exposed sheep and may relate to cross-reactivity due to similarities between the two organisms. These results may relate to infection with M. avium or other unknown reasons.

The analysis of test sensitivity and specificity as a test for exposure of sheep to Mptb was conducted using data from the experimental infection trials and field trial sheep in P.PSH.0576.A in EpiTools. Fig. 14 shows a summary of the test data. As expected, there were outliers in the unexposed population, shown on the box plot. The cut-point results for various target test sensitivity and specificity levels are shown in Table 9. Sensitivity and specificity of 81% were obtained at the optimum cut-off value. The ROC (receiver operating characteristic) curve analysis is shown in Fig. 15. The area under the curve was 0.906 (95% confidence interval 0.87-0.943).

Fig. 14. Box plot of IFNγ^{Plus} results in sheep by exposure status.

Table 9. Cut-point results for target sensitivity in sheep

Target Se	Cut-point	Sensitivity	Se Lower 95% CL	Se Upper 95% CL	Specificity	Sp Lower 95% CL	Sp Upper 95% CL
0.999	0.008	1	0.96	1	0.133	0.092	0.189
0.995	0.008	1	0.96	1	0.133	0.092	0.189
0.99	0.008	1	0.96	1	0.133	0.092	0.189
0.98	[illegible]	[illegible]	[illegible]42	0.998	0.404	0.337	0.476
0.95	[illegible]	[illegible]	[illegible]395	0.983	0.644	0.573	0.709
0.9	0.083	0.903	0.826	0.948	0.739	0.672	0.797
0.8	0.12	0.806	0.715	0.874	0.809	0.746	0.858

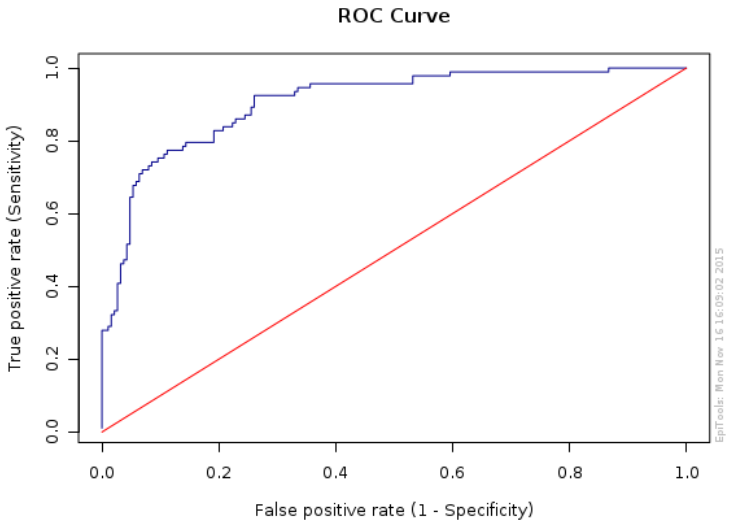

Fig. 15. ROC curve of sensitivity versus specificity for IFNγ^{Plus} results from sheep

Discussion

The IFNγ assay for Johne's disease is an excellent predictive tool in the research setting. It appears to offer great advantage in the detection of early stages of paratuberculosis infection and at early time points post-exposure in sheep. However, its real use will lie in detection of exposure to Mptb, because sheep that have resisted infection or recovered still have responses in the test.

These results will need to be further validated using field samples from a range of Mptb exposed and non-exposed farms. The applicability of this assay lies in the ability to identify farms which may have stock that are infected with Johne's disease, when exposure history is unknown, and prior to detection of faecal shedding. The IFNγ^{PLUS} assay enables a blood sample to be collected and transported to a laboratory within a realistic time-frame for this purpose.

As yet, insufficient samples have been evaluated from cattle.

Further studies on Mptb-specific antigens for stimulation of immune cells to improve specificity may be beneficial and there have been significant efforts to examine this (see Table 8). When a promising candidate antigen(s) is identified this can be trialed in the IFNγ^{Plus} assay. International research efforts are ongoing to identify a sensitive, Mptb-specific alternative antigen for use in IFNγ response assays for Johne's disease. Collaborative approaches that have maximised the available resources without duplicating effort need to continue.

4.9 Subprogram 1.6. Part 1. Evaluating antibody isotype assays - faeces

Introduction

Detection of the host's immune response to Mptb is most frequently performed via antibody detection ELISA carried out on serum or milk samples; these assays are high throughput and relatively low cost. However, the sensitivity of Mptb-specific ELISAs are generally well below 50%. Most of the commercially available Mptb ELISA tests including the IDEXX Pourquier serum antibody ELISA detect the IgG isotype of antibody. Studies have been done to examine other antibody isotypes such as IgG1, IgM and IgA in various ruminant species; the findings indicate that Mptb-specific serum antibodies are of the IgG isotype, specifically IgG1. Recently, studies on tuberculosis in humans have suggested that using a combination of antigens to detect multiple antibody isotypes may improve the predictive diagnostic outcome.

IgA is thought to play an important role in the immune protection of mucosal surfaces and is the predominant isotype in the intestinal mucosa. In ruminants IgG is transferred into the intestinal mucosa, but is thought to be degraded by proteases which may limit its protective efficacy. Interestingly antibody isotypes IgG, IgA and IgM specific to viral infections can be recovered from the faeces of cattle. These findings indicate that a faecal sample from an Mptb infected animal might contain immunoglobulins specific for Mptb. There are no published studies in which faeces from Mptb-infected animals have been examined for antibodies that may be specific to the infection.

Examination of faecal IgA and IgG responses may provide information regarding JD pathogenesis, in particular mucosal immunity, throughout the course of the disease. Additionally, the diagnostic potential of detecting these antibody isotypes should be determined. This longitudinal study examined Mptb-antigen specific IgG and IgA responses in faecal samples collected from sheep experimentally exposed to Mptb compared to unexposed control animals. Faecal samples were also cultured to detect the level of Mptb shedding, and matching serum samples were tested for antigen-specific IgG.

Methods

Experimentally infected Merino lambs in trial P.PSH.0311.1 were used in this study (Table 1). Faecal and blood sample collection was performed prior to inoculation with Mptb then repeated every 1–3 months post inoculation until necropsy to monitor the progress of the infection, and the controls were also sampled. Faecal samples were stored at -80°C until required. Serum from the blood samples was stored at -20°C until required. Animals culled for weight loss were confirmed to have clinical JD by detection of gross and histopathological lesions consistent with the disease and were shown to be infected with Mptb as determined by culture of intestinal tissues. Twelve gut tissues, including ileum, jejunum and associated lymph nodes were collected from each sheep for analysis. Faecal samples were cultured and IDEXX serum antibody ELISA was conducted.

For ELISA on faeces, samples were removed from the -80°C freezer and thawed at room temperature. Using aseptic technique, 0.5 g (+/- 0.05 g) of the faecal sample was removed and placed into a 5 mL tube. A 1/10 dilution of the faeces was made by adding 4.5 mL of PBS to each half gram of faeces. The samples were then mixed vigorously on a vortex mixer, incubated at 4°C overnight and mixed again. A sterile wooden stick was used to

break up any clumps in the sample and the sample was mixed vigorously. Using a transfer pipette, 600 µL was then placed in a 1.5 mL tube. The samples then were either frozen at -20°C until required or processed immediately.

ELISA plates were coated with 50 µL per well of 2.5 µg/mL Mptb 316v French pressed antigen diluted in carbonate buffer (0.1 M, pH 9.6) and stored at 4°C overnight. The ELISA plates were then machine washed 5 times using wash buffer (reverse osmosis water with 0.05% v/v Tween 20). The 1/10 diluted faecal samples were thawed if required and centrifuged at 3000 x g for 5 minutes. An aliquot of the supernatant (10 µL) was removed and diluted into 790 µL of PBS from which duplicate 50 µL aliquots were added to wells of the ELISA plate as required. The plates were then incubated at 37°C for 1 hour. The plates were washed and the conjugated antibody, either anti-ovine IgG or anti-ovine IgA was added at 50 µL per well and the reaction was developed using standard methods. Data for the longitudinal animal trial were standardised by calculating the S/P% (as for the serum ELISA) using known positive and negative controls for IgG or IgA on all plates. The controls were sourced from a Mptb exposed sheep with high levels of IgG and IgA specific to Mptb in its faeces and from an unexposed animal. Aliquots of these faeces were stored and used on every test plate.

Results

Eight of the 20 Mptb-inoculated sheep developed clinical disease, confirmed by gross and microscopic lesions associated with JD and positive culture for Mptb from intestinal tissues. This group is referred to as "clinical cases". One animal (No 29) at necropsy 30 months post inoculation, while not having clinical disease, did have gross and microscopic lesions consistent with JD and Mptb was cultured from its tissues and faeces; this animal with subclinical disease was removed from the analysis of the surviving animals. The remaining 11 "survivors" were all negative by tissue culture and did not have gross pathology consistent with JD. One of these 11 animals (No 27) had a grade 2 microscopic lesion in an anterior to middle mesenteric lymph node, although all the other 11 gut associated tissues sampled in this sheep had no significant histopathological lesions associated with JD.

Mptb-specific serum antibody levels in the inoculated sheep increased gradually throughout the trial until 12 months post inoculation. The faecal IgG antibody levels specific to Mptb were similar between the un-inoculated controls, the clinical cases and the survivors until the sampling point at 16 months post inoculation. At this time the survivors had a significant ($P<0.001$) spike in the faecal IgG level which remained high at 17 months post inoculation. However, by 19 months post inoculation the response had returned to baseline levels, similar to the control animals. The Mptb-specific faecal IgA antibody levels showed a similar pattern to the Mptb-specific faecal IgG responses. A significant ($P<0.001$) peak was seen at the 16 month post inoculation time point and by 19 months, the response was similar to the level observed from the faecal samples of the unexposed controls.

Discussion

The results from this study show for the first time that faecal samples from ruminants in an Mptb exposed flock contain Mptb-specific IgG and IgA, although it is only measurable by ELISA at certain times. The Mptb inoculated sheep with increased amounts of faecal

antibodies at 16 months post inoculation were not clinical cases, shedding no detectable or low levels of Mptb in their faeces and did not develop clinical disease in the next 3-4 months.

Faecal antibodies were detected in Mptb inoculated animals soon after removal of sheep that had been shedding high numbers of Mptb onto pasture and had to be culled due to clinical disease. The faecal antibody response dropped to background levels several months after removal of these clinical cases. The detection of mucosal antibodies in these sheep may be indicative of a protective response triggered by environmental exposure, that had the effect of sequestering Mptb passing through the gut to prevent or limit the infection. This theory is supported by an experiment conducted to test the Mptb antigen binding capacity of faecal antibodies. The addition of exogenous Mptb to a faecal sample containing a high level of IgG and IgA antibodies resulted in a reduction in the level of free antibodies in a dose responsive manner (data not shown).

The results led to speculation: i) that Mptb ingestion leads to stimulation of mucosal IgG and IgA, ii) the more Mptb ingested the more that is adsorbed by the mucosal Ig and that iii) the amount of faecal Igs detected by ELISA is determined by the level of stimulation and the level of adsorption. Ways to examine this hypothesis in more detail could involve using experimentally or naturally Mptb exposed animals that are not shedding Mptb in faeces, and remove them from further Mptb exposure. Later the animals could be exposed to different sized oral boluses of viable or non-viable Mptb. This would determine if antigen or viable Mptb exposure is required to stimulate the mucosal Ig response, and whether a measurable Ig response is abolished by adsorption and further our understanding of immune protection against Mptb infection. The amount of faecal Igs detected by ELISA maybe determined by the level of stimulation and the level of adsorption by recently ingested Mptb, suggesting that mucosal immunity may play a role in protecting against re-infection.

The findings show that sheep exposed to Mptb have a mucosal immune response, detectable as Ig in the faeces. The assays developed to detect faecal IgG and IgA are not of use in the diagnosis of JD as the Mptb-specific faecal antibodies were only detected under certain conditions.

4.10 Subprogram 1.6. Part 2. Evaluating antibody isotype assays - saliva

Introduction

Serum antibody ELISAs are commonly applied for the diagnosis of Johne's disease, but the sensitivity of these tests is low. It is known that other secretions from animals also contain specific antibodies that are protective against local viral and bacterial infections; one of these secretions is saliva. Immunoglobulin A is the most abundant isotype found in mucosal secretions and at mucosal surfaces. Mucosal IgA has been shown to play a role in protection against respiratory mycobacterial infections.

This study examined Mptb-antigen specific IgA and IgG responses in saliva samples collected from sheep experimentally inoculated with Mptb, compared to unexposed control animals. It was similar in its design to the faecal antibody study reported above.

Methods

Saliva samples were collected from a selection of 35 sheep in the P.PSH.0576.2 trial at the time of necropsy. Care was taken to ensure clean saliva samples were taken without contamination.

The IgA and IgG ELISAs were performed as described above, with minor modifications.

Results and discussion

Optimisation of the ELISAs was first performed to ensure the IgG and IgA assays were able to identify differences between Mptb inoculated and unexposed sheep. Dilutions of the saliva were prepared from a small cohort of the Mptb exposed and unexposed animals.

The Mptb exposed animals did not show an increased specific IgG response compared to the unexposed animals with unexposed animals having a greater response at lower dilutions (1/5 to 1/80) than the exposed animals, suggesting high non-specific responses. As a result of these findings, it was decided to cease examination of the salivary IgG responses.

However, in the IgA ELISA run on the same cohort of Mptb exposed and unexposed animals, two of the inoculated animals showed a response. Both of these animals were Mptb tissue culture positive animals. Further testing was conducted on saliva collected from a larger cohort of sheep. A set of 27 Mptb exposed animals was chosen, of which 12 had active infection as determined by tissue culture and histology. However, only one animal had a response in the assay which was stronger than the unexposed controls (data not shown). This animal was also found to be infected with Mptb at necropsy. The remaining 11 Mptb exposed animals of the 27 that had active infection were not detected. Thus there was a low rate of positive results with this assay.

Due to high non-specific responses for IgG detection in saliva and the low rate of identification of Mptb exposed animals for the IgA assay, no further work is warranted on these assays.

4.11 Subprogram 1.6. Part 3. Evaluating antibody isotype assays - serum

Introduction

Specificity issues in the commercial ELISAs for Johne's disease have been dealt with by pre absorbing the serum against *M.phlei*. In 2005 an IgG1 ELISA was described in New Zealand for deer, where antigens are analysed that represent Mptb specific and non-specific responses (Griffin et al., 2005). The aim of this study was to develop a similar IgG1 ELISA test for sheep and cattle using multiple antigens to increase the sensitivity of antibody detection for Mptb infected animals.

Methods

Two hundred and seven serum samples from sheep naturally or experimentally exposed to Mptb were used. Within the set there were animals of different histological lesion types, Mptb exposed or unexposed and tissue culture positive or negative. The data set also included

142 samples from experimental infection trials in which 4 repeated samples were collected from the same animals.

One hundred and ninety serum samples from cattle were used. The animals were categorised as: unexposed (sourced from multiple bleeds in the cattle trials P.PSH.0297.2 and P.PSH.0576.4), experimentally exposed (sourced from the 2 last bleeds from these trials), or naturally exposed animals (faecal culture data only as necropsies were not performed) collected in Tasmania.

Histopathology, culture of Mptb and a commercial serum ELISA were used to classify the disease outcome in all animals from which samples were available.

ELISA plates were coated with four antigens in individual wells, 50 µL per well of 5 µg/mL of Mptb 316v French pressed antigen, purified protein derivative from *Mycobacterium avium* (PPDA), heat-killed *M. phlei*, and protoplasmic antigen A (PPA). Serum samples were added to duplicate wells for each antigen. The plates were then incubated, washed and the conjugate anti IgG1 was added. The reaction was developed using standard methods. The serum controls were sourced from an Mptb exposed or unexposed sheep with high levels or reactivity as identified by the IDEXX ELISA.

Results and discussion

Unlike traditional ELISAs for the detection of Mptb infection, the version of the IgG1 assay developed in this study did not involve pre-absorbtion of the serum against *M.phlei*, but instead used this as an antigen in test. The use of the M.phlei and PPDA antigens in the assay gives an indication of non-specific reactivity. Two Mptb-specific antigens were used in an effort to increase the sensitivity of the assay. Responses of the test serum to the Mptb-specific antigens (316v or PPA) that are greater than those against the non -specific antigens should indicate an infected animal.

The IgG1 assay detected more exposed sheep than the IDEXX assay (Table 10), with 64 of the Mptb exposed sheep identified compared to 58. Neither assay detected an animal that was not exposed to Mptb. Of the Mptb exposed sheep the IgG1 and IDEXX/Porquier assays detected a similar number of tissue culture negative animals, 14 and 13 respectively. The IgG1 and IDEXX assays detected similar numbers of animals with different histological lesions. The IgG1 assay did detect 7 more of the 3a to 3b lesioned sheep. The IDEXX assay detected one more animal than the IgG1 assay for each of the histological lesion grades 1, 2 and 3c.

Table 10. Detection of Mptb exposed or unexposed sheep by the IgG1 and IDEXX assays

	IgG1 positive	IDEXX/Porquier Positive	Serum samples tested
Unexposed	0	0	72
Mptb exposed	64	58	135
Total	64	58	207

In cattle, three different positive:negative cut-points were applied to the data to explore the effect on sensitivity. Due to the mild form of BJD in the Trial P.PSH.0297.2 and the short

post inoculation monitoring time in the P.PSH.0576.4 trial, which resulted in low antibody responses, these data have been separated from those of the naturally infected animals.

In the unexposed cattle, the only positive ELISA responses came from the IgG1 assay using the first cut-point. The other two cut-points selected did not identify any unexposed animals as ELISA positive. In the experimentally inoculated animals very few animals were identified as ELISA positive no matter which test was used; the IDEXX ELISA detected 3 serum samples as positive, IgG1 ELISA identified 4-6 samples depending on the cut-point.

For the naturally exposed cattle there was agreement between the IDEXX ELISA and the three cut-points used for the IgG1 assay, with all detecting 15 of the 18 faecal culture positive animals (Table 11). The IgG1 assay detected 14 to15 of the IDEXX positive serum samples irrespective of the cut-point criteria.

Table 11. Detection of Mptb naturally exposed cattle by the IgG1 assay and the IDEXX assay

Naturally exposed cattle	Total Number of samples	Total Faecal culture positive	Total IDEXX ELISA positive	IgG1 ELISA cutpoint 1	IgG1 ELISA cutpoint 2	IgG1 ELISA cutpoint 3
All samples	40	18	17	17	17	16
Faecal culture positive	18	18	15	15	15	15
IDEXX ELISA positive	17	15	17	14	15	15

The cut-off used in the assay was arbitrary, set by visual examination of the data, and might be improved. This may mean that not all of the antigens are required to be used which will reduce the cost of the assay. As other antigens are discovered, either specific to infection or as better detectors of non-specific responses, they may be included in the assay. These could improve the sensitivity and specificity of the assay.

Conclusions

The IgG1 assays for sheep and cattle appeared to be as good as the IDEXX Porquier ELISA on these sets of serum, despite use of crude antigens. There is room for improvement in both assays as statistically relevant cut-points need to be made when a larger data set is available. Antigens can be added or removed as required to improve the sensitivity of the final assay giving flexibility not currently observed in the commercial assays.

4.12 Subprogram 1.7. Developing an immune signature of disease resistance and vaccine efficacy using multiplex cytokine assays

Introduction

A multiplex cytokine assay enables the detection of several cytokines simultaneously in a single sample. The principle of this method is shown in Fig. 16. The intensity of the fluorescent signal detected by a flow cytometer is used to quantify the amount of cytokine in a sample. This multiplex assay was designed to enable testing of multiple, and an increased range of cytokines, in individual samples, allowing the development of a test for measuring a profile of cytokine production.

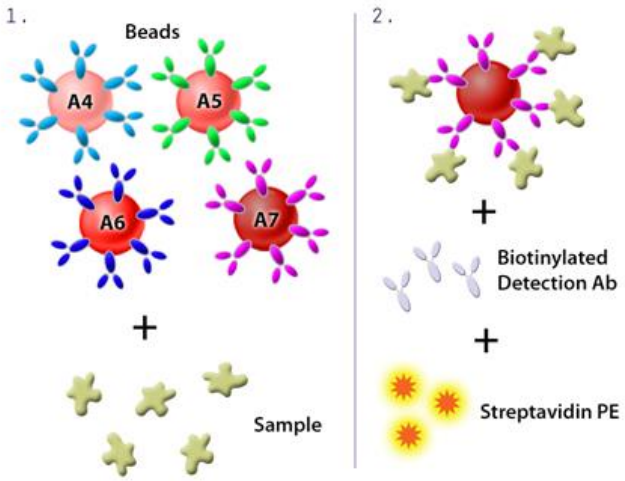

Fig. 16. Flow cytometric bead array technology (from http://www.biolegend.com/legendplex). Fluorescent beads are coated with antibodies that recognise the cytokine. Antibodies are shown as Y-shaped components. Sample is added to a mix of beads, each bead coated with antibodies to a specific cytokine. Biotinylated detection antibodies and fluorescent streptavidin PE are added and fluorescence data are collected by flow cytometry. The intensity of the fluorescent signal detected is associated with the quantity of cytokine present in a sample.

Method

Antibody pairs against cytokines of interest, IFNγ (IFNγ), tumour necrosis factor alpha (TNFα), transforming growth factor beta (TGFβ), and the interleukins (IL) IL-10, IL-2, IL-4 and IL-17A were selected based on their suitability for ELISA tests and cross-reaction with both bovine and ovine samples. For each of the pairs, one antibody ('capture') was coated onto fluorescent beads. Coated beads were assessed to ensure that the coating process had been successful. Biotinylated antibodies were used as the second 'detection' reagent to allow signal amplification and detection of molecules of low-medium abundance. The multiplex assay was carried out on filter plates at room temperature, protected from light exposure. Data were acquired in a Guava EasyCyte 8HT flow cytometer and analysed using InCyte software. Each bead population was selected based on the inherent fluorescence intensity for a particular set of beads. The median fluorescence intensity (MFI) for phycoerythrin (PE) for each bead population was calculated. For each cytokine, a standard curve using a recombinant cytokine was used to enable quantification of cytokine in a sample.

As the sheep and cattle trials described in Table 1 progressed, cell cultures were set up and supernatants were stored for subsequent testing. Briefly, blood was collected from the jugular vein of sheep into lithium heparin-coated tubes. Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation and cultures were set up with medium alone or with one of three antigens: Mptb, pokeweed mitogen (PWM) or concanavalin A (con A) and incubated at 37°C in 5% CO_2 for up to three days for initial tests. Replicate cultures were set up to enable harvesting of the entire culture supernatant daily. For experimental trials cultures were set up with medium alone, Mptb antigen or PWM.

Culture supernatants were stored at -20°C and cytokines were detected by the multiplex assay as described above.

Results and discussion

A multiplex cytokine assay was developed for the simultaneous detection of bovine or ovine IFNγ and interleukin 10. The inclusion of tumour necrosis factor alpha (TNFα), transforming growth factor beta (TGFβ) and interleukin IL-2, IL-4 and IL-17 was assessed but the detection of these cytokines in the multiplex system was not successful.

At the time the multiplex assay was being developed, an antibody that detects ovine IL-17 was not available; therefore the commercially available anti-bovine IL-17 reagents were evaluated for use with ovine blood samples. Initial tests indicated that the bovine reagents cross-reacted with samples from sheep and could be used to detect ovine IL-17 produced from antigen stimulated (pokeweed mitogen and Mptb antigen) peripheral blood or purified mononuclear cell culture supernatants. Therefore these reagents were included in the development of the multiplex cytokine assay.

The first step in the development of the multiplex cytokine assay was ensuring that the bead coating step had been successful. This was assessed by using fluorescently labelled molecules that bind specifically to the antibody coated onto the beads. The next step was to determine the limits of detection for each coated bead-detection antibody combination using a positive (supernatants from PBMC cultures with PWM) and negative (supernatants from PBMC cultures with medium alone) sample or recombinant cytokines. Recombinant cytokines were also detected by IL-17, IL-4, TNFα and TGFβ conjugated bead-detection antibody pairs. Further optimisation of these assays was required prior to assessment of trial samples to increase the detection signal (fluorescence intensity). A number of factors were varied including the incubation time, number of coated beads per sample, detection antibody concentration and steptavidin-PE concentration.

Detection of IL-2 was unsuccessful and despite investigation the reason for this remains unknown. This cytokine was removed from the multiplex cytokine assay.

The final step in the development of the multiplex cytokine assay was to test all coated beads in combination. For cytokines other than IFNγ and IL-10, recombinant proteins were only detected at high concentrations. To ensure that the low level of cytokine detection by the multiplexed assay was not due to technical issues the cytokines of interest were also assessed by ELISA. Detection by ELISA was successful, but similar to the multiplex assay, the limit of detection of each cytokine varied.

In preliminary tests, PBMCs from two sheep were tested, and IFNγ and IL-10 were the only cytokines detected in cell culture supernatants. The other cytokines included in the multiplex assay (IL-4, TNFα, TGFβ and IL-17) were not detectable in stimulated culture supernatants. As expected the level of cytokines stimulated by the presence of the general mitogens was greater than with the Mptb antigen.

IFNγ responses during infection were described in a previous section of this report. IL-10 was tested in infected sheep and cattle by ELISA. There was no significant difference in the Mptb-specific IL-10 response in Mptb exposed sheep or cattle compared to non-exposed controls (Fig. 17 and Fig. 18).

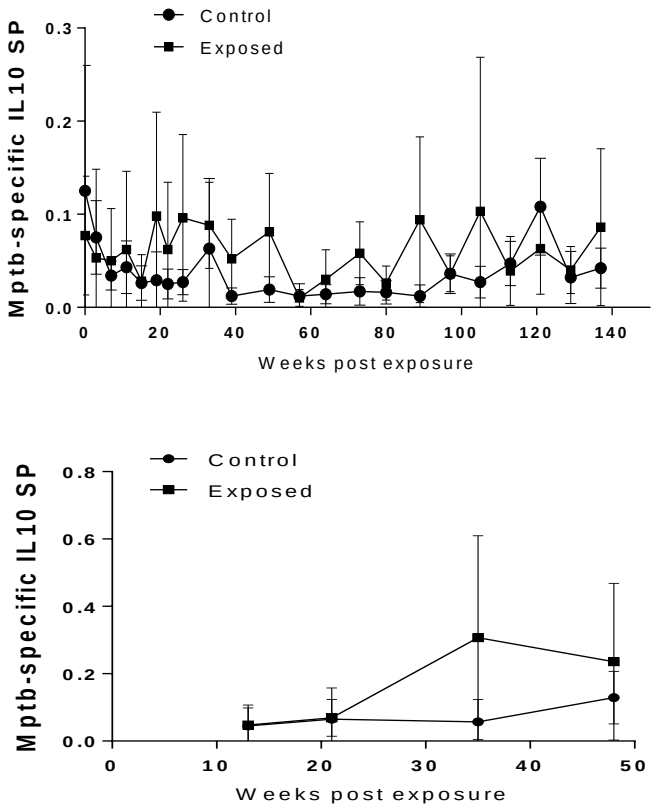

Fig. 17. The Mptb-specific IL-10 response in sheep measured by ELISA. Mptb-specific IL-10 was calculated by subtracting IL-10 in unstimulated cultures. The top panel shows mean+/- sd for trial P.PSH.0311.1 and the lower panel shows similar data for Merino sheep from trial P.PSH.0576.1.

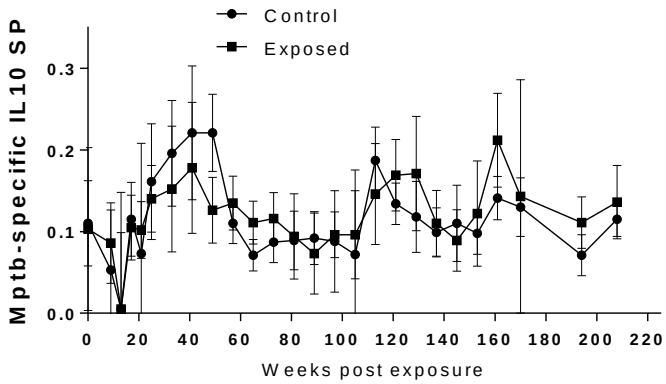

Fig. 18. The Mptb-specific IL-10 response in cattle measured by ELISA. Mptb-specific IL-10 was calculated by subtracting IL-10 in unstimulated cultures. Mean+/- sd for Trial P.PSH.0297.2.

Samples from Gudair® vaccinated controls (n=10) and vaccinated-Mptb exposed (n=31) sheep from two trials (P.PSH.0576.1 and P.PSH.0576.2) were tested by ELISA for the cytokines IL-4, TNFα and IL-17 at 4-5 months post exposure. IL-4 was not detected in any of the samples. TNFα was detected in one animal (vaccinated-Mptb exposed). An Mptb antigen-specific IL-17 response was detected in vaccinated Mptb-exposed sheep. Cytokine secretion from stimulated cells occurs in a time-dependent manner. Cytokines that are stored within a cell can be released soon after stimulation while others that require de novo synthesis take longer. Thus even though several time points were assessed these may not have been optimal for some of the cytokines included in the multiplex assay. While the Mptb antigen-specific IL-17 response tended to be higher in vaccinated Mptb-exposed sheep there was no significant difference between the two groups.

Conclusion

IFNγ and IL-10 are the most suitable cytokines for assessment using a multiplexed cytokine assay. IFNγ responses appear to be specific whereas IL-10 responses may not be. The IFNγ and IL-10 data will be analysed further and used to compare multi-cytokine profiles post-vaccination and in early disease and the results will be detailed in an Addendum to this final report. This may allow for characterization of resistant and susceptible animals and form part of the 'immune signature'. We will compare whether vaccinated animals have a similar multi-cytokine profile as the 'resistant' animals or animals with less severe disease.

4.13 Subprogram 1.8. Incorporating genomics data into the immune signature

Introduction

This project was unique in that microbiological, pathological and immunological data were collected from infected and vaccinated animals, as well as state of the art gene expression data, all in relation to an accurately determined final disease outcome. It was hoped that all of these data could be combined into a predictive 'immune signature' through multivariable statistical approaches. It was envisaged that such a signature could be used in a diagnostic setting, as well as to inform vaccine development.

Methods

Data from all time points for the P.PSH.0311.1 trial were used for analysis of an immune signature. This trial was chosen for this analysis as it is the longest known experimental infection trial in sheep; the animals were held for 2.5 years. This duration is most likely to accurately identify those animals that remain free of infection. Gene expression data were acquired as described in Section 4.14. Univariable binary logistic regression analyses were conducted to evaluate unconditional associations of gene expressions and immunological variables with the selected outcome variable (infected/non-infected). We tried to further evaluate associations of significant variables using multivariable models but the models did not converge due to a smaller sample size.

To generate an immune signature for Gudair® vaccine efficacy, data from the earliest time point (4-5 months post Mptb exposure) from the trials P.PSH.0576.1 and P.PSH.0576.2 were used. Other time points were not included in this vaccinate data set as the main purpose of this immune signature was to generate an early protective immune signature

which could be used when screening potential vaccine candidates in short-term trials. This was based on previous analysis which had demonstrated that lymphocyte dysfunction could be detected as early as 13 weeks post Mptb exposure in Gudair® vaccinated sheep that eventually become infected. Similar analyses to those for the infection trial were conducted for the vaccination dataset to identify variables predicting eventual infected/non-infected status of animals.

Disease outcome was classified as: infected or non-infected based on tissue culture result at necropsy (infected = positive culture result); infectious or non-infectious based on a positive faecal culture result (infectious = positive culture result at any time during the trial period); susceptible or resistant based on the criteria described in Section 4.1. For the P.PSH.0311.1 dataset these three disease outcome variables classified animals in the same manner. Therefore, only one outcome variable was used for the regression analysis. For the 'vaccine efficacy' dataset infected animals were also susceptible; however not all infected animals were infectious and some non-infected animals were infectious. The infectious/non-infectious disease outcome has not been analysed yet. These results will be included in an addendum to this final report.

Results

There were numerous significant associations from the univariate analysis of data from exposed non-vaccinated sheep in trial P.PSH.0311.1. The majority of the significant explanatory variables were gene expression markers. The many significant findings were collapsed into a short list by targeting the most significant associations from the univariate model, using only the earliest significant time point for genes that were identified to be significant at multiple time points, targeting genes that had been identified in the animal genomics subprogram as being associated with particular disease outcomes (multibacilliary, paucibacilliary disease), and collapsing explanatory variables that were found to be significantly negatively or positively correlated with another into the single most significant variable. This resulted in a short list of 24 genes. Unfortunately a multivariable model for the gene expression data could not be fitted due to lack of convergence, probably associated with the limited number of observations. For the traditional microbiological and immune parameters, faecal culture at 6 months post exposure and serum antibodies at 12 months post exposure were significant, however, these results had a limited number of observations.

Similarly, for the dataset from vaccinated sheep in trials P.PSH.0576.1 and P.PSH.0576.2 there were numerous significant associations from the univariate analysis. These were collapsed into a short list as described above. Then, in the multivariable model, IL-10 and IFNγ along with three genes were significant explanatory variables.

Discussion

The combination of immunological and other data with genomic data to identify variables associated with disease outcome in Mptb infection or vaccination is novel. The statistical models used in this research need to be checked further, and other data may be able to be added to increase sample sizes. Changes in outcome will be reported in an Addendum to this final report.

4.14 Subprogram 2. Animal genomics

This subprogram addressed the following project objective:

To develop new genetic approaches to predict accurately which herds/flocks are resistant, which particular animals are resistant and which infected animals will get sick, contaminate the environment or recover. As part of this objective, to determine the relative susceptibility to JD of different breeds, such as pure British breed and Merino sheep.

The operational plan agreed with MLA for this subprogram included research on the following aspects:

1. Gene expression profiling (microarray)
2. Breed susceptibility trial
3. Gene expression profiling across breeds
4. Gene expression profiling in vaccinated sheep
5. Candidate gene analysis by single nucleotide polymorphism

The immune system has evolved to cope with and to eradicate pathogens and an understanding of the mechanisms could identify correlates of protection leading to the formulation of more effective diagnostics and vaccines. This requires a Systems Immunogenetics approach as the response to infection results from complex interactions between host immunity and the pathogen. Focus on an individual immune component within the larger process may not provide the full story. Gene interaction networks derived from gene expression analysis may provide a framework for modeling the complex molecular interactions within cells, and these data when analysed in combination with measured immune outcomes such as IFNγ, IL-10, lymphocyte proliferation and serum antibody levels may identify key factors associated with the host response and inform targeted research on molecular targets for diagnosis, treatments or vaccine development.

Single nucleotide polymorphisms (SNP) are small changes in the genome sequence of an animal that are linked to genes. They can be used as markers to detect useful or deleterious genes.

4.15 Subprogram 2.1. Part 1. Gene expression profiling (microarray) in sheep

Introduction

Microarray chip technology is a tool for probing the activity of thousands of genes in a single experiment. It determines whether each gene is turned off or on at a particular time. A microarray chip works by exploiting the ability of messenger RNA to bind to the DNA template of the gene from which it originated. By comparing samples collected from control animals and animals with Johne's disease it is possible to determine how hard each gene is working, and which groups of genes are working together, and from that it is possible to infer how the animal is responding to the infection. This understanding could lead to approaches to predict the course of the disease for management purposes, to alter the eventual clinical

outcome through new treatments, and to develop vaccines by targeting specific parts of the immune system.

The aim of this study was to assess early gene expression changes in Mptb exposed Merino sheep from trial P.PSH.0311.1 to identify disease-associated gene markers and genes specific to animals with demonstrable resistance or susceptibility to Johne's disease.

Methods

Briefly, 20 Merino sheep aged between 2 to 4 months were inoculated orally with Mptb S strain to induce Johne's disease in trial P.PSH.0311.1. Blood samples were collected prior to inoculation and at monthly intervals thereafter (Table 12). Tests conducted on samples collected from live animals were faecal culture and HT-J faecal PCR. Necropsy was conducted later (Table 13) enabling tissue culture and histopathology tests to be used to accurately classify disease outcome as defined in the resources subprogram (see above).

White blood cells (WBC) were isolated from peripheral blood samples using ammonium chloride lysis and centrifugation. Total RNA was isolated from WBC samples and its quantity and integrity was verified by spectrophotometry and using an Agilent 2001 Bioanalyser analysis (acceptable RIN number 6.0-10). RNA samples were stored at -80ºC until required for processing. The Affymetrix GeneChip Bovine Genome Array, containing 23,000 gene transcripts and including approximately 19,000 UniGene clusters was used for the microarray analysis. Briefly; 1µg of total RNA from each sample was reverse transcribed and hybridised to a GeneChip. A total of 125 GeneChips were used for the study (Table 12).

Table 12. Animal sampling across the timepoints in trial P.PSH.0311.1. Data are the number of animals and gene chips used.

Group	Months post exposure to Mptb				
	2	4	6	9	12
Control	5	5	5	5	5
Mptb exposed	20	20	20	20	20

Affymetrix genechip operating software (GCOS) derived raw expression values were obtained as .CEL files and transferred to Partek Genomic Suite 6.6 software (Partek Inc). All samples demonstrated characteristics of high quality cRNA. The raw data were normalised using the RMA (Robust Multichip Averaging) algorithm. In addition, RMA normalisation applied PM distributions across all the chips and a robust probe-set summary of the log 2 normalised probe-level data by median polishing. Array data quality was confirmed by Principle Component Analysis (PCA). ANOVA with a nominal alpha value set to 0.05 was then used to determine those probe sets significantly different between disease classification associated variables in comparison to unexposed controls both within each timepoint and consistently expressed across all timepoints. Variables tested include Mptb exposed, Perez score defined outcome (multibacillary, paucibacillary and designated recovered i.e. Perez score of ≤2) consistently across all time points to the exclusion of the other variables, Mptb exposed with resistant/non-infected/non-infectious or susceptible/infected/infectious classification in comparison to Mptb unexposed controls. This was followed by a Benjamini and Hochberg Multiple testing correction to reduce the false positive rate. To ensure the

accuracy of the results, only differentially expressed genes with a false-discovery rate (FDR) less than or equal to 0.05 and a fold change (FC) ≥ 1.4 or ≤ -1.4 were further analysed.

Significant gene lists were prepared from each comparison group and identification of cellular functions was achieved utilising the Partek pathway analysis tool as well as online bioinformatics tools including the National Institute of Allergy And Infectious Disease Database for Annotation Visualisation and Integrated Discovery (DAVID) and Ingenuity® Pathway Analysis (IPA, QIAGEN). Within Partek pathway analysis, DAVID and IPA the significantly enriched functional categories were derived from Gene Ontology, KEGG and the Ingenuity® Knowledge Base databases (http://www.ingenuity.com/science/knowledge-base). A stringent cut-off value was applied to this analysis and the functional categories highlighted are all of statistical significance with a high fold enrichment score paired with a low P-value. Within the IPA framework the Partek software generated ANOVA files were analysed to identify statistically relevant genes of interest. These modified gene lists were subjected to core analysis for identification of upstream regulators, functional analysis to identify functional groups associated with genes defined by clinical outcome and finally, biomarker analysis to identify genes that may be of potential use as early biomarkers of disease outcome. The results of all these tests were cross-compared to ensure consistency. The Chilibot specialised search software (http://www.chilibot.net/) was utilised to mine PubMed literature database to identify relationships between genes and proteins.

Quantitative real time PCR (qRT-PCR) analysis was carried out on selected differentially regulated genes to verify the array findings and to assess genes of interest as biomarkers for use in predictive tests of disease outcome. A total of 30 samples were subjected to qRT-PCR analysis from each time point/Mptb exposure status combination including those samples in the array experiments. Initially 5 non-changing genes were selected as reference markers from each array dataset; this follows Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines. The reference primer suitability was assessed by geNorm. Twenty nine genes of interest were selected for validation of the array data. Forward and reverse primer pairs were designed for the gene regions of interest using Primer 3, ensuring no amplification of genomic DNA contaminants. Selected RNA samples (5µg) were DNase treated to remove genomic DNA, using 10µl RQ1 DNAse (Promega) and 1µl RNasin Plus RNase inhibitor (Promega), then reverse transcribed to cDNA using oligo(dt) primers and the AffinityScript qPCR cDNA synthesis kit (Stratagene, Agilent) according to the manufacturers' instructions. qRT-PCR was performed using an Mx3000P Real-time PCR system (Stratagene, Agilent) using the QuantiTect SYBR Green PCR kit (Qiagen). Assays were prepared in 96 well plates and included duplicates of each sample. Reaction volumes of 25µl (including 10µl of target cDNA at a 1/100 dilution) were prepared and amplified under the following conditions: 95°C for 15min, then 40 cycles of 95°C for 20s, 52-60°C for 30s and 72°C for 30s, with fluorescence acquisition at the end of each annealing step. The specificity of the reaction was confirmed using melting curve analysis and standard curves were performed on each plate for each primer set. Data collected from the qRT-PCR were analysed using qBASE+ analysis software (Biogazelle) utilising a modified Comparative Ct ($\Delta\Delta$Ct) method.

Results and discussion

Each GeneChip contained 23,000 probe sets. A total of 552,000 expression data points were generated from arrays. The PCA analysis showed that the samples within each group

were distinctly separated from each other, indicating that the quality of the microarray data was good. The PCA results also showed that the global expression patterns within each timepoint were similar and individually clustered. There was delineation between samples at the earliest timepoint (2 months post Mptb exposure) in comparison to the remaining timepoints (4, 6 9 and 12 months post Mptb exposure).

The disease outcome classifications of animals that were used for genomic analyses are shown in Table 13.

Table 13. Disease classification outcomes for each animal in Merino sheep trial P.PSH.0311.1 used for gene expression analysis with Affymetrix GeneChip Bovine Genome Array.

Key List	Treatment	Month of death post Mptb	Survival (months)	Event	Faecal Culture	Histopathology grade[a]	Disease severity (Perez defined)	Non-infected /infected[b]	Resistant /susceptible	Non-infectious /infectious
1	Unexposed	20	50	control	0	0	Control	Control	Control	Control
2	Unexposed	20	50	control	0	0	Control	Control	Control	Control
3	Unexposed	20	50	control	0	0	Control	Control	Control	Control
4	Unexposed	20	50	control	0	0	Control	Control	Control	Control
5	Unexposed	14	50	control	0	0	Control	Control	Control	Control
11	Mptb	20	50	lived	0	0	Recovered	non-infected	resistant	non-infectious
12	Mptb	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
13	Mptb	14	14	died	1	3b	Multibacillary	Infected	susceptible	infectious
14	Mptb	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
15	Mptb	14	14	died	1	3a-c	Paucibacillary	Infected	susceptible	infectious
16	Mptb	14	14	died	1	3a-3c	Paucibacillary	Infected	susceptible	infectious
17	Mptb	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
18	Mptb	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
19	Mptb	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
20	Mptb	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
21	Mptb	14	14	died	1	3a-c	Paucibacillary	Infected	susceptible	infectious
22	Mptb	14	14	died	1	3b	Multibacillary	Infected	susceptible	infectious
23	Mptb	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
24	Mptb	12	14	died	1	3b	Multibacillary	Infected	susceptible	infectious
25	Mptb	14	14	died	1	3b	Multibacillary	Infected	susceptible	infectious
26	Mptb	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
27	Mptb	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious
28	Mptb	17	17	died	1	3b	Multibacillary	Infected	susceptible	infectious
29	Mptb	20	20	died	1	3b	Multibacillary	Infected	susceptible	infectious
30	Mptb	20	50	lived	0	0.5	Recovered	non-infected	resistant	non-infectious

[a] Severity of disease defined by histopathological lesion score using established criteria (Perez et al., 1996) [b] Inoculated animals were classified as infected if any tissue at necropsy was culture positive for Mptb

Identification of genes associated with Mptb exposure. Some genes met the criteria for differential expression consistently across all five sampling times and were associated with evidence of Mptb infection in comparison to the uninfected control sheep. The greatest number of significant genes was associated with three different functions: lipid metabolism, cellular proliferation and cellular transport. Analysis revealed that exposure to Mptb drives the animals to an immune profile in which the overall expression of IFNγ is suppressed in comparison to unexposed control sheep.

Identification of genes associated with susceptibility (infected/infectious) or resistance (non-infected/non-infectious) to Johne's disease. Susceptibility to Johne's disease is defined by evidence of live mycobacteria in intestinal tissues i.e. positive tissue culture. Resistance in experimentally exposed animals is defined as animals with no positive histology or tissue culture. Ontological analysis identified 45 genes for susceptibility and 60 for resistance in a number of significantly enriched functional groups. Both the susceptible and resistant cohorts had genes associated with cellular movement and migration however, deeper mining of the data revealed that there were significant differences in the downstream effects of the genes associated with the two cohorts.

Biomarkers of susceptibility (infected/infectious) or resistance (non-infected/ non-infectious) to Johne's disease. Comparisons of gene lists, data mining to identify genes with altered expression unique to each of the pathology classifications and analysis of the mechanisms of actions associated with each gene resulted in the selection of a subset of 21 genes that are suggested as potential biomarkers for diagnostic purposes.

Identification of genes associated to Johne's disease severity as defined by histopathology. The histopathology score was developed to differentiate the severity of disease and it is a useful tool in the identification of highly infectious sheep and cattle. Genes were consistently differentially regulated across all five sampling times associated with severity i.e. sheep displaying multibacillary, paucibacillary or recovered (no significant lesion scores) outcomes at time of necropsy.

Gene lists were created from analysed data and examined to reveal differential functional and mechanistic (canonical) pathways associated with the outcomes as well as identifying gene groupings that are specific to clinical outcome and are suggestive of previously unseen molecular interactions. Groups of genes differentially regulated in the disease outcome cohorts fitted within a known tuberculosis pathway. Gene expression profiles in diseased sheep led to enhanced accumulation of mycobacteria and inhibition of mycobacterial clearance whereas the recovered/resistant sheep had gene expression profiles leading to the opposite result. The paucibacillary associated gene lists suggested a response similar to the recovered cohort and not the multibacillary cohort. These and other findings suggest that there is much similarity in the pathogenesis mechanisms of tuberculosis and Johne's disease. There is a clear distinction between animals exhibiting multibacillary and paucibacillary characteristics. The difference in the expression profile between multibacillary and paucibacillary animals is further defined by the finding that genes associated with the antigen presenting pathway are differentially regulated only in sheep with multibacillary disease. We have previously reported the involvement of genes in the antigen presentation pathway in Mptb exposed cattle exhibiting high IFNγ at four months post Mptb exposure (Purdie et al., 2012) and other groups have since confirmed this finding in cattle.

There is evidence that expression of IFN is inhibited in the multibacillary cohort of sheep. This links to the findings that early evidence of low IFNγ expression in the blood of sheep soon after Mptb exposure is predictive of animals developing infectious Johne's disease. In contrast, sheep exhibiting a paucibacillary profile show a very different pattern of gene expression.

Comparisons of gene lists, data mining to identify genes with altered expression unique to each of Perez score defined classifications and analysis of the mechanisms of actions of each gene has resulted in the selection of a subset of 131 genes that are suggested as potential biomarkers for diagnostic purposes.

qRT-PCR validation of array findings and selected genes of interest. Five reference genes and 29 genes of interest were selected to validate the array findings. Following optimisation the primers were individually run against all of the sheep in trial P.PSH.0311.1 (Mptb exposed n=20 and unexposed control n=10). Both the array findings and the predicted biomarker specificity were validated.

Conclusion

Transcriptomic analysis by microarray allows for a snapshot of the status of an animal's gene expression at the time the sample was sourced. These data provide a basis for understanding disease pathogenesis, serving to identify targets for diagnostics and vaccination. Potential biomarkers that are specific to Johne's disease resistance, susceptibility and to specific disease outcomes i.e. multibacillary or paucibacillary were identified and the validity of the microarray findings were confirmed by qRT-PCR.

4.16 Subprogram 2.1. Part 2. Gene expression profiling (microarray) in cattle

Introduction

The primary objective of this study was to evaluate early gene expression changes in cattle exposed to Mptb with a view to identifying biomarkers of resistance or susceptibility to Johne's disease.

Methods

Briefly, 20 Holstein and Holstein Red calves aged between 2 to 4 months were inoculated with Mptb in trial P.PSH.0297.2. Samples collected were collected from these and 10 unexposed control animals prior to inoculation and at monthly intervals thereafter. Tests conducted on live animals were IFNγ, faecal culture and faecal HT-J PCR (Table 14). Necropsy was conducted later (about 4.5 years after Mptb exposure) enabling tissue culture and histopathology tests to be used to accurately classify disease outcome as defined in the resources subprogram (see above). Blood cells were isolated and RNA was isolated as described above. The samples analysed by microarray are listed in Table 14. Microarrays were performed and data were analysed as described above.

Table 14. Animal sampling across the timepoints in trial P.PSH.0297.2. Data are the number of animals and gene chips used.

Group	Months post exposure to Mptb				
	2	4	6	9	12
Control	5	5	5	5	5
Mptb exposed	20	20	20	20	20

Quantitative real time PCR (qRT-PCR) analysis was carried out on selected differentially regulated genes to verify the array findings and to assess genes of interest as biomarkers for use in predictive tests of disease outcome. All samples from each timepoint were analysed by qRT-PCR. Previously reported reference genes were utilised (Purdie et al., 2012) and nine genes of interest were selected for validation of the array data as described above.

Results

Principle component analysis of the gene expression findings showed that the samples within each group were closely located apart from those 12 months post exposure to Mptb, which displayed a more scattered pattern of expression; however this pattern was within the acceptable parameters.

Classification of Johne's disease outcome. As there were only two cattle within the Mptb exposed cohort with evidence of disease as determined by histopathology, the bulk of the gene expression analysis was reliant upon the IFNγ response as a predictor of future clinical outcome (Table 15). This follows findings in sheep and those from microarray analysis on 4 cattle from this trial in which evidence of enhanced IFNγ at 4 months post exposure to Mptb was reported (Purdie et al., 2012).

Table 15. Classification of cattle from trial P.PSH.0297.2

Key List #	MAP status	Histopathology outcome (Perez score)	IFNγ at 4 months post exposure
1	unexposed	control	control
2	unexposed	control	control
3	unexposed	control	control
4	unexposed	control	control
5	unexposed	control	control
11	exposed	NVLS	high
12	exposed	NVLS	high
13	exposed	NVLS	high
14	exposed	NVLS	low
15	exposed	NVLS	low
16	exposed	NVLS	high
17	exposed	early Pauci	low
18	exposed	NVLS	low
19	exposed	NVLS	low
20	exposed	NVLS	high
21	exposed	NVLS	high
22	exposed	NVLS	low
23	exposed	early Pauci	low
24	exposed	NVLS	low
25	exposed	NVLS	high
26	exposed	NVLS	low
27	exposed	NVLS	high
28	exposed	NVLS	high
29	exposed	NVLS	high
30	exposed	NVLS	low

NVLS – no visible lesions

Identification of genes of interest related to exposure to Mptb. Data were analysed to identify consistently regulated genes of interest in the Mptb exposed cohort of cattle in comparison to the unexposed controls. This resulted in an annotated genelist that was further explored using bionformatic tools. The most significant interactions were associated with the inhibition of IFNγ, inhibition of IL-10 and functions associated with lipids and fatty acids. These findings mirror those of the sheep trial (see above) and confirm findings associated with predictive immunological measures as reported elsewhere in this report.

Identification of genes of interest associated with high IFNγ at 4 months post exposure to Mptb. A list was created identifying genes of interest associated with high or low IFNγ at 4 months post exposure to Mptb as measured by ELISA. Bioinformatic analysis of these data confirmed previous findings and identified novel pathways. High IFNγ measured at 4 months post exposure to Mptb in sheep is predictive of an animal unlikely to succumb to clinical Johne's disease. Although it was not possible to carry out this method of prediction using the cattle data as none developed clinical disease, it was possible to utilise the collated dataset to inform the predicted outcome should the cattle have lived for longer, and to marry these data with those of the larger sheep trial to identify common or alternate patterns of expression with a view to gaining an understanding of the pathogenesis of the disease.

Aligning significant genes to pathways associated with diseases and functions revealed overall inhibition of cell death and survival functions in contrast to activation of cell trafficking, cell-to-cell signalling and inflammatory responses.

The gene expression over the early stages of infection (2 to 12 months post exposure) in the IFNγ high cohort of cattle suggested inhibition of trafficking, cell death and apoptosis and conversely there was evidence of enhanced cell movement suggesting that in the IFNγ high cohort the immune cells were capable of increased movement to potential sites of infection. However, these cells may not survive for long enough to carry out their immune function. Similar to findings in the sheep, there was gene expression for enhanced fatty acid metabolism and synthesis of lipids.

Identification of genes of interest associated with low IFNγ at 4 months post exposure to Mptb. The genes utilised in this analysis were identified as differentially expressed in the IFNγ low cattle at 4 months post exposure. This cohort includes the two cattle that showed evidence of early disease at necropsy 4.5 years after exposure to Mptb. Preliminary analysis revealed that genes in this cohort aligned to the antigen-presenting pathway in a pattern that confirmed results first reported in 2012 (Purdie et al., 2012) and since validated by the publication of supporting research from Canada (David et al., 2014). This is a very significant finding since we can now confirm that the cohort of animals with histologically confirmed disease show consistent modulation of MHC associated genes. In addition, this finding shows partial agreement in the CD8+ T Lymphocyte pathway with the multibacillary sheep gene expression results.

Deeper mining to align significant genes to pathways revealed predicted enhancement or activation of inflammatory responses and cell trafficking and inhibition of lipid metabolism. This suggests that within the cohort of cattle with susceptibility to developing Johne's disease as determined by low IFNγ there is the potential for increased inflammation, and like the IFNγ high cohort of cattle, there is potential for increased cellular movement/transport. However, the gene expression related to lipid metabolism in this cohort contrasted with that of the IFNγ high cattle in that there was suppression of both fatty acids and lipids.

Identification of potential biomarkers. Genes (n=31) were selected as potential biomarkers for identification of cattle that will respond to Mptb exposure with a high or a low IFNγ profile. A selection of nine genes were successfully validated by qRT-PCR analysis.

Discussion

The objective of this study was to analyse early transcriptomic changes in a cohort of Mptb-exposed cattle in comparison to an age matched herd of non-exposed cattle with the view of identifying markers for resistance or susceptibility. The samples used in this study were obtained from the P.PSH.0297.2 cattle trial in which there were individuals with a clearly defined susceptibility to Johne's disease. Only two animals developed histological lesions but seven were infected. The trial provided a realistic degree of disease severity for cattle, with 10% prevalence at 4.5 years of age. The data were analysed taking into consideration IFNγ responses which may be predictive of clinical outcome. This enabled the identification of potential biomarkers for use in the identification of cattle that are predicted to succumb to Johne's disease. In addition this study has provided a unique opportunity to analyse the host

animals in the subclinical phase, prior to progression to clinical Johne's disease, but with reasonable knowledge of the eventual clinical outcome.

Conclusion

The differential regulation of genes evident in response to Mptb is a significant finding in the understanding of the mechanisms of pathogenesis and may lead to a means to determine disease susceptibility of individual animals. MHC genes are polymorphic; many bovine alleles have been identified and some are associated with susceptibility to infections. Very little is known regarding potential MHC allele-based susceptibility in paratuberculosis, although a study carried out on Merino sheep exposed to Mptb identified MHC alleles with possible associations to susceptibility or resistance to Johne's disease (Reddacliff et al., 2005). We propose that these genes are consistently regulated and these early changes may persist in the ongoing sub-clinical infection within the adult host, although to a much lesser degree. In addition to the MHC, the genes associated with lipid function and cell movement emerged as commonly changed in both sheep and cattle.

4.17 Subprogram 2.2. Breed susceptibility trial

Introduction

Some producers have stated that some breeds of sheep are more resistant to Mptb infection than others and a cross sectional survey of farmers suggested that fine wool Merino sheep were more likely to develop clinical disease (Lugton, 2004). However, information on breed susceptibility is limited, as no trials have been conducted. It is known that many different breeds of sheep can develop clinical Johne's disease including Merino, Churra Blackface, Texel, Bleu du Maine, East Friesian, Romney and Highland cross breeds and Rocky Mountain bighorn sheep. The aim of this study was to examine susceptibility to Mptb infection in four different breeds of sheep.

Methods

Relevant sheep breeds were identified following consultation with the Sheepmeat Council of Australia (SCA). Merino (n=46), Poll Dorset (41), Border Leicester (41) and Suffolk first cross Merino lambs (41) were used in the experiment (total 169 sheep). All the supplying farms were from the Armidale region of New South Wales, Australia, and had a market assurance program score of MN3[1]. The farms had a similar lambing time and all lambs were approximately 3 months of age. The animals were managed under conventional Australian sheep farming conditions by grazing in open paddocks on unimproved pasture and the lambs from different breeds were grazed together. Following experimental inoculation, the animals were monitored for clinical disease development up to 14 months post inoculation when the trial was terminated. Tests conducted were gross and histopathological examination, culture and PCR of faeces and immune measures. An animal was classified as having clinical disease if it lost greater than 10% of its body weight over a 1 month period

[1] Three negative sample tests of flock over a period of at least 4 years plus Flock Management Plan (Anon 2013)

and Mptb was cultured from tissues after necropsy and/or as infected if one tissue was culture positive for Mptb

Results

Clinical cases were seen in all breeds (Fig. 19). In the Poll Dorset breed, visual assessment alone was unable to identify individuals with clinical disease, and weighing was needed (Fig. 19.D). Clinical disease was seen in the Merino (44%) and Suffolk first cross Merino (37%) breeds more often than in the Border Leicester (12%) and Poll Dorset (11%) sheep (Fig. 20).

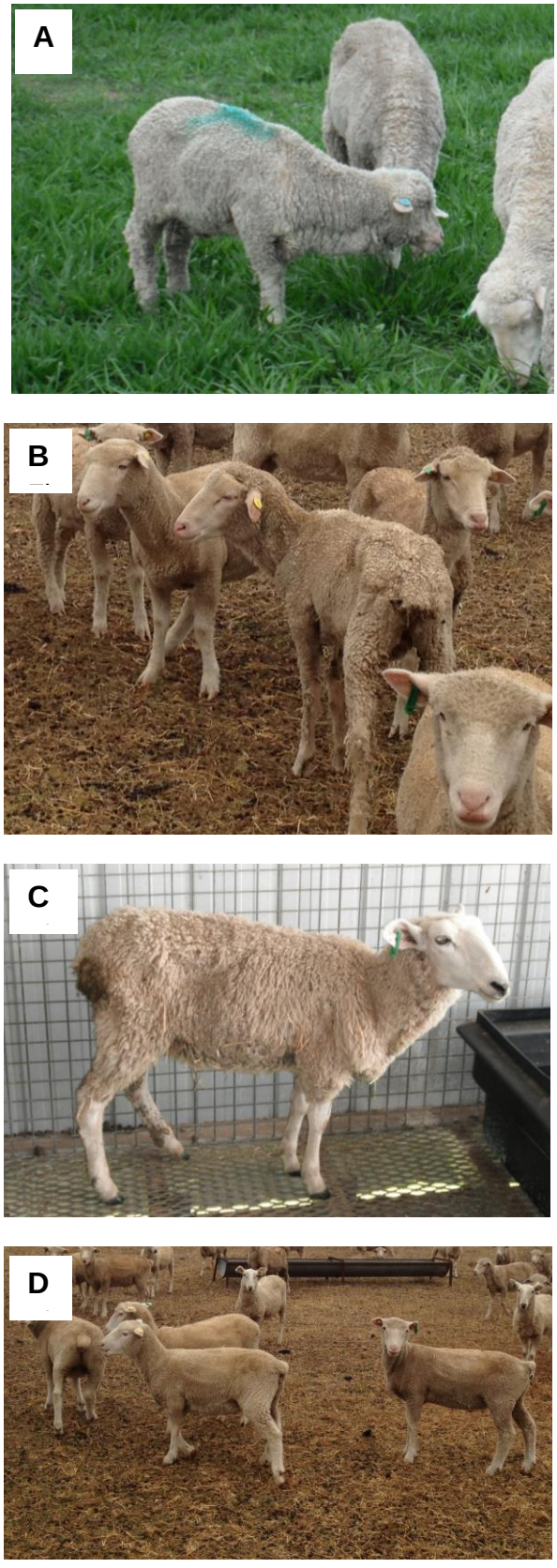

Fig. 19. Clinical cases in different sheep breeds. A. Merino, B. Suffolk first cross Merino, C. Border Leicester and D. Poll Dorset

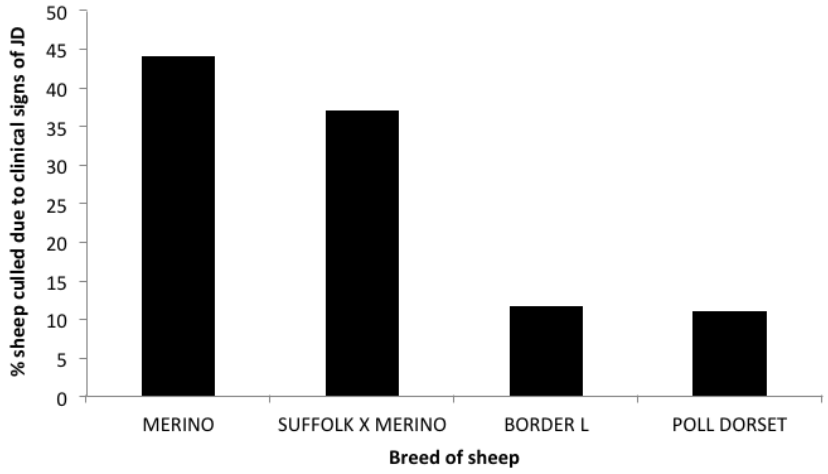

Fig. 20. Clinical disease in different breeds.

The tissue culture results indicated that at least 40% of sheep from each breed were infected at necropsy. Merino (69%) and Suffolk first cross Merino (75%) sheep had greater numbers of animals with viable Mptb in their tissues while Border Leicester had 55% and Poll Dorset 44% infection rates. Dissemination of Mptb to tissues outside of the gut was examined by culture of a section of liver and rates varied from 19 - 59%, in most cases matching the animals which developed clinical disease.

Slightly fewer animals had Johne's disease lesions in their intestines than had Mptb isolated from their tissues. The Merinos and Suffolk first cross Merinos were more likely to have multibacillary lesions (Fig. 21.B).

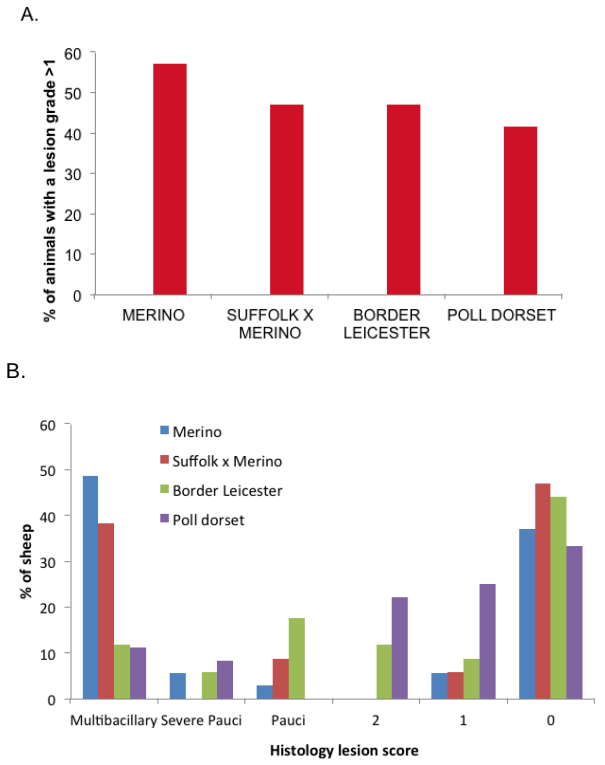

Fig. 21. Histological lesions observed in gut and mesenteric lymph node tissues from sheep of different breeds. A) The percentage of animals with lesions greater than grade 1. B) The percentage of sheep of each breed with different lesion scores, based on the most severe lesion observed in an animal.

The Merinos and Suffolk first cross Merinos were the only ones to be shedding Mptb in their faeces at 3 months post inoculation. As the trial progressed, the Border Leicesters and Poll Dorsets had increasing numbers of culture positive pools, indicating increasing faecal shedding. At 12 months post inoculation the number of pooled faecal cultures of the Suffolk cross Merino breed decreased due to removal of individuals with clinical disease (Fig. 22).

The amount of Mptb shedding in the faeces was measured using the HT-J faecal PCR test and was not significantly different between breeds (Fig. 23). Clinical cases in all breeds were equally infectious and shedding large numbers of Mptb.

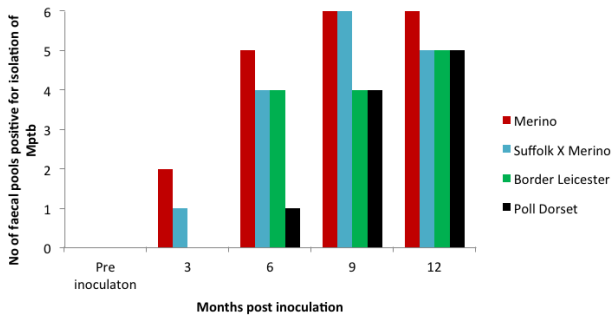

Fig. 22. Faecal shedding from the different breeds of sheep based on pooled faecal cultures with 6 animals per pool.

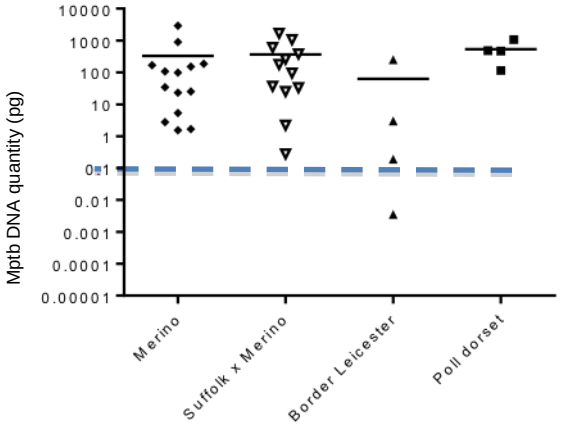

Fig. 23. Faecal shedding of Mptb in sheep that developed clinical disease from each breed, as measured by HT-J PCR. Results above the dotted line are equivalent to more than 10,000 Mptb per gram of faeces.

The ELISA antibody responses of all the breeds increased at a similar rate for the first 9 months post inoculation. At 12 and 14 months post inoculation, the responses from the Merino sheep increased while the Suffolk cross Merino decreased. By 14 months both breeds had lost significant numbers of animals due to clinical disease. Half of the clinically affected animals of each breed were classified as ELISA positive.

Discussion

All of the breeds examined were susceptible to develop Johne's disease after exposure to Mptb, but the Merino and Suffolk first cross Merino breeds developed the disease earlier than the other breeds. When the trial was terminated, each of the breeds tested had infection rates of 44-75% and had had some individuals develop clinical disease. All of the breeds had individuals that were infectious. High quantities of Mptb DNA were detected in the faeces of clinical cases independent of the breed. Animals were assessed only up until 14 months post Mptb exposure; more sheep of each breed would likely have gone on to develop clinical disease.

Most of the clinical cases had multibacillary lesions and ELISA had low sensitivity in detecting these cases, irrespective of breed.

In this study the within breed variability to Mptb infection was not examined as the sheep of each breed were sourced from a single farm. All breeds examined could present a biosecurity risk.

4.18 Subprogram 2.3. Gene expression profiling across breeds

Introduction

The aim of this study was to examine potential variations in gene expression responses to Johne's disease in different sheep breeds. The expression of genes that were selected as predictive of disease outcome in Mptb exposed Merinos were tested by qRT-PCR in

samples sourced from Mptb exposed Poll Dorset, Border Leicester and Suffolk first cross Merinos in trial P.PSH.0576.1.

Methods

Please refer to the previous sections for trial design and methodological information for gene expression. Quantitative real time PCR (qRT-PCR) analysis was carried out on genes informed from gene expression analysis research described above. Twenty nine genes were tested to determine expression in the Poll Dorset, Border Leicester and Suffolk first cross Merino sheep at four months post exposure to Mptb in comparison with unexposed breed matched controls. Reference gene primer suitability was assessed by geNorm. Fold change calculations were carried out with reference to animals of each breed displaying resistance to disease or susceptibility; the susceptible animals were subgrouped as having either paucibacillary or multibacillary disease.

Results and discussion

Fifteen genes had statistically significant P-values and two were potential markers for tendency to paucibacillary disease across all breeds. Four genes were predictive of resistance in Border Leicester, White Suffolk cross Merinos and Merinos but not Poll Dorset sheep. Data presented above suggested that while Poll Dorset sheep developed clinical disease, they took longer to succumb to the infection and this may explain their variation in gene expression.

It is clear that there are variations in gene expression between breeds, however several genes were identified as markers of resistance for potential use in a multi-gene screening panel. This would require extensive validation in naturally infected flocks to ensure specificity.

4.19 Sub-program 2.4. Gene expression profiling in vaccinated sheep

Introduction

Samples derived from sheep trials P.PSH.0576.1 and P.PSH.0576.2 were analysed by microarray to identify immunomodulatory effects of Gudair® vaccine. This study served to identify indirect measures of vaccine efficacy and the data were analysed in a systems immunogenic approach to identify correlates of protection.

Methods

This study included sheep from two trials (P.PSH.0576.1 and P.PSH.0576.2) and comprised Mptb exposed/Gudair® vaccinated sheep (n=28) and Mptb unexposed/Gudair® vaccinated sheep (n=6).

Blood samples for array analysis were sourced at 4 months post Mptb exposure. Tests conducted on live animals were faecal culture and HT-J faecal PCR assay (HT-J). Necropsy was conducted at about 14 months post Mptb exposure and tissue culture and histopathology tests were performed to accurately classify disease outcome. Methods were as described in previous sections of this report. Disease outcome classifications were determined as described above. For the purpose of analysis, gene expression files from

non-vaccinated Mptb exposed and unexposed Merinos at 4 months post exposure from trial P.PSH.0311.1 were included.

Results and discussion

Quality of microarray. Principle component analysis of the gene expression data showed clustering of data points associated with vaccination regardless of Mptb exposure. Separation of the clusters was not due to variation in processing since data points associated with P.PSH.0576.1 and P.PSH.0576.2 were closely situated and mean probe expression values were consistent across all trials.

Classification of disease outcome (P.PSH.0576.1 and P.PSH.0576.2). Disease outcome classifications are provided in Table 16 and include Merino sheep from two trials that were vaccinated with Gudair® with or without oral exposure to Mptb. In addition to the tabulated animals, the gene expression data from control sheep (n=5) and Mptb exposed sheep (n=20) from trial P.PSH.0311.1 at 4 months post Mptb exposure were incorporated into the analysis as controls for subtractive analysis. Statistically relevant gene expression analysis requires the data from at least 4 gene chips per classification. This required the Perez score determined disease classifications of multibacillary and paucibacillary to be merged to provide a larger dataset (n=8) for comparison with the Mptb unexposed vaccinated controls (n=6) and 'recovered' (resistant) vaccinated sheep (n= 19).

Table 16. Disease outcomes for Gudair® vaccinated Merino sheep in trials P.PSH.0576.1 and P.PSH.0576.2.

Key List number[a]	Trial # (P.PSH.0)	Vaccinated	Exposure status	Severity[b]	Infection status[c]
124	576.1	yes	control	control	control
125	576.1	yes	control	control	control
126	576.1	yes	control	control	control
133	576.1	yes	control	control	control
135	576.1	yes	Infected	Recovered	non-infected
137	576.1	yes	Infected	Recovered	non-infected
139	576.1	yes	Infected	Paucibacillary	Infected
140	576.1	yes	Infected	Multibacillary	Infected
141	576.1	yes	Infected	Recovered	non-infected
142	576.1	yes	Infected	Recovered	non-infected
144	576.1	yes	Infected	Recovered	non-infected
145	576.1	yes	Infected	Recovered	non-infected
148	576.1	yes	Infected	Recovered	non-infected
149	576.1	yes	Infected	Paucibacillary	Infected
150	576.1	yes	Infected	Paucibacillary	Infected
153	576.1	yes	Infected	Recovered	non-infected
2001	576.2	yes	control	control	control
2002	576.2	yes	control	control	control
2007	576.2	yes	Infected	Paucibacillary	Infected
2009	576.2	yes	Infected	Paucibacillary	Infected
2010	576.2	yes	Infected	Recovered	non-infected
2011	576.2	yes	Infected	Recovered	non-infected
2012	576.2	yes	Infected	Recovered	non-infected
2013	576.2	yes	Infected	Recovered	non-infected
2015	576.2	yes	Infected	Recovered	non-infected
2016	576.2	yes	Infected	Recovered	non-infected
2017	576.2	yes	Infected	Recovered	non-infected
2018	576.2	yes	Infected	Recovered	non-infected
2019	576.2	yes	Infected	Recovered	Infected
2020	576.2	yes	Infected	Paucibacillary	non-infected
2022	576.2	yes	Infected	Recovered	non-infected
2023	576.2	yes	Infected	Paucibacillary	non-infected
2024	576.2	yes	Infected	Recovered	non-infected
2025	576.2	yes	Infected	Recovered	non-infected

[a] Identification number of individual animals
[b] Severity of disease defined by histopathological lesion score using established criteria (Perez et al., 1996)
[c] Inoculated animals were classified as infected if any tissue at necropsy was culture positive for Mptb

Identification and bioinformatics analysis of a protective gene expression profile. Gene lists were created for the vaccinated but non-infected sheep compared to the vaccinated but infected sheep (in trials P.PSH.0576.1, P.PSH.0576.2) excluding genes that were differentially regulated in the non-vaccinated Mptb exposed control sheep (in trial P.PSH.0311.1). This list contained 785 genes.

There was a large proportion genes that influence signalling, suggesting that an effective immune response to Mptb exposure requires inhibition of these cell processes at the early stage of exposure. The mechanism of actions of vaccines are commonly associated with T and B cell function. Functional differences between the Mptb exposed vaccinated sheep with no infection in contrast to the Mptb exposed vaccinated sheep with infection revealed a predicted enhanced proliferation of lymphocytes, which is in agreement with the findings reported in lymphocyte proliferation assays.

Identification of genes for potential use as markers. The gene list was informed by bioinformatic analysis to identify genes whose expression is associated to known immune-function relevant molecular pathways to identify 66 genes for use as indirect measures of vaccine efficacy. These selected genes are further discussed above (Section 4.13) in which data were also collated for the immune parameters IFNγ, IL-10, antibodies, proliferation and faecal shedding (live/dead numbers/ratio) in relation to the ultimate disease outcome.

Conclusion

There are likely to be many factors contributing to vaccine efficacy or conversely failure of a host to develop a protective immune response. The results from this study suggest that a large proportion of the genes specific to the Mptb exposed yet recovered (resistant/protected) vaccinated sheep is associated with cellular signalling. Clearly the vaccine targets this function. B cells are an integral part of the immune response but their role within Mptb and progression to Johne's disease is not well characterised. There was evidence of decreased lymphocyte proliferation in animals that were not protected by the vaccine and this may be a mechanism employed by Mptb to decrease the immune response. In Subprogram 3.1 below we report an assessment of the immune parameters IFNγ, IL-10 and lymphocyte proliferative responses and serum antibody levels in the same sheep; 72% of non-vaccinated sheep and 24% of vaccinated sheep were infected, at 49 weeks post exposure. There were significant differences in the proliferation of $CD4^+$, B and γδ T-cells over time in vaccinated sheep in which the vaccine failed to protect against infection compared to the non-infected vaccinated sheep. There were no significant differences in the IFNγ response or serum antibody levels between the vaccinated Mptb infected and vaccinated non-infected sheep. Importantly, the conclusions on lymphocyte function in vaccination are supported by the results in this study of gene expression. That the two lines of investigation, using completely different laboratory disciplines (genomics and immunology) agree provides confidence in the discovery.

4.20 Subprogram 2.5. Candidate gene analysis by single nucleotide polymorphism

Introduction

The purpose of this study was to identify single nucleotide polymorphisms (SNPs) within the sheep genome that are associated with susceptibility or resistance to Johne's disease as a consequence of infection with Mptb.

Methods

DNA samples from 461 sheep from the Merino, Border Leicester, White Suffolk first cross Merino and Poll Dorset breeds were evaluated. The sheep were from 11 separate trials carried out at the University of Sydney between 2007 and 2015 (animal trial numbers OJD.031.3, OJD.031.4, OJD.031.5, OJD.031.6, OJD.031.A, OJD.031.E, OJD.031.G, P.PSH.0311.1, P.PSH.0311.A2, P.PSH.0576.1 and P.PSH.0576.2) as shown in Table 17.

The sheep were exposed to Mptb under varying conditions: either naturally exposed or experimentally inoculated with either gut homogenate from a sheep with Johne's disease or an inoculum of laboratory cultured Telford 9.2 S strain Mptb. Sheep were culled if they lost 10% of their body weight or more in a one month period. All remaining animals were culled at the conclusion of the trial or at 12 months post inoculation as indicated in Table 17. Tests conducted were histopathology, culture of Mptb from faeces and tissues, and IDEXX antibody ELISA. The disease outcomes in each animal were categorised as described above.

SNP genotyping was undertaken with the Ovine Infinium® HD SNP BeadChip (International Sheep Genomics Consortium (ISGC) in conjunction with FarmIQ (www.farmiq.co.nz)). This comprises 603,350 callable SNPs, the majority of which are equally spaced across the genome and selected from groups differing in their minor allele frequency across an international sample of breeds. The chip also contains SNPs reported in the literature as affecting function, those that change protein structure, and those on the OvineSNP50 BeadChip. The processing of the DNA samples with the BeadChips was carried out by the manufacturer under contract and the data were provided to the University of Sydney.

The data were analysed by two independent bioinformaticians. Initial analysis was carried out under contract at the University of Queensland bioinformatics service QFAB (http://www.qfab.org). The analyses were carried out to identify SNPs associated with the following variables: infected/non-infected, resistant/susceptible, infectious/non-infectious. Quality control filtering and data cleaning were performed to filter the SNPs. Briefly, samples with a call rate <95% were removed, as were SNPs with a call frequency <98%. SNPs with minor allele frequency (<5%) were removed to avoid genotyping errors and SNPs with variance <0.1 were removed as these were not informative. This involved a three-step process incorporating supervised univariate and multivariate analysis followed by unsupervised multivariate analysis. The supervised univariate logistic regression was performed for each variable of interest on each SNP separately (single-marker association tests). The p-values obtained were adjusted by Bonferroni correction to adjust for multiple testing. The SNPs with the smallest p-values in the logistic regression were then tested in a supervised multivariate analysis with variable selection (penalized logistic regression via elastic net). The variable selection method sought the SNPs showing the strongest

association with the outcome of interest. As an exploratory approach, unsupervised data analysis (Multiple Correspondence Analysis) was then performed on the SNPs pre-selected by elastic net to study the structure of the data and identify a possible natural separation between the groups based on the genetic information. No p-values can be obtained from this method but a list of potential SNPs contributing to the separation between the groups was drawn for each classification variant.

Further statistical analysis was undertaken at the University of Sydney. This involved the three step analysis detailed above but the statistical stringency was significantly increased. An analysis of breed variation and SNP association with disease severity as defined by histopathological analysis commenced. These data are undergoing further analysis and will be reported as an addendum to this final report.

Results

After data cleaning, the genotype data included 606,006 SNPs for 459 animals. The data were grouped by the variables infected/non-infected, resistant/susceptible, infectious/non-infectious and SNPs associated with each outcome were identified.

SNPs associated with an infectious versus non-infectious outcome. Animals were classified as either infectious (n=95) or non-infectious (n=101). Statistical analysis identified 73 SNPs associated with an infectious versus a non-infectious outcome.

SNPs associated with a Johne's disease resistant versus a susceptible outcome. Animals were classified as either resistant (n=141) or susceptible (n=210) to Johne's disease. Statistical analysis identified 58 SNPs associated with a resistant versus a susceptible outcome.

SNPs associated with sheep that are infected with Johne's disease in contrast to those that are uninfected following exposure to Mptb. Mptb exposed animals were classified as either infected (n=212) or non-infected (n=135). Statistical analysis identified 136 SNPs associated with an infected versus a non-infected outcome.

Table 17. Animal trial data for sheep samples utilised within the SNP study.

Trial number (P.PSH.)	Breed	Mptb exposure method		Sex		Classification							
		Experimental inoculation (IN) or natural exposure (NE)				Histopathology severity score		Infection status		Infectivity status		Resistance status	
031.A	Merino	NE	13	Female	13	Paucibacillary	4	Non-infected	4	Non-infectious	5	Resistant	2
				Male	0	Multibacillary	2	Infected	9	Infectious	8	Susceptible	11
						Recovered	7						
031.G	Merino	Control	10	Female	10	Paucibacillary		Non-infected		Non-infectious		Resistant	
				Male	0	Multibacillary		Infected		Infectious		Susceptible	
						Recovered							
031.E	Merino	NE	33	Female	33	Paucibacillary	17	Non-infected	11	Non-infectious	27	Resistant	11
				Male	0	Multibacillary	4	Infected	22	Infectious	6	Susceptible	20
						Recovered	12						
031.3	Merino	Telford IN	23	Female	14	Paucibacillary	6	Non-infected	13	Non-infectious	14	Resistant	10
				Male	9	Multibacillary	4	Infected	10	Infectious	6	Susceptible	10
						Recovered	13						
031.4	Merino	Telford IN	17	Female	12	Paucibacillary	4	Non-infected	9	Non-infectious	11	Resistant	7
				Male	5	Multibacillary	3	Infected	8	Infectious	4	Susceptible	9
						Recovered	10						
031.5	Merino	Telford IN	12	Female	9	Paucibacillary	8	Non-infected	5	Non-infectious	6	Resistant	3
				Male	3	Multibacillary		Infected	7	Infectious	4	Susceptible	7
						Recovered	4						
031.6	Merino	Telford IN	18	Female	0	Paucibacillary	16	Non-infected	10	Non-infectious	20	Resistant	10
		Gut homogenate IN	19	Male	57	Multibacillary	10	Infected	27	Infectious	17	Susceptible	25

Trial number (P.PSH.)	Breed		Mptb exposure method		Sex		Classification							
			Experimental inoculation (IN) or natural exposure (NE)				Histopathology severity score		Infection status		Infectivity status		Resistance status	
			Control	20			Recovered	11						
0311.1	Merino		Telford IN	20	Female	0	Paucibacillary	3	Non-infected	11	Non-infectious	11	Resistant	11
			Control	10	Male	30	Multibacillary	6	Infected	9	Infectious	9	Susceptible	9
							Recovered	11						
0311.A2	Merino		NE	18	Female	18	Paucibacillary	9	Non-infected	2	Non-infectious	7	Resistant	2
					Male	0	Multibacillary	5	Infected	16	Infectious	11	Susceptible	14
							Recovered	4						
0576.1	Merino	74	Telford IN	155	Female	76	Paucibacillary	51	Non-infected	68	Non-infectious	-	Resistant	68
	Border Leicester	42	Control	45	Male	124	Multibacillary	33	Infected	87	Infectious	17	Susceptible	86
	White Suffolk x Merino	40					Recovered	70						
	Poll Dorset	44												
0576.2	Merino		Telford IN	38	Female	0	Paucibacillary	13	Non-infected	17	Non-infectious	-	Resistant	17
			Control	10	Male	48	Multibacillary	7	Infected	21	Infectious	13	Susceptible	20
							Recovered	17						

Discussion

The basis for this study lay in published observations of probable genetic effects in other mycobacterial diseases in species such as mice and humans, in paratuberculosis in cattle, sheep and deer and from observations of breed differences in Johne's disease incidence in cattle and sheep and anecdotal evidence from farmers.

An aim of genetics is to find an association between phenotype and genotype. Gene variation forms the basis for biodiversity and evolution, and this variation can underlie traits like disease susceptibility due to potential effects on gene expression or regulation and hence protein function. Some traits are complex, associated with the action of a number of different genes and environmental factors. Genome variations manifest in the form of single nucleotide polymorphisms (SNP) are deletions, mutations or insertions of nucleotides or whole genes or gene clusters; gene or whole chromosomal rearrangements; gene duplications and; copy number polymorphisms or variants. Analysis of gene variation is complemented but not replaced by the analysis of gene expression (transcriptomic analysis, see other sections of this report as we have also studied this aspect). Although consistent differential gene expression patterns in an experimental disease model such as that used in this project can provide clues to underlying gene variation patterns, this cannot be used in isolation since gene expression is not a static process; it is dependent on external and internal influences including vaccination status, the environment, physiological state, management and nutrition.

A genome association study is one in which a group of genetic markers that are representative of a phenotype are analysed for variation within a population of animals using DNA samples obtained from each animal. A genome-wide association study (GWAS) was conducted for this study, in which genetic variation across an entire genome is analysed to identify genetic association with observable traits. The GWAS approach has been made possible in cattle and sheep by the recent development of whole genome arrays and in this study we have used the most advanced array available anywhere in the world, the Ovine Infinium® HD SNP BeadChip that comprises over 600,000 SNPs.

The authors of most studies on paratuberculosis susceptibility have been aware of the limitations in deriving accurate estimates of the size of genetic effects, which are imposed by inaccurate classification of the phenotype of both infected animals and controls. We reviewed this in 2011 (Purdie et al., 2011), leading to stringent phenotypic classification in the present study. The specificity and accuracy of the disease classification as well as the use of samples from multiple trials reduces the risk of inaccurate classification. The disease phenotype ranges from mild to severe, possibly associated with differential susceptibility between individuals, but also with infectious dose and time since exposure (Whittington and Sergeant, 2001).

The SNPs that were identified in this study may be used in the future in marker-assisted breeding approaches for paratuberculosis. The variety of host responses in JD reflects the fact that it is a complex disease with outcomes most likely determined by many genes. We do not know how many genes. However, this concept of the genetic basis for disease seems to be reflected by the number of SNPs discovered in this study; a panel of SNPs would be needed for selection for resistance. Further research is recommended to verify that the SNPs are representative of populations present in Australian flocks.

4.21 Subprogram 3. Improving vaccine technology

This subprogram addressed the following project objective:

Conduct research towards a safer, more effective vaccine for sheep and cattle

1. To develop an indirect measure of vaccine efficacy, based on blood and faecal testing, to facilitate the development of a safer more effective vaccine
2. To improve the safety of vaccines, by reducing their tendency to cause severe tissue reactions in livestock and humans
3. To trial a prototype vaccine through experimental infection in sheep and cattle

The operational plan agreed with MLA for this subprogram included research to address the above aspects.

4.22 Subprogram 3.1. To develop an indirect measure of vaccine efficacy, based on blood and faecal testing, to facilitate the development of a safer more effective vaccine

Introduction

In general terms a vaccine can be effective if it enhances natural immune processes to resist an infection. Therefore knowledge of these natural processes is extremely valuable and the results from Subprogram 1.1. (see above) may be directly relevant here. To reiterate from that section of this report, although Mptb exposed sheep have an IFNγ response, the strength of the early IFNγ response reflects future disease outcome: sheep that had a weaker early IFNγ response were the ones that were more likely to be infectious (i.e. shed Mptb in their faeces), more likely to be truly infected (i.e. have viable Mptb in intestinal tissues) and more likely to have severe multibacillary disease pathology. An IL-10 response at 4 months p.i. was also associated with increased likelihood of disease resistance and decreased likelihood of infection. Similarly, shedding of Mptb in faeces at high levels at an early stage is predictive of severe disease later. Vaccination may induce very different immune responses, and these factors may or may not influence protection. For this reason a study of vaccinated sheep was conducted.

Gudair® is a widely-used vaccine in sheep and goats and is the only vaccine approved for use in sheep in Australia and New Zealand. This vaccine is able to reduce Mptb-infection associated mortality by up to 90%. It also delays and reduces prevalence of faecal shedding, but some animals remain highly infectious. Gudair®-vaccination stimulates cell-mediated and humoral immune responses but the immune mechanisms responsible for vaccine protection are not understood. The aim of this work was to characterise differences in the host response to Mptb-infection in vaccinated sheep to identify early markers associated with success versus failure of protective immunity in vaccination against Mptb-infection.

Methods

Seventy-six Merino lambs that were part of trials P.PSH.0576.1 and P.PSH.0576.2 were drafted into four groups using systematic random sampling and either left unvaccinated or vaccinated with Gudair®. Animals in the Mptb-exposed groups were challenged with Mptb

four weeks after vaccination at 4-5 months of age giving rise to four groups based on vaccination and Mptb-exposure: Controls = non-vaccinated non-Mptb exposed (n=15); Non-vaccinated Mptb-exposed (n=36); Vaccinated controls = vaccinated non-Mptb exposed (n=8); vaccinated Mptb-exposed (n=17).

Blood, serum and faecal samples were collected at intervals throughout the trial. Tissue samples were collected at 49 weeks post-Mptb-exposure following euthanasia. The following assays were undertaken: IFNγ assay, IL-10 assay, IDEXX antibody ELISA, lymphocyte proliferation assay, total leucocyte counts, faecal culture and Mptb DNA quantification. After necropsy, tissue culture and histopathology were performed, animals were classified as infected if there was a positive Mptb culture result for intestinal tissue and/or lymph node samples (terminal ileum and anterior jejunum) taken at the end of the trial period. Each parameter measured at multiple time points was examined using restricted maximum likelihood (REML) in a linear mixed model.

Results

No infection, histological lesions associated with Mptb infection or faecal shedding of Mptb were detected in the Control and Vaccinated Control groups (both non-Mptb-exposed). Disease outcomes for Mptb-exposed sheep are shown in Table 18.

Table 18. Distribution of disease outcomes for Mptb-exposed sheep

Treatment Group	Tissue culture positive	Faecal culture positive[a]	Faecal Mptb quantity (pg)[b]	Clinical disease[c]	Histological lesions[d] Multi	Pauci	Disseminated disease[e]
Non-vaccinated (n=36)	72% (26)	72% (26)	0-56.95 (36)	47% (17)	47% (17)	8% (3)	36% (13)
Vaccinated (n=17)	24% (4)	18% (3)	0-11.49 (17)	12% (2)	6% (1)	12% (2)	6% (1)

[a]Detected by culture on at least one occasion;
[b]Range at 39 weeks post Mptb-exposure in 1.2 +/- 0.3 g of faeces for each animal;
[c]culled due to weight loss compared to flock mates;
[d]Histological lesions based on Perez et al where Multibacillary (Multi) = type 3b and Paucibacillary (Pauci) = types 3a and 3c;
[e]Liver culture positive

The four vaccinated-infected sheep had a diverse range of disease manifestations. Two lost weight and had viable Mptb in faeces; one had disseminated disease as indicated by positive culture from liver samples (Table 18).

For the purposes of this objective, the most important observations in this experiment relate to a comparison between vaccinated-infected sheep and vaccinated-non-infected sheep.

An early, strong IFNγ response was observed following Gudair®-vaccination ($P < 0.05$) and Mptb-exposure further increased this response ($P < 0.05$). There were no significant differences between vaccinated-infected and vaccinated-non-infected sheep. Gudair®-vaccination significantly increased Mptb-specific serum antibody levels ($P < 0.05$) but there was no significant difference between vaccinated-infected and vaccinated-non-infected sheep.

Gudair®-vaccination boosted a significant increase in Mptb-specific IL-10 responses but at the earliest time point the IL-10 response was significantly lower in the vaccinated-infected sheep compared to the vaccinated-non-infected ($P < 0.05$). However, at later time points IL-10 responses increased in both these groups.

Gudair®-vaccination significantly increased Mptb-specific PBMC proliferation compared to the non-vaccinated controls up to the final sampling point ($P < 0.05$) (Fig. 24). However, vaccinated-infected sheep had significantly decreased PBMC proliferation ($P < 0.05$) compared to the vaccinated-non-infected (Fig. 24.A). There were no significant differences in the concentration of total leucocytes, mononuclear cells or granulocytes in peripheral blood at any of these sampling times between vaccinated-infected and vaccinated-non-infected sheep. When vaccination did not protect against disease, significantly lower proliferation ($P < 0.05$) was seen in $CD4^+$ and B-cells by 13 weeks and in γδ T-cells by 21 weeks post Mptb-exposure (Fig. 24.C-E).

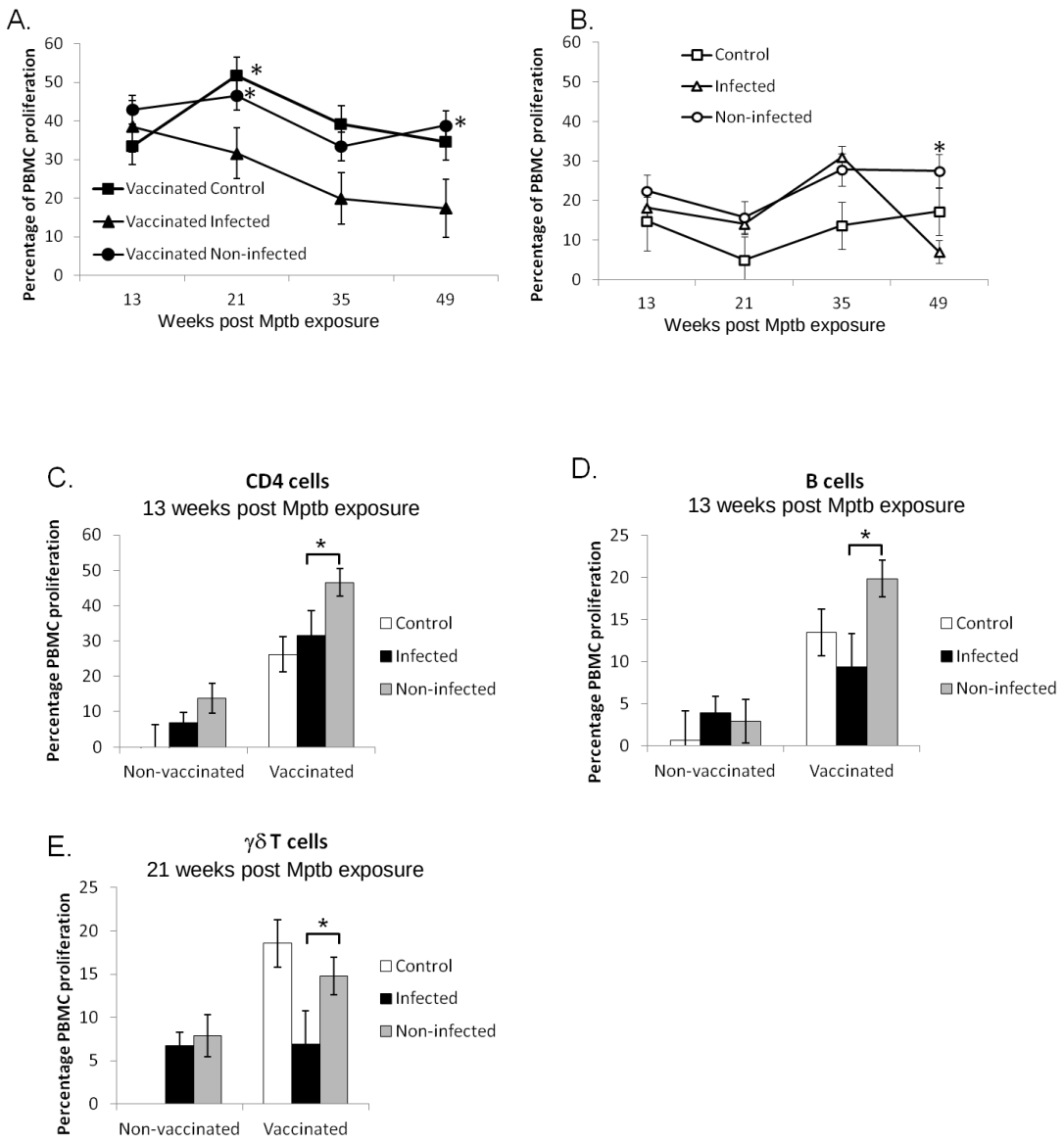

Fig. 24. Mptb-specific PBMC proliferative response. Percentage of proliferating cells over time for cultured PBMC from unexposed controls, and sheep that were exposed to Mptb that became infected or remained non-infected in vaccinated (A) and non-vaccinated (B) groups. Significant differences in proliferation of specific subsets at early time points post Mptb-exposure are shown in C-E. * P<0.05 compared to infected in each group.

Discussion

The aim of vaccination is to induce memory of a protective immune response in order to mount an effective and immediate path of action when a particular pathogen is encountered. Thus, to develop an effective vaccine, it is useful to understand the nature of the protective response. The exact nature of the protective immune response in Gudair®-vaccinated animals is poorly defined and the purpose of this study was to try to understand why some animals experience a lack of protection from disease despite vaccination.

The ability of Gudair®-vaccination to provide a degree of protection against Mptb-infection in our experimental sheep model was similar to studies in naturally-infected flocks. Disease

outcomes in vaccinated-infected sheep varied from a typically 'silent' profile where no overt signs of disease were detectable (no faecal shedding of Mptb, no weight loss) to those that could be easily detected as being infectious and therefore a threat to the health of the flock.

Specific IFNγ and serum antibody responses are commonly used as surrogates of vaccine efficacy and IFNγ is also an early predictor of disease outcome. However, neither of these two parameters was significantly different between the vaccinated-infected and vaccinated-non-infected sheep suggesting that they are indicators of vaccine administration rather than vaccine protection. Thus it is clear that there is a need to look beyond immune parameters that are customarily used when assessing the ability of a vaccine to protect against Mptb-infection.

Short-term monitoring of the antigen-specific IL-10 response and lymphocyte proliferation, especially of particular subsets, are most likely to be useful when testing novel vaccines as these parameters were significantly different as early as 13-21 weeks after Mptb-exposure in animals that were not protected by vaccination. An early elevated Mptb-specific IL-10 response in peripheral blood is also associated with lack of future disease in non-vaccinated sheep.

While $CD8^+$ T-cell proliferation was also lower in vaccinated-infected sheep compared to the vaccinated-non-infected, this difference did not reach statistical significance at early time points.

γδ T-cells, a major cell population in young ruminants, are non-MHC restricted and are able to directly respond to pathogen associated molecular patterns allowing these cells to react early. Thus the ability to generate an IFNγ response at the site of infection early, prior to T-cell migration, could be crucial to effective clearance of Mptb-infection in the host. While our study shows that Gudair®-vaccination does induce an antigen-specific memory response in γδ T cells (the antigen-specific proliferation was only in Gudair-vaccinated or Mptb-exposed animals), how vaccines can be used to generate and maintain γδ T-cell memory are questions that require further research.

While antibodies to Mptb are universally measured in relation to Mptb-infection, the B-cells that are required for secreting these molecules are generally forgotten in terms of their functional relevance to disease pathogenesis. Our data suggest that B-cell function is important in the protection of vaccinated sheep against Mptb-infection. At 13 weeks post Mptb-exposure when there was a strong antibody response to vaccination, B-cell proliferation was significantly lower in the vaccinated sheep that became infected compared to those that remained non-infected.

In non-vaccinated sheep, an early reduced IFNγ response was associated with the likelihood that an animal would shed Mptb in faeces and progress to severe disease (de Silva et al., 2013). Since all the Gudair®-vaccinated sheep had a strong IFNγ response other, as yet to be defined, factors may influence this aspect of the infection and need to be identified. Faecal shedding is probably a useful marker of efficacy, but can't be used at early time points. An ideal vaccine is one that prevents both faecal shedding and tissue infection.

4.23 Subprogram 3.2. To improve the safety of vaccines, by reducing their tendency to cause severe tissue reactions in livestock and humans

Introduction

Gudair® vaccine is effective in reducing clinical Johne's disease occurrence by up to 90% although vaccinated sheep can still develop infection and some shed large numbers of Mptb in faeces. Johne's disease vaccines in general are based on an antigen (whole cell heat killed Mptb) mixed with oil adjuvant; they can cause lesions at the site of injection and in regional lymph nodes, and in the case of Gudair ® this can be in a large proportion of animals. From a safety perspective, accidental self-injection may require multiple medical treatments often involving surgical intervention. The high degree of tissue reactivity is due to the adjuvant, and some think this is required to stimulate a protective immune response. Recently, new highly refined mineral oil emulsion adjuvants have become available. There are several types: water in oil (W/O), water in oil in water (W/O/W) and oil in water (O/W), and there are studies to show that the type of immune response elicited by a vaccine is adjuvant dependent. Therefore it may be possible to select an adjuvant based on a desired immune profile, combined with reduced tissue reactivity. There is a strong indication for a trial of new adjuvants for paratuberculosis vaccines.

In this study, we characterised the immunological responses to Mptb antigens and tissue reactions in relation to a range of adjuvants.

Methods

Ninety Merino wethers aged 24-36 months were sourced from a flock in Armidale, New South Wales, for trial P.PSH.0576.adj (Table 1). Sheep were systematically randomised into 18 groups of five. The first eight groups were allocated a single dose of the novel vaccines including one with no adjuvant (Table 19). The next eight groups were allocated a double dose of the same novel vaccines. One group received Gudair® in a single dose. A negative control group was not vaccinated. The design is shown in Table 19.

Mptb cellular antigen was emulsified with adjuvant under aseptic conditions. Mptb inactivation was confirmed by liquid culture. All novel vaccines were tested for sterility by aerobic culture on sheep blood agar incubated ay 37°C for 48 hours, prior to use.

Table 19. Vaccine formulations used in the trial.

Group	Vaccine	Adjuvant	Antigen
1	A	A	Heat killed Mptb
2	B	B	Heat killed Mptb
3	C	C	Heat killed Mptb
4	D	D	Heat killed Mptb
5	E	E	Heat killed Mptb
6	F	F	Heat killed Mptb
7	G	G	Heat killed Mptb
8	H	PBS	Heat killed Mptb
9	Gudair®	As supplied by the manufacturer	Killed Mptb as supplied in the vaccine by the manufacturer
10	Negative control Unvaccinated	None	None

Note: Vaccines A to H were tested both as single dose and double dose (with a booster dose 4 weeks apart) in 2 groups of 5 animals each, making a total of 18 groups in the trial.

The vaccines were administered by subcutaneous injection, 1 mL dose, behind the right ear. At one month post primary administration, groups requiring a booster were given a second dose of the same vaccine. Gudair® vaccine was administered as a single dose according to the manufacturer's instructions on the right side of the neck.

Blood samples were collected from all animals immediately before vaccination and at 2, 3, 4, 5, 6, 7, 8, 10, 14, 18, 22 and 26 weeks post primary vaccination for antibody ELISA and the IFNγ assay.

The site of injection was monitored weekly until 10 weeks post vaccination and then monthly until 6 months post vaccination. The area around the injection site was palpated for the presence of any adverse reactions such as swelling, hard lumps, open lesions or abscess formation. Injection site lesions were defined as having a diameter greater than 0.5 cm, measured in one axis.

Results

Gudair® vaccinated sheep had a greater probability of having an injection site lesion than did sheep given the other vaccines (Table 20, Fig. 25). Sheep receiving two doses of the novel Mptb vaccines had a greater probability of having an injection site lesion present than did the animals that received only one dose (25% compared to 7%). There were also significant differences in the size of the lesions (Table 20). Gudair® vaccine produced larger lesions overall ($P<0.05$). The animals that received the double dose of the modified vaccines had significantly larger lesions than the sheep that received a single dose ($P< 0.05$).

Table 20. Injection site lesions in sheep given a single dose of the formulations of killed Mptb and different adjuvants.

Vaccine	No of animals observed with lesions	Number of lesion observations for the treatment group	Mean lesions size (CM)	Mean weeks to first recorded lesion	Mean weeks to last recorded lesion	Mean weeks between first and last lesion observation
A	3	15	1.3	2	21	19
B	1	2	0.9	1	6	5
C	1	1	0.8	3	1	1
D	1	3	1.1	2	26	24
E	1	11	1.3	1	26	25
F	1	3	2.0	8	14	6
G	2	22	1.4	1.5	26	24.5
H	0	0	0.0	-	-	-
Gudair	5	46	2.2	2.2	26	23.8
Unvaccinated	0	0	0.0	-	-	-

The immune responses of the sheep to the different vaccine formulations were different. Vaccines formulated with the adjuvants D and G did not stimulate an antigen-specific IFNγ response with either a single or a double dose. Vaccines B, E, and F resulted in an increased antigen-specific IFNγ response in sheep given a second dose. Vaccines A and C resulted in lower antigen specific IFNγ production after the booster vaccination compared to the group that were given a single dose. The antigen-specific antibody responses were significantly greater ($P<0.05$) in sera collected from Gudair® vaccinated sheep compared to sheep vaccinated with the other vaccine formulations.

Overall, the results indicated that the immune response profile to heat-killed Mptb antigen was altered by the adjuvant in the formulation. With no adjuvant, the heat-killed Mptb (Group H) did not induce a significant immune response, i.e. responses were similar to those found in the unvaccinated control sheep.

The development of injection site lesions was not always associated with a strong immune response. Of the sheep vaccinated with a single dose of the combination of killed Mptb and adjuvant G, injection site lesions were observed at 33% of the recordings, predominantly in 2 sheep, both of which had low levels of antigen-specific IFNγ and antibodies.

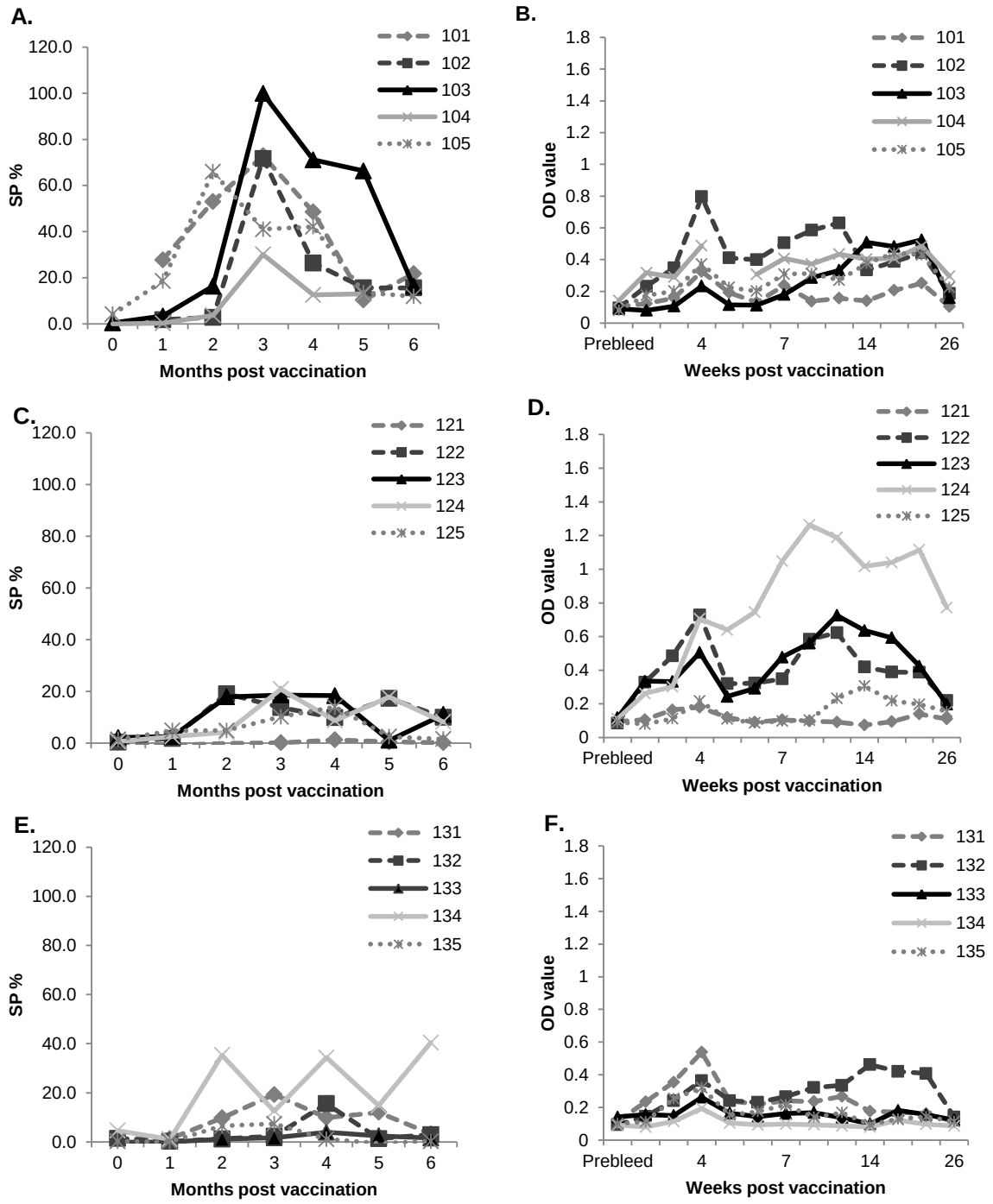

Fig. 25. Antigen specific IFNγ and antibody responses from individual animals vaccinated with formulations of Mptb and adjuvants. panel A: IFNγ responses, one dose of vaccine A; panel B: Antibody responses, one dose of vaccine A; panel C: IFNγ responses, one dose of vaccine E; panel D: Antibody responses, one dose of vaccine E; panel E: IFNγ responses, one dose of vaccine G; panel F: Antibody responses, one dose of vaccine G. SP% = sample-to-positive %.

Discussion

Testing the immunogenicity of the same mycobacterial antigen with different adjuvants is logical, and based on precedent in vaccine development. Immunisation of sheep with such formulations resulted in different immunological profiles. The immune response was also altered by the use of a second dose of the same vaccine. In this study, the testing of adjuvants was done with a complex whole cell mycobacterial antigen, and led to a range of unexpected results.

The theoretical optimal immune profile (i.e. the dogma) proposed for protection against mycobacterial infections including JD is a cell mediated/ IFNγ biased response. The commercially available Mptb vaccine provides incomplete protection, but results in strong mixed cellular IFNγ and humoral immune responses. This study has shown that by altering the adjuvant, different immunological profiles can be achieved ranging from cellular or humoral biased responses to mixed responses. Such widely differing immune responses to a single antigen have not previously been observed in JD vaccine development, due to the limited number of adjuvants that were tested previously. This finding has significance for JD vaccine development as novel antigens can be tested with a range of adjuvants. Furthermore previously evaluated and discarded antigens maybe now be re-examined to determine if an altered immune profile and protection can be established using different adjuvants.

All of the vaccines tested in this study resulted in fewer injection site lesions compared to Gudair®. These lesions are considered to be due to the interaction between the adjuvant, the antigen and the immune response of the host. However, there were a number of adjuvants where lesions were found in animals or groups with a low systemic immune response. One of the adjuvants, G, resulted in injection site lesions but the measurable acquired immune response in these sheep was negligible.

Conclusion

This study demonstrated that altering the adjuvant mixed with killed Mptb in a vaccine modifies the immune response and reduces the incidence of injection site lesions compared to Gudair®. With this knowledge should now be possible to test the efficacy of vaccines that produce different immunological profiles, such as those that create more of a cellular IFNγ or more of a humoral antibody profile.

4.24 Subprogram 3.3. To trial a prototype vaccine through experimental infection in sheep and cattle

This objective required a large body of systematic work: i) antigen discovery and confirmation of immunogencity, and ii) vaccine formulation and animal trials: P.PSH.0576.Ag, P.PSH.0576.2, P.PSH.0576.3, P.PSH.0576.4.

1. Antigen discovery and confirmation of immunogenicity

This aspect of the research program has been a long term endeavour linked through previous MLA projects (OJD.031, P.PSH.0311, P.PSH.0297) and PhD students and involved numerous collaborations to identify immunogenic proteins and lipids, as summarised in

Table 8. Briefly, studies by PhD students Sanjeev Gumber and Satoko Kawaji involved the identification and cloning of a range of Mptb stress/dormancy proteins, based on the hypothesis that stress and/or dormancy-associated pathways may be triggered during disease pathogenesis and amenable for diagnostics and vaccines (Gumber et al., 2009a, Gumber et al., 2009b, Gumber and Whittington, 2009, Kawaji et al., 2010, Kawaji et al., 2012a, Kawaji et al., 2012b). This work continued in the PhD program of Ratna Gurung who used *in silico* epitope analysis for the prediction of T and B cell epitopes to select 25 stress proteins (Gurung et al., 2012a, Gurung et al., 2012b). A meta-analysis of the published literature relating to the Mptb stressome was conducted mining available data archives to identify potential future targets for research, and synthetic peptides of Mptb stress-regulated proteins were also examined (Gurung et al., 2014c). Additional recombinant proteins that had been cloned in the USA and Canada were checked for immunogenicity through collaborations (Gurung et al., 2014a, Gurung et al., 2013, Gurung et al., 2014b) as were lipid antigens through the PhD program of Shyamala Thirunavukkarasu (Thirunayukkarasu et al., 2013). This body of research enabled selection a several candidate antigens for evaluation in novel vaccines, to demonstrate an approach for validation of subunit vaccines for Johne's disease using immune correlates of protection.

Further assessment was performed in cattle. Two hundred and ten serum samples from cattle were used for the analysis of antigens using ELISA. The 90 unexposed samples were collected pre-inoculation from the P.PSH.0297.2 and P.PSH.0576.4 trials and at the last 2 bleeds from the unexposed control animals. Samples collected from the Mptb inoculated animals that were experimentally exposed to Mptb in these trials comprised a further 80 samples. The remaining 40 samples were collected from a farm with natural infection. An in-house ELISA was developed using French pressed Mptb316v antigen generously provided by Elizabeth Macarthur Agricultural Institute, Australia and used to evaluate the sera. The naturally exposed animals had the highest immune responses to the candidate antigens but for every candidate antigen, several unexposed serum samples would have high responses. Setting a cut-point to include the 2 highest unexposed responses to the antigens meant that very few inoculated and naturally exposed animals were detected. It should be noted that the ELISA was not optimised for each antigen. Two immunogenic antigens MptbAg1 and MptbAg2 contained a higher number of T-cell epitopes than the other candidate antigens in -epitope prediction and were chosen to take forward to be formulated into vaccines. This information combined with the immunoreactivity of these antigens in exposed sheep and cattle indicated these would be "best bets" for the purposes of demonstrating a vaccine development pathway using immune correlates of protection.

2. Vaccine formulation and animal trials: P.PSH.0576.Ag, P.PSH.0576.2, P.PSH.0576.3, P.PSH.0576.4.

Animal trial P.PSH.0576.Ag in sheep

Introduction

Recombinant Mptb vaccines have merits over killed or attenuated vaccines in terms of antigen production and human safety. The most commonly evaluated recombinant proteins for Johne's disease have been Hsp 70, antigen 85, 74F, SOD, 35 kDa, mpt, 95 kDa, P22, 65 kDa and 16.8 kDa. Many of the recombinant vaccines were reported to induce strong cellular as well as antibody mediated immune responses. Some of the recombinant vaccines also

induced partial protection from infection. There is a need to evaluate more Mptb antigens to identify potential vaccine candidates.

We found MptbAg1 and MptbAg2 to be upregulated under *in vitro* stress conditions and *in silico* analysis suggested that MptbAg1 and MptbAg2 proteins contained relatively more T and B cell epitopes than other stress regulated Mptb proteins. These proteins were detected by antibodies and induced recall of cell mediated immune responses from Mptb infected sheep suggesting that they are expressed *in vivo* and recognised by the host immune system. The aim of this study was to evaluate cellular as well as humoral immune responses in sheep against the recombinant antigens MptbAg1 and MptbAg2 when they were administered in various antigen/adjuvant formulations.

Methods

A total of 34 Merino wethers, between 24 and 36 months of age were randomly divided into eight groups of four animals for vaccination and the remaining two sheep were used as unvaccinated controls. Recombinant Mptb antigens MptbAg1 and MptbAg2 were cloned expressed and purified as previously described and mixed with four adjuvants for formulation of recombinant vaccines (Table 21). The animals were given 1 ml of the required vaccine subcutaneously behind the left ear. Four weeks after primary immunisation, a booster was given on the right side of the neck. Blood samples were collected regularly after immunisation and injection site areas were palpated and lesions recorded. Blood tests conducted were IFNγ and antibody ELISA.

Table 21. Vaccine composition and the number of animals with cellular and humoral immune responses to recombinant vaccine.

Group	Antigen	Adjuvant	No. of animals responding to vaccine			
			IFNγ response		Antibody response	
			A[1]	B[2]	A[1]	B[2]
I	MptbAg1	B	1	1	0	3
II	MptbAg1	C	0	2	0	2
III	MptbAg1	E	0	0	0	4
IV	MptbAg1	F	0	3	0	2
V	MptbAg2	B	1	1	4	4
VI	MptbAg2	C	2	3	4	4
VII	MptbAg2	E	0	0	3	4
VIII	MptbAg2	F	1	0	3	4

[1]316V specific response
[2]Vaccine antigen specific response

Results

Mptb 316v specific IFN-γ responses of the sheep in response to vaccination with the formulation of MptbAg1 were similar between the adjuvants (Table 21). The sheep vaccinated with MptbAg2 + adjuvant C showed the highest Mptb 316v specific IFN-γ responses among the different formulations. However, great variation was observed between the groups of sheep vaccinated with MptbAg2 vaccines depending on the adjuvant.

The vaccine antigen (MptbAg1) specific IFN-γ response was highest for formulations prepared from MptbAg1 + adjuvant C and MptbAg1 + adjuvant F (Table 21). Similarly,

formulations prepared from MptbAg2 + adjuvant C induced the highest vaccine antigen specific IFN-γ responses followed by the MptbAg2 + adjuvant B.

The humoral immune response to recombinant vaccine was evaluated by measuring Mptb 316v and vaccine antigen specific serum antibody levels. Mptb 316v antibody responses from sheep vaccinated with MptbAg1 and all four adjuvant combinations were low. The Mptb 316v antibody responses from the vaccines formulated with MptbAg2 and all four adjuvants showed significant responses which remained high until 8 weeks post primary vaccination.

The vaccine antigen specific antibody responses were strong for MptbAg1 as well as MptbAg2 vaccines. The formulations of MptbAg1 with the four adjuvants showed similar patterns of vaccine antigen specific antibody responses. In contrast, antibody responses to MptbAg2 formulations were similar to those of Mptb 316v specific antibody responses and were significantly higher than those pre-vaccination at all sampling time-points.

The protective level of IFN-γ response using a validated animal infection model was $\geq$ 38% SP of IFNγ response (this project). A cut-point of 70% SP antibody as an indicator of humoral immune response to exposure was considered. These two thresholds were used to examine the number of animals responding to recombinant vaccine. A higher proportion of animals had responses to MptbAg1 + adjuvant C, MptbAg1 + adjuvant F and MptbAg2 + adjuvant C 61VG. The proportion of animals showing vaccine antigen specific IFN-γ responses was higher than that of Mptb 316v specific responses. None of the animals that received MptbAg1 vaccine formulations showed Mptb 316v specific antibody responses > 70% SP.

Lesion prevalence was analysed for each antigen/adjuvant combination. The majority of the animals (75%) developed injection site lesions > 0.5 cm. The animals that developed the most lesions were those vaccinated with MptbAg2 + adjuvant C (87.5%) and MptbAg2 + adjuvant F. Average lesion prevalence in animals vaccinated with MptbAg1 (33%) was lower than in those vaccinated with the MptbAg2 vaccines (45%) and lesions were smaller.

Except for the animals vaccinated with MptbAg1 + adjuvant C, the mean lesion size for all other adjuvant groups were smaller than 2.2 cm and decreased over the study period. The mean lesion size for MptbAg1 + adjuvant C was 2.35 cm and rapidly decreased over the study period. In animals that received MptbAg1 + adjuvant F, the injection site lesions had completely resolved by the end of the study period. The injection site lesion recovery in animals that received MptbAg2 vaccine was similar between different formulations. The mean lesion size was greatest for the MptbAg2 + adjuvant C group and persisted at 1.73 cm until the end of the study period. Some of these lesions resulted in wool loss and a discharging sinus at the injection sites in animals vaccinated with MptbAg2 + adjuvant C.

Discussion

The evaluation of recombinant Mptb antigens as vaccine candidates in this study was focused on cell mediated and humoral immune responses in sheep following vaccination with different formulations prepared from combinations of recombinant antigens and four different adjuvants. Expression of cytokines such as IFNγ is believed to contribute to protection against intracellular pathogens including Mptb but it is not known what level of response is adequately protective. In this study the recombinant vaccine formulations were found to induce strong IFNγ responses, as high as 90% SP, in some vaccinated animals.

The assessment of vaccine formulations in this study was based on five criteria: (a) antigen specific IFNγ response, (b) Mptb 316v specific IFNγ response, (c) adjuvant effect on IFNγ response, (d) injection site lesions and (e) antibody response. Recombinant vaccine formulated with MptbAg1 antigen and adjuvant F induced stronger Mptb 316v as well as recombinant antigen specific IFNγ responses in vaccinated animals compared to the unvaccinated controls and pre-vaccination. The vaccine also produced lower lesion prevalence and severity compared to other formulations.

Lesions at the vaccine injection site have been observed in a high proportion of animals following vaccination against paratuberculosis. It is not just the adjuvants that are responsible for the injection site lesions but the combination between adjuvant and antigen. Sheep vaccinated with some of the MptbAg1 formulations showed substantial levels of IFNγ responses and low prevalence of injection site lesions. The vaccines formulated with MptbAg2 showed a strong antibody response for all adjuvant groups and caused more severe lesions.

Conclusion

Due to the likely protective immune function of IFNγ and the relatively lower degree of lesion development at the site of vaccine injection from MptbAg1 + adjuvant F vaccine compared to that of other formulations, this vaccine should be evaluated further in sheep. A longitudinal study in a larger cohort of animal is required to evaluate whether it induces protective immunity against Mptb infection.

Animal Trial P.PSH.0576.2 in sheep

Introduction

From the results of the P.PSH.0576.Adj trial, four novel vaccines were selected that promoted different immune responses in sheep, from weak to strong mixed responses and biases towards Th1 (IFNγ) or Th2 (antibody) type responses and these were given to 4 month old Merino lambs, and compared with a Gudair® control group. The sheep were then inoculated with Mptb and the disease outcomes were monitored to assess protective immune responses associated with vaccination.

Methods

One hundred and eighty Merino wether lambs aged 4 months were randomly allocated into 7 treatment groups (Table 22).

Table 22. Treatment groups of the sheep in trial P.PSH.0576.2

Vaccine Treatment Group	Antigen	Adjuvant	No. of doses (1mL)	No. of sheep inoculated (No. controls)
Unvaccinated (Inoculation control)	-	-	-	20 (10)
Gudair®	Mptb	Gudair	1	20 (5)
Treatment 1	Mptb	A	1	20 (5)
Treatment 2	Mptb	E	1	20 (5)
Treatment 3	Mptb	B	2	20 (5)
Treatment 4	Mptb	F	1	20 (5)
No adjuvant control[a]	Mptb	None	1	20 (5)

Gudair® (Zoetis, Australia) was administered as per the manufacturer's recommendations. [a] No adjuvant control contained only heat killed Mptb in PBS.

Primary vaccinations were given to lambs in all treatment groups (1-4), as well as the Gudair® and No Adjuvant control groups. One month after the primary dose, a booster dose was given to the animals in Treatment group 3 to create a predicted strong mixed Th1/Th2 response, based on findings in section 4.23 (P.PSH.0576.adj trial). Three oral Mptb inoculations over one month commenced 6 weeks after primary vaccination, or 2 weeks after the booster vaccination. Blood and faecal samples were collected at regular intervals (2-4 months). Sheep were culled from the experiment if they lost 10% of their body weight or more in a one month period. All remaining animals were culled at 13-14 months post inoculation and tissues were sampled. Tissue cultures, histopathology, faecal HT-J, IDEXX ELISA, IFNγ assay, and lymphocyte proliferation assays were performed.

Inoculated animals were classified as infected if one tissue collected at necropsy was culture positive for Mptb. An animal was classified as having clinical disease if it lost greater than 10% of its body weight over a 1 month period and Mptb was recovered from tissues. If no tissues could be obtained (one animal), Mptb faecal shedding at the last sampling prior to death was used to determine infection status.

Results

The clinical disease rates for the different novel vaccines indicated that they provided partial protection. The vaccine that gave best protection against the development of clinical Johne's disease was Gudair®. Only one of 20 (5%) Gudair® vaccinated inoculated animals developed clinical disease and this animal was shedding high numbers of Mptb in its faeces. The worst clinical case rate was seen in the inoculated control group (no vaccination), in which 45% of the animals were removed from the trial. In the no adjuvant control group, where only heat killed Mptb was administered, 25% of the animals developed clinical disease. The prototype vaccine treatments tested had disease rates of between 15-20%, i.e. less than half that seen in the unvaccinated animals.

Recovery of Mptb from the tissues by culture showed that between 20 and 80% of inoculated animals were infected with viable Mptb at the conclusion of the trial. The inoculated control group had the highest tissue infection rate. Animals given the vaccine Treatment 2 had the highest infection rate of vaccinated treatment groups at 65%. Gudair® vaccinated animals had the lowest infection rate at 21%, while the novel vaccine treatments had between 40-45% of animals infected at the conclusion of the trial.

Histopathological lesions were most frequently observed in the inoculated control group followed by the animals given in Treatment 2 (Table 23). The lesion rate was the lowest for the Gudair® vaccinated group (5%) while the novel vaccine formulations had 45-52% of the animals with lesions.

Table 23. Severity of histological lesions from animals given different Mptb vaccine treatments.

	Lesion Score					
	0	1	2	3a	3b	3c
Inoculated control	20	5	5	10	55	5
Gudair®	68.4	26.3	0.0	0.0	0.0	5.3
Treatment 1	50	5	0	10	10	25
Treatment 2	20	15	15	5	35	10
Treatment 3	40	15	15	0	25	5
Treatment 4	42.1	5.3	15.8	5.3	21.1	10.5
No Adjuvant	30.0	20.0	0.0	10.0	35.0	5.0

Data are % of sheep

Faecal culture positives were seen in all groups and the results aligned closely with the percentage of tissue culture positives in the different treatment groups. The HT-J faecal qPCR results showed that the level of shedding was at high levels in all groups, with a similar overall profile independent of whether the animals were vaccinated or unvaccinated. The major contributors to the total quantity of Mptb DNA were the clinical cases, though multiple individuals in each treatment group were positive in the HT-J test. All uninoculated controls were HT-J negative.

Gudair® produced the strongest IFNγ and antibody responses specific to Mptb. The novel vaccines all elicited a similar elevated Mptb-specific IFNγ response, compared to the control groups (unexposed and no adjuvant), with profiles that were more similar to each other than expected.

There was variation between the mean Mptb specific antibody responses from the sheep vaccinated with the different novel vaccines. The Treatment 2 group had a trend towards a higher antibody response, as predicted by the earlier adjuvant study. The Treatment 1 animals had a higher than expected antibody response. Sheep in Treatment 3 had a low antibody response and those in treatment 4 were the lowest. Unsurprisingly, the No adjuvant control group and unvaccinated unexposed control animals had low background IFNγ and antibody responses.

Gudair® vaccination induced the strongest Mptb-specific lymphocyte proliferative response and similar to previous work proliferation was lower in vaccinated infected sheep compared to vaccinated uninfected sheep. In addition, for all adjuvanted vaccine formulations tested, CD4+ T cell proliferation was lower in the vaccinated infected animals compared to the vaccinated non-infected animals. Of the other vaccine formulations tested, Treatment 1 and Treatment 2 showed the highest Mptb-specific total lymphocyte proliferation. CD4+ T cell proliferation was induced by Treatments 1, 2 and 4. B cell proliferation was induced by the Treatment 4 vaccine and γδ T cell proliferation by the Treatment 1 vaccine. None of the adjuvanted vaccine formulations tested enhanced the Mptb-specific proliferative response of

all the lymphocyte subsets considered important for a protective immune response, using Gudair® as the standard for comparison.

The incidence of injection site lesions were measured at 2 and 6 months post inoculation. The Gudair® vaccinated group had the most lesions, with 32% of the animals affected. Treatment 3 resulted in 12% of the animals with injection site lesions, Treatment 2 resulted in 4% lesions. In the other novel vaccine and control groups no injection site lesions were observed. One of the Gudair® vaccinated animals at necropsy had an open caseo-liquefactive injection site lesion (Fig. 26).

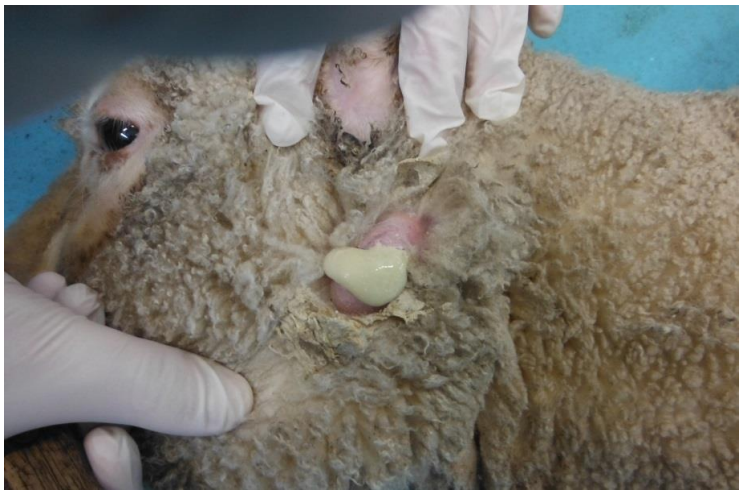

Fig. 26. An injection site lesion from animal 2015, 15 ½ months post vaccination with Gudair®.

Discussion

Protection against Johne's disease and Mptb infection was best achieved in this trial by the use of Gudair® vaccination. The commercial Gudair® vaccine performed according to expectations with a 89% reduction in clinical disease compared to the unvaccinated cohort (5% versus 45% respectively). Infection and histological lesions were also reduced by Gudair® . It is known that Gudair® does not prevent infection or disease and has other problems such as a high number injection site lesions which can be an animal welfare and human safety issue. A high number of injection site lesions was observed in this trial. The injection site lesions observed in Gudair® vaccinated sheep are due to the adjuvant used in the vaccine because injection of Mptb alone does not cause them.

The No Adjuvant control group offered some protection against disease. Just by injecting a tenth of the amount of Mptb used in Gudair® mixed with saline, a reduction in disease rates of 44% was observed and infection rates were reduced by 25%. This indicated that heat killed Mptb alone is sufficient to elicit a mild protective response.

Interestingly the immune responses generated in this trial did not match those in the P.PSH.0576.Adj trial. The immune responses appeared to be more Th1 or IFNγ biased or mixed responses than Th2 biased. The reason for these differences may be in the age of the sheep (4 month old in this trial, 2-3 years of age in previous trial). Another difference is that the previous trial did not involve live Mptb challenge, and this factor alone could explain the

differences. We know that Mptb infection generates a Th1 response. This may have skewed the responses of all vaccines towards IFNγ.

A protective vaccine-induced immune response involves CD4+ T cells, B cells and γδ T cells (see this report above). In all the adjuvanted vaccine formulations tested, CD4+ T cell proliferation was lower in vaccinated infected animals compared to the vaccinated non-infected animals. Thus similarly to Gudair® vaccine, none of the vaccines tested were able to maintain an Mptb-specific CD4+ T cell proliferation when disease progressed. This immune parameter is an important one that should be checked in future short-term vaccine studies as it will indicate if the vaccine being tested is likely to provide a protective immune response superior to that of Gudair®. Unlike the other vaccine formulations, B cell and γδ T cell proliferation was elevated in vaccinated infected animals compared to non-infected animals given the Treatment 3 and 4 vaccines. While this indicates that there are other as yet unknown factors influencing vaccine-induced protection, these two formulations certainly boost immune parameters that are considered important for protection based on the Gudair® study. Like Gudair® they did not prevent infection.

Gudair® contains approximately 10 times the number of Mptb per dose than was used in the novel vaccines. This is likely to explain the stronger immunogenicity of Gudair®, but not the injection site lesions. While the novel vaccines may not be as good as Gudair®, they did provide some protection which was achieved with minimal injection site lesions. Improvements can be made to these novel vaccines. This study is indicative of the integration of design aspects required. From this study, we know that greater than 50% protection can be achieved with non-optimised vaccines. The results show that some of the adjuvants mixed with Mptb provided better protection than others. Treatment 2 vaccine, provided the least amount of protection, and should not be used for further studies. The Treatment 1 vaccine, also cannot be used for further studies because the manufacturer has discontinued production of this adjuvant due to biosecurity issues. This leaves Treatment 3 and Treatment 4 as possible candidates for further research. Treatment 4 is the best candidate as it had the greatest amount of protection of these two novel vaccines. Further research should include altering the Mptb adjuvant ratio, adjusting the number of killed bacteria in each dose and the number of doses given. These studies would require further examination of injection site lesion development as well as protective efficacy using the immune predictors of efficacy developed in this project.

Animal Trial P.PSH.0576.2 (subset) in sheep

Introduction

The aim of this trial was to examine the efficacy of a prototype candidate subunit antigen vaccine in sheep in the context of the new vaccine evaluation strategy.

Methods

The study was designed by including an additional group in trial P.PSH.0576.2, such that the control groups were common. Two groups were those from the P.PSH.0576.2 trial, and a third group was added, Treatment 5 (Table 24). All these sheep were managed concurrently. All other aspects were as for the P.PSH.0576.2 trial.

Table 24. Vaccine candidate antigen study treatment groups of the sheep in trial P.PSH.0576.2.

Vaccine Treatment Group (All animals were Mptb exposed)	Antigen	Adjuvant	No. of doses	No. of sheep inoculated (No. of controls)
Unvaccinated (Inoculated control)	-	-	-	20 (10)
Gudair®	Mptb	Gudair®	1	20 (5)
Treatment 5	MAPAg1	F	2	20 (5)

Results

The clinical disease rates of the animals given the vaccine containing candidate antigen MAPAg1 (Treatment 5) 39%, was slightly lower compared to that for the inoculated controls, 45%. A similar pattern was seen for the other parameters of infection recorded such as the percentage of animals with Mptb recovered from their tissues, disseminated infection rate, and the distribution of histological lesions. Faecal culture positives were seen in all groups, aligning closely with the results of tissue culture. Faecal shedding at high levels was seen in all groups, with major contributors to the total quantity of Mptb DNA being the clinical cases.

Immunological testing on blood from the vaccinated animals showed that the treatment 5 vaccine induced IFNγ specific responses 1 month after primary vaccination. Treatment 5 vaccination did not induce a detectable whole Mptb (316v)-specific IFNγ or antibody response in the absence of exposure. This was in strong contrast to Gudair® vaccinates.

Discussion

There was minimal protective efficacy of the Treatment 5 vaccine when compared to the unvaccinated controls, in contrast to Gudair® vaccination. This trial was of 13 months duration, which was several years shorter than that usually undertaken for efficacy testing in the context of Johne's disease. We have shown that this trial duration is sufficient to assess vaccine efficacy, using the experimental infection model developed at the University of Sydney, in this case ruling out a candidate antigen relatively quickly.

Australia is in a unique position having a validated infection model for Johne's disease in the natural host, where animals can be held until the development of clinical disease. This is an important tool to which many other research groups do not have access. An important aspect of the vaccine trials conducted in this project was to develop the tools and processes to enable future vaccine trials to be conducted, using this infection model as well as indirect measures of vaccine efficacy. Other research groups have potential vaccine candidates but due to the short nature of their trials or the animal species used efficacy data is lacking. Future research could be aimed at utilising both international research and examination of other protein candidates to find improved vaccine options.

Animal Trial P.PSH.0576.3 in sheep

Introduction

There are no published data on repeatability of Johne's disease vaccine efficacy. Such information is critical to have confidence in vaccine development in general and evaluation of candidate antigens and adjuvants in particular. Two prototype vaccines that appeared to

have differing outcomes in P.PSH.0576.2 were examined for their protective efficacy. Appropriate control groups of Unvaccinated and Gudair® vaccinated animals were included. The immune response of sheep to all of the remaining prototype vaccines without infection challenge was examined in small groups, to determine if Mptb specific antibody and IFN-□ responses were similar to those in the previous trial.

Methods

One hundred and fifteen Merino sheep aged 4 months were randomly allocated into 7 treatment groups, with four groups containing 25 sheep and three groups of five sheep (Table 25).

Table 25. Treatment groups of the sheep in trial P.PSH.0576.3

Vaccine Treatment Group	Antigen (HK Mptb strain)	Adjuvant	No of doses (1mL)	No. of Sheep inoculated (No. controls)
Unvaccinated (Inoculation control)	-	-	-	20 (5)
Gudair®	Mptb	Gudair	1	20 (5)
Treatment 1	Mptb	A	1	0 (5)
Treatment 2	Mptb	E	1	20 (5)
Treatment 3	Mptb	B	2	20 (5)
Treatment 4	Mptb	F	1	0 (5)
No adjuvant control[a]	Mptb	None	1	0 (5)

[a] No adjuvant control contained only heat killed Mptb in PBS.

Vaccines were injected subcutaneously behind the left ear of each animal by a single operator. One month after the primary dose, a booster dose was given to the Treatment 3 animals to create a predicted strong mixed Th1/Th2 response, based on findings in section 4.23 (P.PSH.0576.adj trial). Sheep were challenged by oral dosing with Mptb 6 weeks after primary vaccination or 2 weeks after the booster vaccination. Three doses were delivered over a one month period. Blood and faecal samples were collected at regular intervals (2-4 months). Sheep were culled from the experiment if they lost 10% of their body weight or more in a one month period. All remaining animals were euthanased at 12 months post inoculation. Tests conducted were histopathology and tissue culture, faecal culture, faecal HT-J, IDEXX ELISA and IFN-γ. Other methods were as described above for the previous trial. Inoculated animals were classified as infected if one tissue was culture positive for Mptb. An animal was classified as having clinical disease if it lost greater than 10% of its body weight over a 1 month period, had gross and histological lesions associated with Johne's disease and Mptb was recovered from tissues.

Results

In this trial only two animals developed clinical disease signs of weight loss; both of these animals were from Treatment 3 group and were detected at 12 months post inoculation.

Infection, as determined by histological lesions and culture of Mptb from tissues, was most prevalent in the inoculated controls. Gudair® provided the best protection with 10% of the inoculated animals having infection as determined by culture or histology. The two novel vaccines had infection rates of 40-42% as determined by tissue culture and 37-35% of animals with histological lesions. There was little difference between the two prototype

vaccines (Treatment 2 and Treatment 3) and the inoculated control group in the percentage of animals with more severe histological lesions 3b and 3c.

Faecal shedding was less prevalent in this trial compared to trial P.PSH.0576.2. However, faecal culture positives were seen in all groups. As an example, the unvaccinated inoculated control group had 13 faecal culture positives at 9 months post inoculation, compared to 6 faecal culture positives within the same treatment group at 12 months post inoculation in this trial.

The HT-J faecal qPCR results were interesting in that, despite lower clinical disease rates and a lower prevalence of faecal culture positives in this trial compared to the previous trial (P.PSH.0576.2), there was still a high burden of Mptb shed as evidenced by the quantity of DNA detected. The number of HT-J positives was less than in the previous trial, similar to the finding for culture positives.

All of the novel vaccines induced an Mptb specific IFN-γ response to vaccination. Animals given the Treatment 2 vaccine had the lowest mean IFN-γ response of the vaccinated animals. This was lower than that seen in the No adjuvant control group. With the exception of the Gudair® treated sheep, the animals produced very little Mptb specific antibody in response to vaccination only. Of the novel vaccines, the Treatment 3 had the highest Mptb specific antibody response.

Discussion

The aim of this trial was to repeat the P.PSH.0576.2 study on a smaller scale to check repeatability. However, slower development of disease was seen than in the P.PSH.0576.2 trial: slower IFN-γ, antibody response and clinical disease onset. In the P.PSH.0576.2 trial clinical disease was observed as early as 8 months post inoculation while in this trial the first cases were seen at 12 months. The reason for the slower disease development would have been predominately due to the lower inoculation dose of Mptb, an almost 100 fold reduction. Slower development of disease may have resulted in the reduction of infection seen in the unvaccinated inoculated controls, Gudair® and Treatment 2 animals.

Without Mptb infection, the immune response generated in response to the various vaccines differed slightly compared to previous trials. Treatment 1 and Treatment 2 groups had lower mean antibody responses than those seen in trial P.PSH.0576.2, while the responses of the Treatment 3 group were about the same. The mean IFN-γ response of the animals in Treatment 2 was also lower in this trial, although the pattern of response in inoculated animals was similar to that seen in trial P.PSH.0576.2. All of the novel vaccine treatments had low Mptb-specific antibody responses and this was very similar to the results from the previous trial. As expected, Gudair® vaccination provided the best protection. This was followed by Treatment 3 and Treatment 2, which was the same hierarchy seen in trial P.PSH.0576.2.

Small differences were seen in the responses to the different vaccines, including Gudair®, between the two trials but ultimately the order of protective efficacy of the vaccines was the same indicating reproducible results can be achieved even with differences in the rate of disease development.

Animal Trial P.PSH.0576.4 in cattle

Introduction

This trial was designed to test a novel vaccine in calves as a demonstration of the new strategy for evaluating vaccine efficacy using the experimental infection model and immune correlates of protection. The formulation of the novel vaccination for the cattle in this trial was informed by the P.PSH.0576.Adj adjuvant trial and the P.PSH.0576.2 prototype vaccine trial in sheep. From the P.PSH.0297.2 trial, there was evidence that we could experimentally infect cattle with Mptb, but clinical disease was not expressed in a 4.5 year time frame. So that we might be able to observe differences between the treatment groups, a decision was made to use a higher dose of cattle (C) strain Mptb for oral inoculation. This was aimed at achieving a higher rate of infection and possibly other disease parameters.

Methods

Thirty calves were sourced from the University of Sydney dairy farm in Camden, and allocated into two groups of 15. One group was vaccinated with a single 1 ml dose of cattle vaccine CV1 subcutaneously behind the right ear. One month later the animals from each group were divided into 2 groups, one of 5 Unexposed controls (n=5) and another to be inoculated with Mptb (n=10) (Table 26). The 20 animals to be inoculated were dosed orally using the same schedule as described previously using a cattle strain of Mptb.

Table 26. Treatment groups of cattle in trial P.PSH.0576.4.

Vaccine Treatment Group	Antigen	Adjuvant	No. of doses (1mL)	No. of cattle inoculated (No. controls)
Unvaccinated control	-	-	-	10 (5)
CV1	Mptb	B	1	10 (5)

From each animal blood and faecal samples were collected at regular intervals (1-3 months). All animals were monitored by visual inspection, more than three times weekly. All animals were culled at 9 months post inoculation. Tests conducted were histopathology, culture of tissues, culture of faeces, IDEXX ELISA, IFNγ.

Results

All animals tolerated the vaccination well and there were no adverse reactions or injection site lesions observed.

Vaccination with CV1 induced a weak antibody response detectable in the vaccinated control (uninoculated) animals and also the vaccinated inoculated animals from 3 months post-vaccination (2 months post-inoculation). The CV1 vaccinated cattle that were inoculated with live Mptb tended to have a stronger antibody response than all other groups (Fig. 27). The unvaccinated inoculated animals had low levels of antibody that increased from 6-9 months post-inoculation. Two animals, both inoculated, one vaccinated and the other not, had a positive test result in the IDEXX ELISA.

The IFNγ responses were stronger after vaccination and the CV1 vaccine induced a strong early response in association with exposure (Fig. 28). There was a higher IFNγ response in

vaccinated animals that were not exposed to Mptb compared to unvaccinated control animals that were not exposed to Mptb.

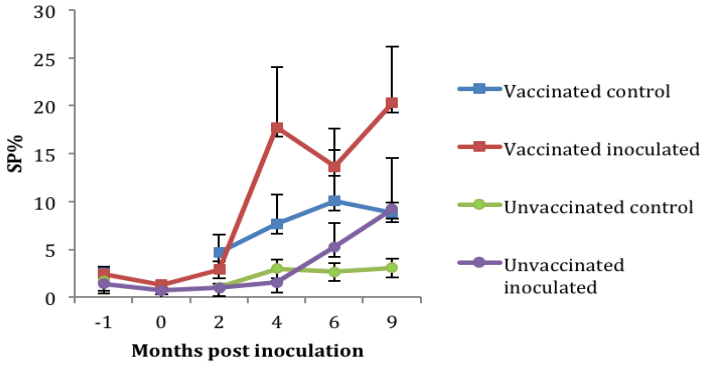

Fig. 27. Mptb-specific antibody responses from animals in the P.PSH.0576.4 trial in which cattle were vaccinated with a prototype vaccine and inoculated. Error bars show the standard error of the mean.

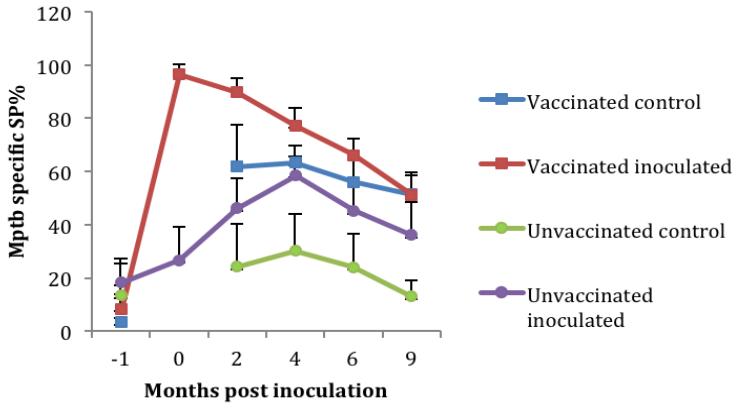

Fig. 28. Mptb-specific IFNγ responses from the trial P.PSH.0576.4 in which cattle were vaccinated with a novel vaccine and inoculated. Error bars show the standard error of the mean.

Faecal shedding of Mptb was seen intermittently from 2-6 months post inoculation but only in the inoculated animals. Of the 8 shedding animals, 7 were from the unvaccinated control group. The vaccinated animal that shed was faecal culture positive at 2 months post inoculation but not at 4, 6 or 9 months.

Histopathological lesions were observed from the Mtpb inoculated cattle. These were in 6/10 unvaccinated animals and 1/10 vaccinated cattle. The lesions were more severe in the unvaccinated animals with half the inoculated animals having 3a lesions. Only one vaccinated Mptb inoculated animal had a 3a lesion. All 6 of the unvaccinated animals with lesions were found to be faecal shedding, but not the vaccinated animal with the 3a lesion.

In both the vaccinated and unvaccinated groups, 9/10 Mptb inoculated animals had Mptb cultured from their tissues. Mptb was not cultured from any of the tissues of the animals not exposed to Mptb.

Discussion

In this trial, vaccination with prototype vaccine CV1 was shown to give a positive benefit in terms of immunological markers and disease severity measures.

The results from this trial indicate that the results from the P.PSH.0297.2 and P.PSH.0576.2 trials were useful. After the P.PSH.0297.2 trial, a decision was made to increase the inoculation dose by an estimated 10 times. This resulted in an increase in infection rate. While it is not possible to compare the two cattle trials directly due to the difference in length of the trials and consequently the times when the necropsy or biopsy samples were collected, it seems that trial P.PSH.0576.4 had more infected animals, with 5/10 of the unvaccinated, inoculated cattle at 9 months having lesions as severe as those seen at the end of trial P.PSH.297.2 after 4.5 years. For vaccine efficacy studies we recommend the oral dose used in P.PSH.0576.4.

Cattle vaccinated with CV1 had a markedly enhanced IFNγ response to exposure and also showed elevated antibody responses. The early increase in IFNγ response is of great interest, as an early peak in expression of this cytokine was identified from our predictive studies as a key marker of protection. Based on the P.PSH.0576.adj adjuvant trial data a single dose of vaccine with the B adjuvant was chosen due its ability to induce an IFN-γ response and antibody response. Another decision was made based on immune profile of the P.PSH.0576.2 sheep to also increase the dose of heat killed Mptb in the vaccine to the level used in Gudair®. These decisions, based on the evidence from sequential trials and a stable trial design, have been shown to have an extremely beneficial impact.

The histological and faecal shedding results indicate the animals vaccinated with CV1 had less severe infections and appeared to be protected from disease. The infection rate in this trial was 90%. This high number of tissue culture positives in both treatment and control groups is supportive of the argument that the vaccine is protective, because it is clear that there was tissue invasion/infection in the vaccinated animals but with reduced lesions and faecal shedding. However, without leaving the animals for longer until clinical disease became evident we cannot estimate what the recovery or progression rate might be. A trial duration of 12 months instead of 9 months is recommended if further research is undertaken.

The prototype CV1 vaccine appears to display the traits of a desirable vaccine: protects against disease, reduces faecal shedding and does not cause injection site lesions. The results from this trial indicate that the predictive tools, experimental animal models and vaccine design strategies that have been developed and applied in project P.PSH.0576 have strong potential to lead the development of a safer, more effective vaccine.

4.25 Subprogram 5. Research capacity for livestock health

This subprogram addressed the following project objective:

To maintain and develop capacity in livestock health for the benefit of Australian producers through the training of 4 post doctoral fellows and four postgraduate students whose skills and knowledge will be applicable and available to the broader veterinary and agricultural research community as specialist immunologists, pathologists and microbiologists.

Training and human capacity building

Four post doctoral fellows were associated with and responsible for subprograms within the large project, ensuring a unified approach across complex disciplines. This led to research momentum and diverse outcomes.

Over the course of the project, the research team sought to maintain and develop capacity in livestock health through the training of graduate and postgraduate students whose skills and knowledge will be applicable and available to the broader veterinary and agricultural research community as specialist immunologists, pathologists and microbiologists.

Through the research environment created around the P.PSH.0576 project, five PhDs were awarded in Johne's disease. Note that the student candidatures do not align with the start and end dates of particular research projects. Consequently there has been overlap of students across projects P.PSH.0311, P.PSH.0297 and P.PSH.0576. Each PhD is a minimum 3 year duration progam, typically 3.5 to 4 years. The graduating candidates and their projects were:

- Dr Ratna Gurung carried out research on the immunogenicity of Mptb stress-related proteins.
- Dr Shyamala Thirunavukkarasu carried out research on the involvement of pathogen and host associated lipids in the macrophage immune response to Mptb.
- Dr Satoko Kawaji completed her PhD on the pathogenesis and diagnosis of Johne's disease with emphasis on subclinical infection.
- Dr Kate Bower completed her PhD on aspects of bacteraemia and dissemination in Mptb infection.
- Dr Sally Brown completed her PhD on apoptotic responses during the pathogenesis of Mptb in sheep.

Current PhD students

- Hannah Pooley is studying for a PhD on indirect vaccine efficacy measures and macrophage function in Johne's disease following the award of a prestigious MLA Scholarship.
- Matt Johansen is a PhD candidate studying pathogenesis of Johne's disease following the award of an APA Scholarship.
- Kamal Acharya is currently studying for his PhD on disseminated infection in animals with Johne's disease. He is also conducting a survey of the opinions of Australian veterinarians towards the disease.

In addition to PhD training, eight honours students have carried out short-term research projects associated with the P.PSH.0576 research project:

- Emma Gulliver completed BVSc Honours on the histopathology of the delayed type hypersensitivity (skin test) reaction in Johne's disease.

- Kathyrn Wright completed Bachelor of Animal and Veterinary Bioscience (AVBS) Honours on the expression of Toll like receptor and major histocompatibility complex associated genes in Johne's disease using an *in vitro* cell infection model.
- Portia Westall completed AVBS Honours on the candidate MHC II genes associated with susceptibility or resistance to paratuberculosis in Australian sheep.
- Kylie Wai completed AVBS Honours focused on the outcomes from the breed susceptibility trial, focusing on diagnostic test outcomes for clinically diseased sheep from different breeds.
- Assyl Naddi completed an AVBS Honours on the selection of an appropriate adjuvant for vaccine development.
- Hannah Pooley completed AVBS Honours examining immune response and efficacy of novel Johne's disease vaccines in sheep and has since continued this research as she studies for a PhD.
- Matt Johansen completed AVBS Honours on the change in host cell gene expression related to cholesterol catabolism associated with Mptb infection in Johne's disease. Matt continues with the group as a PhD candidate.
- Shannon Smith completed AVBS Honours examining immune responses and efficacy of novel Johne's disease vaccines in sheep.

The University of Sydney Johne's disease laboratory hosted two DVM student recipients of the Tufts University Cummings School of Veterinary Medicine Summer Vacation Studentship:

- Ms Yamini Chalam completed research investigating differential antibody responses of Mptb exposed sheep from different breeds.
- Ms Carla Stoffel completed research to evaluate genetic predisposition to Johne's disease in gut and lymph node derived tissues from Mptb exposed Australian sheep breeds through analysis of gene expression.

The research group sponsored a summer scholarship program for Faculty of Veterinary Science undergraduate students in the 2nd and 3rd years of the BVSc and BVAnBioSc degree programs and each year up to 2 students spent the summer months working in the project to gain hands on experience with research.

All graduate, honours, and undergraduate students participated in both field and laboratory research to obtain a well rounded experience.

Physical and staff resources

An invaluable fully documented and traceable sample archive containing blood, serum, tissue (lymph node, intestine, liver, skeletal muscle, and bone marrow), faeces, Mptb strains, nucleic acid (DNA and RNA and microRNA) and a legacy dataset has been developed. A data archive has been created including time point specific immune parameters, gene expression data, genome wide SNP and MHC sequence allelic variants.

We have made investments in PC2 laboratories and purchased cutting-edge laboratory equipment. We have also made investments in infrastructure on the research farm at Camden including fencing, shade, water reticulation and other improvements for pasture-based research in both sheep and cattle. Funding for all of this has been sourced externally (not through MLA).

We maintain a highly skilled group of professional and technical staff capable of carrying out diagnostic tests for Johne's disease as well as other specialised techniques that can be utilised for research on this and a variety of other livestock health issues. Techniques include pathology, microbiology, quantitative PCR, ELISA, flow cytometry, cell mediated immune assays, fluorescence microscopy, transcriptomics and proteomics.

Services to other laboratories

The research laboratory manufactures culture media for Johne's disease and distributes it to animal health labs across Australia. Development of the HT-J diagnostic assay and technical expertise in the conduct of other routine diagnostic tests has resulted in the provision of services as a Johne's disease diagnostic laboratory together with expert professional advice to submitters and their clients.

Quality assurance and accreditation

The research team has sought to ensure that testing is 'best practice' through involvement in quality assurance alongside national (ANQAP) and international (USDA NVSL) laboratories. The laboratory will be accredited to ISO17025 under NATA in 2016.

The team has remained involved in extension activities with industry including committees, working groups and webinars.

International collaborations

Linkage and collaborative partnerships have been sought to maintain a cutting edge research scope and to minimise duplication of effort. The group has developed successful collaborative endeavours with the following international research scientists and public and private entities:

- Prof. John Bannantine, United States Department of Agriculture (USA)
- Dr. Torsten Eckstein, Colorado State University (USA)
- Dr. Antonio Figueras, Spanish National Research Council (Spain)
- Dr Frank Biet, INRA Centre Val de Loire (France)
- Prof. Cath Reese, University of Nottingham (UK)
- Dr Ben Swift, University of Nottingham (UK)
- Prof. Horatio Bach, University of British Colombia (Canada)
- Prof. Greg Keefe, University of Prince Edward Island (Canada)
- Prof. Chris Davies, Utah State University (USA)
- Prof. Gregers Jungersen, Danish Technical University (Denmark)
- Prof. Y Mori, National Institute of Animal Health (Japan)
- Dr Satoko Kawaji, National Institute of Animal Health (Japan)
- Prof. Eichi Momotani, Tohto College of Health Sciences (Japan)
- Prof Cord Heuer, Massey University (NZ)
- Prof. Alan Murray, Massey University (NZ)
- Dr Peter Little, Zoetis Australia

5 Discussion

5.1 Johne's disease landscape

Johne's disease (JD) is a chronic infection of the intestines and associated lymph nodes caused by *Mycobacterium avium* subsp. *paratuberculosis* (Mptb). Most infections are acquired soon after birth but progress slowly, and signs of disease are usually not seen for 1 to 3 years in sheep and for 3 to 10 years in cattle. During this long incubation period diagnostic tests are inaccurate. Subclinically infected animals can be moved to other farms, thereby spreading the infection. Eventually the signs appear: weight loss, and in cattle also diarrhoea, followed by death. Initially only a few animals are affected, but inevitably the number affected increases over time. Vaccination does not prevent infection, and so-called supershedders emerge among vaccinates in some flocks. The results of project P.PSH.0565, which reported the results of a 12 year study of vaccinating flocks to MLA in January 2016, confirmed that in most flocks eradication is unlikely using Gudair® vaccine alone, and spread from these flocks is possible through trade in vaccinated sheep. Vaccination of cattle has recently become possible in Australia with release of Silirum® vaccine, but there are no long term efficacy data in the public domain. As it is of similar composition to Gudair® and as JD behaves in a similar way in both sheep and cattle, Silirum® is likely to have similar outcomes to Gudair® (but it appears to be less tissue reactive). The current commercial vaccines are a very useful adjunct in JD control, but they are not the final solution.

JD has become a controversial issue in Australia because of control measures taken since 1995 that were seen to be unfair and that became unpopular in some industry sectors and regions. However, the distribution of JD in flocks and herds in Australia is very uneven, which creates an opportunity to reduce spread while better tools are developed to detect, control and prevent the disease. At the time of writing (January 2016) a review of bovine JD control options was being conducted by Animal Health Australia and it seemed likely to recommend deregulation of the disease. This will shift responsibility to the sellers and buyers of livestock to avoid the infection. The future is difficult to predict but under this scenario, access to reliable diagnostic tools and high quality technical advice will be crucial for those producers who wish to maintain herds and flocks free of JD through biosecurity measures including pre-purchase testing. Using ELISA with the primary objective to not diagnose Mptb infection, which has occurred in some circumstances and which has been a criticism of the current JD program, will diminish. Testing with the primary objective of identifying exposure to Mptb and faecal shedding so as to avoid importing the disease may increase in importance for domestic livestock movements and for live export.

For producers who already have the problem of JD in their herd/flock, access to better control options may become more important in the future. Scenarios under which this may apply include being able to meet putative future international market access requirements for live animals and animal products. Accurate individual animal tests for JD, as distinct from or as well as herd level tests may be required. For affected producers who wish to reduce production losses, a safer more effective vaccine will also be useful, as will new strategies to break the infection cycle.

The objectives and the results of this project should meet a range of future scenarios, because they relate to improving basic understanding of the biology of JD. Armed with this

understanding, the technologies available "on the day" can be exploited to tackle the infection. As will also be demonstrated, the project has resulted in high level technical training of a group of professionals who will be a resource for the livestock sector into the future if jobs are available to accommodate them. To some extent this is an issue of national science and innovation policy, but the livestock industries can have a direct role in this too.

This discussion will return to application scenarios after consideration of the scope and main findings of the project.

5.2 Scope of Project P.PSH.0576 and its main outcomes

This is a large program of basic and applied research, commissioned to drive understanding of the biology of JD in order to provide a foundation for better tests and future control options, while ensuring that diagnostic tools remain available and are as accurate as possible. The project was divided into three major research subprograms, and an animal trial program, and it contained an explicit objective to build animal health research capacity for the benefit of the livestock industries. The components of the project were:

1. Predictive and diagnostic tools - to enable producers to identify exposed and infected herds/flocks/individuals, to predict those which are likely to spread the disease.

2. Animal genomics - to identify resistant animals, and to determine the susceptibility of different breeds of sheep.

3. Improving vaccine technology – to conduct research towards a safer, more effective vaccine for sheep and cattle.

4. Develop animal models

5. Research capacity for livestock health - to maintain and develop capacity in livestock health for the benefit of producers.

In this project we concentrated on the disease in the period soon after exposure to Mptb (< 12 months), while animals had early subclinical infection, because we were most interested in determining early predictors of future disease outcome. This work required long term experimental infection trials in both sheep and cattle. Most of the pathogenesis research conducted over the last 100 years internationally has focussed on the end stage of the disease, possibly because it is easier to identify and work with. But it has hindered true understanding of the biology of the infection. Based on findings in this project and hints in the results of earlier MLA projects OJD.031, P.PSH.0311 and P.PSH.0297, it is a reasonable hypothesis that the outcomes of Mptb infection in individual sheep and cattle are determined in the first few months after infection. There were remarkable discoveries and practical achievements in this area in the current project. The highlights from each research subprogram are discussed below.

Predictive and diagnostic tools

We discovered that an early elevation in faecal Mptb DNA quantity and a lower IFNγ response identified susceptible sheep. Conversely, early low faecal Mptb DNA and higher IL-10 responses predicted immunity. In research flocks these tests had 75% accuracy. These tests can be used at 4-5 months of age to predict future disease outcomes. We showed that

the IFNγ^{Plus} assay can detect Mptb exposure in sheep before any other test, as early as 4-5 months after Mptb exposure and unlike standard IFNγ assays, it can be performed 2 days after sample collection, allowing sufficient time for shipment of blood samples from farm to laboratory. Sensitivity and specificity was 81%. There is insufficient information about assay validation in cattle, but the test has great potential.

We developed and validated M7H9C medium which has been adopted by animal health diagnostic laboratories around Australia to replace BACTEC medium and we also developed an efficient qPCR method for confirming Mptb in liquid culture media. As a direct result, these laboratories continue to be able to provide Mptb culture as a diagnostic service to the national livestock industry.

We trialled other diagnostic test platforms and rejected them on grounds of poor practicality or low sensitivity: lymphocyte proliferation assay; ELISA on saliva or faeces. However, one test may be critical to maintain even though it is cumbersome: we introduced a phage assay from the UK to detect viable Mptb in blood and milk. This assay was used recently by others to detect Mptb in human infant milk formula (Botsaris et al., 2016) and it is important that the technology is available in Australia for verification purposes.

"Antigen hunting" for JD has been a theme in research programs in the USA, NZ and EU for many years. We completed a long term discovery program for novel antigens for use in diagnostic tests and vaccines that was a fertile area of research for PhD students who followed a coherent theme of stress and dormancy related proteins. Through international collaborations we were able to evaluate a large number of candidates as well as lipid and other antigens. However, the global quest for better antigens for JD diagnosis will have to continue because we did not find a suitable antigen. Should one or more be found, they can be introduced into the immune assays and evaluated quickly. For example, ELISA can be investigated immediately using our biological sample archive from well characterised cases of JD across the spectrum of disease.

The hope remains that a combined immunological and genomic signature can be found, and statistical analyses will be reported in an addendum to this final report as we seek to verify numerous significant associations. The animal genomics program revealed many opportunities, as is discussed below.

Animal genomics

We found gene expression patterns consistent with a JD resistant or a susceptible outcome in both sheep and cattle and potential biomarkers for JD prediction were identified. Some were common across breeds of sheep (Merino, Suffolk first cross Merino, Border Leicester, and Poll Dorset), all of which were susceptible to Mptb infection, although Poll Dorset and Border Leicester sheep appeared more resilient. We also found gene expression changes that are potential indirect markers of Gudair® vaccine efficacy. These discoveries provide a foundation for strategic research on modulation of immune gene function and biological targets for future vaccine development and as outlined above, for diagnosis. In order to assess tools for selective breeding, we conducted a genome-wide association study and we identified significant SNPs related to JD resistance. These need to be followed-up, and we make recommendations below to do so.

Improving vaccine technology.

The main objective was to devise a strategy for candidate vaccine evaluation in cost effective, short term trials using a defined infection model to evaluate proven correlates of protection. This would overcome impediments to new vaccine development: long term trials (years) to prove efficacy, and a plethora of candidate vaccines to be tested. As vaccines for Johne's disease need to contain two major components, adjuvant and antigen, we conducted research on both to prove the strategy.

We discovered that specific lymphocyte subsets protect against Mptb-infection in vaccinated sheep, and that immune parameters other than the commonly used IFNγ and antibody tests are required when assessing vaccine efficacy. We used these measures to assess progress in a series of trials. We produced novel formulations that resulted in fewer injection site lesions than Gudair® and found that the types of immune response were dependant on the adjuvant. We formulated novel vaccines with recombinant Mptb antigens and different adjuvants. We obtained partial protection, despite low antigen content, with fewer, smaller, less persistent injection site lesions than Gudair®. Through a series of trials we were able to devise a prototype vaccine to prove the efficacy testing pathway. The final trial was conducted in cattle in which the experimental infection model was used together with immune correlates of protection in a study of only 9 months duration. There were no injection site lesions. The novel vaccine induced a weak antibody response and a strong early IFNγ response, identified from our predictive studies as a desirable marker of protection. The novel vaccine protected against JD when assessed using faecal shedding of Mptb and histopathological lesions, but like commercial vaccines did not prevent infection. We demonstrated that the predictive tools, experimental animal models and vaccine design strategies have strong potential to lead to the development of a safer, more effective vaccine.

5.3 Applications of the research outcomes - examples

The national animal health system is undergoing a sea change with respect to control of endemic diseases, and JD is part of this as discussed above. Ultimately it appears that it will be the livestock industries that determine their needs and approaches. A national review of bovine JD is about to hand down its recommendations. What are the main requirements for the future and how does this research program meet those needs? While breeding approaches are now able to be scoped technically, diagnostic testing approaches can be adopted sooner. Several requirements can be forecast, as a way of providing examples of potential future application:

1. The desire to prevent introduction of Mptb onto a farm
2. The need for sellers to provide confidence to buyers of their livestock as to the disease status of those livestock
3. The need to reduce the effect of the disease on affected farms
4. Responses to JD incursions (trace forward investigation)

Prevention of entry of Mptb

Prevention of entry of JD affected livestock can be accomplished through a biosecurity program, important components of which include the exclusion of straying stock, and purchasing only disease free animals. Sources of stock can be certified market assured

flocks and herds. Alternatively, and to open up a wider range of sources, producers could consider buying stock from farms where there is no evidence of Mptb exposure. This can be determined through herd history, and by pre-purchase testing using a test for exposure. The IFNγ^{PLUS} assay is such a test. It might be combined with the HT-J faecal PCR test, which has been fully validated, or use of gene expression markers (after validation).

JD status of livestock offered for sale

This is a parallel objective to the first example of preventing entry of JD. In the future it might operate at several levels: vendor assurance around individual consignments of livestock, and herd/flock certification through a formal market assurance program. Flocks and herds that are currently free of JD may enjoy a competitive advantage in trade in the future, and this may be a driver for obtaining or maintaining an accredited free status. In other cases, and sometimes for intangible reasons, some producers would prefer to stay free of JD. To open up a wider range of markets for live animals, a producer with a non-market assurance program enterprise, where there is no evidence of JD based on herd/flock history, could consider pre-sale testing using a test for exposure such as the IFNγ^{PLUS} assay (which requires further validation) and/or a test for shedding such as the HT-J faecal PCR test, which has been fully validated. In the past market assured flocks and herds have "fallen over" which has been detrimental to uptake of the program. The reasons are eventual emergence of cases of JD after a long silent incubation period. This problem may be forestalled by application of tests for exposure at a much earlier time in the history of the flock/herd. Further validation of the IFNγ^{PLUS} assay is needed particularly in cattle, but in principle it can be applied in this way. Application of a sensitive test such as pooled faecal HT-J or pooled faecal culture would be preferable to use of ELISA for MAP testing.

Reducing the impact of the disease

The impact of JD can be reduced by vaccination of lambs/calves, and over a period of about 5 to 10 years (depending on the age structure of the flock/herd), 90% of clinical cases can be avoided. However, it seems likely that the infection cannot be eradicated using Gudair® vaccine alone (final report Project P.PSH.0565); the same could be surmised for Silirum®. Breaking the cycle of Mptb infection in a flock or herd is an important step. It cannot be achieved by vaccination due to the persistence of supershedders among vaccinates (Reddacliff et al., 2006). It is commonly attempted in cattle by removing young animals from the source of infection, perceived to be the dam. However the environment may be a more important source of infection than the dam for many animals (Eisenberg et al., 2015) and recently it was shown that rearing calves from 1 day of age off site was most effective, presumably by excluding exposure to contaminated dairy environments (Aly et al., 2015). Given that most environmental contamination stems from relatively few individuals, the so-called supershedders, early detection and removal of these can be considered as a means to reduce environmental/pasture contamination. The HT-J faecal DNA test, which was maintained as a research tool during this project, subjected to national quality control, applied in the bovine JD outbreak in northern Queensland, and used tactically for test and cull in a large commercial cattle herd in 2015, is a rapid test that can determine which animals are responsible for the most severe pasture contamination. It can be used in both sheep and cattle. Progress towards reducing the level of Mptb exposure at herd and flock level could be appraised over time by applying a test for exposure, the IFNγ^{PLUS} assay.

While current commercial vaccines cannot prevent infection and shedding, it is possible that pre-exposure vaccination with a more effective vaccine may be able to do so. However, this will require further investment in vaccine development. Currently it is possible to improve the safety of vaccine by changing the adjuvant, but efficacy improvements will require more work. This should be done in partnership with vaccine companies, which would enable further leveraging of industry's research dollars.

Responses to JD incursions (trace forwards)

A common problem when JD is suspected or detected in a recently introduced animal is whether home bred stock have become exposed and infected. A complex risk assessment is usually undertaken. This may be followed by necropsy and tests of both the introduced animals and the home bred stock. The approach varies depending on whether the introduced stock can be located, the duration of contact with home bred stock, the time elapsed since introduction and the degree of segregation of the animals. As an alternative in some situations, a test for exposure may be applied to the home bred stock. The IFNγ^{PLUS} assay is such a test; it can be applied within 4-5 months of putative exposure. The introduced stock can be tested as well with this and other tests. The period of suspicion surrounding the property's JD infection may be shortened by this approach.

5.4 Extent to which project objectives were met

The project objectives are listed here with a statement on the extent of their achievement in the context of the operational plan agreed with MLA.

1. Predictive and diagnostic tools

> *To provide new diagnostic tools to enable producers to determine more accurately whether herds/flocks are infected or uninfected, which animals in particular are exposed and infected and to predict which animals are at high risk of contaminating the environment before they do so.*
>
> This objective was met. A new culture medium to replace BACTEC medium was developed, validated and has been adopted throughout Australia, combined with a high throughput confirmation method for Mptb in liquid culture medium. The HT-J faecal PCR test has been maintained and used in research trials, demonstrating predictive utility. The IFNγ^{PLUS} assay was applied and evaluated. Early diagnostic predictors, including microbiological, immunological, molecular and genomic variables were identified, but need further development before they can be offered by diagnostic service providers.

2. Animal genomics

> *To develop new genetic approaches to predict accurately which herds/flocks are resistant, which particular animals are resistant and which infected animals will get sick, contaminate the environment or recover. As part of this objective, to determine the relative susceptibility to JD of different breeds, such as pure British breed and Merino sheep.*
>
> This objective was met. Gene expression changes that were correlated with exposure/infection, lesion severity and resistance/susceptibility were identified in

trials with sheep and cattle. Gene expression changes related to breeds of sheep and vaccination success were also identified. Single nucleotide polymorphisms (SNPs) related to JD resistance in sheep were identified. Time will tell whether cattle and sheep breeders see the need for this technology to be developed to become practical selection tools.

3. Improving vaccine technology

Conduct research towards a safer, more effective vaccine for sheep and cattle

1. *To develop an indirect measure of vaccine efficacy, based on blood and faecal testing, to facilitate the development of a safer more effective vaccine*
2. *To improve the safety of vaccines, by reducing their tendency to cause severe tissue reactions in livestock and humans*
3. *To trial a prototype vaccine through experimental infection in sheep and cattle*

This objective was met. An indirect measure of vaccine efficacy comprising a test panel was developed. Adjuvants to reduce injection site lesions for a safer immunogenic vaccine were identified. A prototype vaccine was developed through trials in sheep and cattle, illustrating a pathway for adjuvant and antigen evaluation. The overall objective of demonstrating a new strategy to evaluate candidate JD vaccines based on a controlled animal infection model, short duration trial ($\leq$ 1 year) and immune correlates of protection was demonstrated. A final candidate had evidence of protective immunity without injection site lesions in calves in a trial of 9 months duration. This information may stimulate interest in the commercial development of a new vaccine.

4. Research capacity for livestock health

To maintain and develop capacity in livestock health for the benefit of Australian producers through the training of 4 post doctoral fellows and four postgraduate students whose skills and knowledge will be applicable and available to the broader veterinary and agricultural research community as specialist immunologists, pathologists and microbiologists.

This objective was met. Four young post doctoral fellows were responsible for subprograms, ensuring a unified approach across complex disciplines, and 8 PhD students, 8 domestic honours students and 2 DVM summer vacation students were trained. The research team was involved in extension activities with industry. A highly skilled group of professional and technical staff capable of carrying out all diagnostic tests for Johne's disease as well as other specialised techniques that can be utilised for research on this and a variety of other livestock health issues was maintained. A fully traceable biological sample archive and a legacy dataset were created. Physical capacity was created through parallel investments in PC2 laboratories, laboratory equipment and farm infrastructure. The laboratory provides services nationally, participates in quality assurance at national and international levels and will be accredited to ISO17025 under NATA in 2016.

6 Conclusions/recommendations

There are many unanswered questions regarding the pathogenesis, diagnosis and prevention of JD in sheep and cattle. In the context of the national JD problem, a wide range of recommendations for future research can be made. Many of these relate to enhancements and further technology development flowing from this project.

1. Diagnostic tests

- further validation and applications of IFNγ^{PLUS} particularly in cattle
- gene expression data in field validation
- use of predictive markers for on-farm management strategies

2. Vaccines:

- apply correlates of vaccine protection outcomes to enable shorter vaccine trials
- evaluate available vaccine candidates efficiently
- assess candidate antigens including combinations of recombinant antigens
- use of predictive markers for on-farm management strategies
- assess *in vitro* vaccine screening using cells from vaccinated animals
- evaluate effectiveness of vaccine in intestinal parasite co-infections
- evaluate effectiveness of vaccine when used in combination with other vaccines
- make this information available for commercial exploitation.

3. Issues related to Mptb S strain infections in cattle

- evaluate relative host susceptibility to different strains in experimental infection trials
- evaluate multi-strain infection - co-infection with C, S and/or B strains
- survey cattle populations to better understand prevalence of S strain
- conduct risk factor analysis to understand drivers for S strain in cattle
- evaluate genetic shifts in Mptb leading to increased infection rates
- evaluate environmental and management guidelines to avoid grazing calves with sheep with OJD; are the existing guidelines appropriate?
- evaluate variance in diagnostic accuracy for different strains of Mptb at the level of i) pathogen detection and ii) detecting host immune response. (eg. would C strain "out-grow" S strain in culture and thus mask the true level of co-infection)

5. Gastrointesitnal co-infections

- develop better understanding of gut pathology and immune responses, particularly in internal parasites, and BVDV co-infections
- link current capacity in ruminant immunology and pathobiology to research in other economically important diseases of livestock
- evaluate co-infection with parasites and immune modulation (hygiene hypothesis).

5. Food safety and human infection with Mptb

- maintain Crohn's disease literature awareness
- maintain international research linkages to monitor what trade partners may do next
- develop methods for quality assurance testing of animal products (direct PCR/strain typing, phage assay)
- critically evaluate environmental persistence of Mptb and level of contamination at catchment level based on international interest in the presence of Mptb in water supplies
- critically evaluate overseas research outcomes

6. Genomics/genetics

From the current study we (i) established standards for best practice diagnosis and used these for definition of phenotype (ii) conducted pilot genome association studies to find haplotypes associated with disease resistance/susceptibility. We need to take these forward as candidates for a validation and ultimately a selection program, as follows:

- validation. This includes (i) analysis of a very complex phenotype to simplify it for large scale use. (ii) expanding genetic studies to confirm heritability and association (iii) estimate genetic variation for candidate haplotypes by sampling from flocks, including existing resources that have been assembled from previous MLA-funded programs
- develop breeding values. The goal will be to utilize existing systems from sheep EBVs, with the goal of developing genomic breeding values
- implementation: Partner with existing industry organizations, particularly Sheep Genetics and potentially Meat Standards Australia, to transfer into national flocks
- conduct parallel studies for cattle

7. Education and extension on applications of diagnostic tests and vaccines to veterinarians and industry peak bodies to improve producers' capacity to deal with JD in a deregulated environment.

7 Key messages

The livestock sector has a range of new diagnostic tests available or able to be validated that will greatly improve confidence in whether individual animals and herds/flocks have been exposed to Mptb.

Implementation of genetic approaches as an adjunct to JD control require further investigation but it appears that selection markers exist.

Application of these new tests and procedures will depend on the outcome of current national discussions on the future of the JD program in cattle, and ongoing discussions in the sheep industry. It seems likely that the cattle JD program will be changed from a 'control' program involving significant government intervention to a 'management' program where producers will be required to make their own buying decisions. Tools to assist producers make such decisions are currently inadequate.

A vaccine development pipeline based on a new efficient strategy could be established with a suitable commercial partner.

JD is a complex disease with international ramifications. Industry will require expert technical advice to manage changing requirements and local developments. This project has created critical mass in livestock health research and diagnostics for the benefit of the livestock industries.

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9 Appendix

9.1 Acknowledgements

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9.2 Abbreviations

CFSE: carboxyfluorescein succinimidyl ester)

FPTB: Fast plaque TB assay

IFNγ: interferon gamma

IL: interleukin

JD: Johne's disease

MHC: Major histocompatibility complex

MPN: most probable number

Mptb: *Mycobacterium avium* subspecies *paratuberculosis*

Mptb antigen: 316v antigen (French pressed)

PBMC: peripheral blood mononuclear cell

PBS: Phosphate-buffered saline

PMMS: peptide mediated mycobacteriophage separation

PPDA: purified protein derivative from M. avium subspecies avium

PTPA: Protein tyrophosphatase A

PWM: poke weed mitogen

REA: Restriction endonuclease analysis

REML: restricted maximum likelihood

ROC: Receiver operating characteristic

TNFα: tumour necrosis factor alpha

TGFβ: transforming growth factor beta

9.3 Publications arising from this project

Note: publications in peer reviewed scientific publications are listed here. There were also many conference presentations and proceedings arising from this project.

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9.4 List of supplementary appendices

These appendices are not included in this report, either because they are too voluminous, or because they contain commercial-in-confidence information. The appendices can be obtained upon request from MLA. Some appendices will be embargoed until further notice, due to intellectual property concerns.

1. Operational plan
2. Overview of Animal Trials
3. Using early immune markers to predict future disease outcomes
4. P.PSH.0297.2 cattle experimental infection trial outcomes and analysis
5. Further analysis of data from trials P.PSH.0311.1 and P.PSH.0297.2 based on final disease status
6. Development and validation of a liquid medium (M7H9C) for routine culture of *Mycobacterium avium* subsp. *paratuberculosis* to replace modified BACTEC 12B medium.
7. Strategy for use of new culture media and confirmation of Mptb by PCR
8. Application of new culture media and confirmation strategy in research trials to obtain validation data
9. Introduction and validation of the mycobacterial phage assay in sheep and cattle
10. Lymphocyte transformation assay
11. Implementation and validation of the Interferon gamma ($IFN\gamma^{Plus}$) assay including novel antigens
12. Specific faecal antibody responses in sheep infected with Mptb
13. Develop an IgG1 ELISA test for sheep and cattle
14. Specific antibody responses from saliva in sheep
15. Development of immune signature of disease resistance and vaccine efficacy
16. Incorporation of data from Animal Genomics subproject in the 'immune signature'
17. Gene lists
18. Gene expression profiling of sheep trial P.PSH.0311.1
19. Gene expression profiling of cattle trial P.PSH.0297.2
20. Experimental inoculation of different breeds of sheep with Mptb to examine breed susceptibility Trial P.PSH.0576.1
21. Breed variations associated with gene expression

22. Immunomodulatory effects of Gudair® vaccine
23. Candidate gene analysis by single nucleotide polymorphism (SNP)
24. Develop an indirect measure of vaccine efficacy, based on blood and faecal testing, to facilitate the development of a safer more effective vaccine
25. The immunogencity and tissue reactivity of Mptb whole cell vaccines using different adjuvants Trial P.PSH.0576 Adj
26. In silico screened Mycobacterium avium subsp paratuberculosis (Mptb) recombinant proteins upregulated under stress conditions are immunogenic in sheep
27. Lymphoproliferative and IFN-γ responses to stress-regulated *Mycobacterium avium* subspecies *paratuberculosis* recombinant proteins
28. Evaluation of the immunogenicity of candidate antigens in cattle
29. Antigenicity of recombinant maltose binding protein *Mycobacterium avium* subsp *paratuberculosis* fusion proteins with and without factor Xa cleaving
30. Cellular and humoral immune responses in sheep vaccinated with recombinant MAP2698c and MAP3567 proteins from *Mycobacterium avium* subspecies *paratuberculosis* in trial P.PSH.0576.Ag
31. Prototype vaccine trial in sheep (P.PSH.0576.2)
32. Prototype candidate antigen vaccine trial in sheep (P.PSH.0576.2 subset)
33. Second prototype vaccine trial in sheep (P.PSH.0576.3)
34. Prototype vaccine trial in cattle (P.PSH.0576.4)
35. Cellular and humoral immunogenicity of *Mycobacterium avium* subsp. *paratuberculosis* specific lipopentapeptide antigens
36. *Mycobacterium avium* subspecies *paratuberculosis* stressome and diagnostic significance: a review and meta-analysis
37. Antigenicity evaluation of protein tyrosine phosphatase A (PtpA) using sera from sheep infected with *Mycobacterium avium* subspecies *paratuberculosis*
38. Antigenicity in sheep of synthetic peptides from *Mycobacterium avium* subspecies *paratuberculosis*
39. Conference presentations related to this project 2011-2015
40. Sample archive from Johne's disease trials held at the University of Sydney