Can early host responses to mycobacterial infection predict eventual disease outcomes?

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Abstract

Diagnostic tests used for Johne's disease in sheep either have poor sensitivity and specificity or only detect disease in later stages of infection. Predicting which of the infected sheep are likely to become infectious later in life is currently not feasible and continues to be a major hindrance in disease control. We conducted this longitudinal study to investigate if a suite of diagnostic tests conducted in *Mycobacterium avium* subspecies *paratuberculosis* (MAP) exposed lambs at 4 months post infection can accurately predict their clinical status at 12 months post infection. We tracked cellular and humoral responses and quantity of MAP shedding for up to 12 months post challenge in 20 controls and 37 exposed sheep. Infection was defined at necropsy by tissue culture and disease spectrum by lesion type. Data were analysed using univariable and multivariable logistic regression models and a subset of variables from the earliest period post inoculation (4 months) was selected for predicting disease outcomes later on (12 months). Sensitivity and specificity of tests and their combinations in series and parallel were determined. Early elevation in faecal MAP DNA quantity and a lower interferon gamma (IFNγ) response were significantly associated with sheep becoming infectious as well as progressing to severe disease. Conversely, early low faecal MAP DNA and higher interleukin-10 responses were significantly associated with an exposed animal developing protective immunity. Combination of early elevated faecal MAP DNA or lower IFNγ response had the highest sensitivity (75%) and specificity (81%) for identifying sheep that would become infectious. Collectively, these results highlight the potential for combined test interpretation to aid in the early prediction of sheep susceptibility to MAP infection.

KEYWORDS: Paratuberculosis; diagnostic tests; Mycobacterium; faecal DNA; Johne's disease; interferon gamma.

1. Introduction

Disease outcomes following exposure to virulent mycobacteria 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 (American Thoracic Society, 2000). This may partly be due to the nature of the pathogen as virulent mycobacteria are notoriously slow-growing organisms and can switch between dormant and active phases (Magombedze and Mulder, 2012). The host response also plays a pivotal role in orchestrating the progress of mycobacterial disease (Kunnath-Velayudhan and Gennaro, 2011).

Mycobacterium avium subsp. *paratuberculosis* (MAP) causes Johne's disease in ruminants, a chronic debilitating disease resulting in chronic diarrhoea and eventually death. In ruminants the usual route of exposure to MAP is oral, via ingestion of contaminated milk or faecal matter. Once MAP 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, MAP may actively evade intracellular killing mechanisms and take residence within these cells (Weiss et al., 2002). As a result, a more complex host response is required. Antigen presenting cells such as the macrophages and dendritic cells are able to indicate the presence of infection by presenting pathogen-derived antigens on their cell surface. These cells also release a variety of signals in the form of cytokines and chemokines (Weiss and Souza, 2008). The initial response predominantly involves the antigen-specific release of interferon gamma (IFNγ) by T lymphocytes and as disease progresses this response is replaced by an antibody response. In sheep, this classical response is as common as a simultaneous IFNγ and antibody response (Begg et al., 2011). IFNγ activates bystander macrophages and facilitates intracellular killing of MAP. While the IFNγ response is important in the cell-mediated control of intracellular pathogens like MAP it is not always a predictor of disease outcome (Jungersen et al., 2012). To counteract the host's immune response, MAP can also actively induce certain cytokines to suppress and evade immune cells; interleukin (IL)-10 and tumour growth factor (TGF)β are two such cytokines (Weiss and Souza, 2008). IL-10 can also reduce the ability of macrophages to kill intracellular MAP (Weiss et al., 2005). 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 MAP (de Silva et al., 2011).

Infected sheep can shed huge amounts of MAP in their faeces – as high as 10^8 mycobacterial bacilli per gram of faeces (Whittington et al., 2000) – and thus contaminate pasture and act as 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 MAP after vaccination (Reddacliff et al., 2006; Eppleston et al., 2011).

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 (Dennis et al., 2011).

Most of the currently available diagnostic tests for MAP infection have poor sensitivity or specificity in younger age and only detect animals in later stages of infection, when they have already made substantial contributions to contamination of the pasture and have already infected many susceptible animals. Sensitive and specific diagnosis of disease in early age or our ability to identify infected animals prior their becoming highly infectious would be a major step forward in controlling the disease. We conducted this longitudinal study to investigate if there are any such diagnostic indicators that singly and in combination can predict the eventual clinical outcome of sheep. 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.

Identifying indicators of protection against paratuberculosis has benefits in addition to the possible prediction of disease outcome (Berry et al., 2010; Kunnath-Velayudhan and Gennaro, 2011). Understanding the initial changes in immune parameters would inform the design of novel vaccines that drive a similar immune signature leading to the production of a more efficient vaccine.

2. Materials and Methods

2.1. Animals

The use of animals (Merino sheep) in this study was approved by the University of Sydney Animal Ethics Committee.

Merino lambs were obtained from the University of Sydney farms in NSW, Australia where the parent flocks were shown to be free from MAP infection by repeated testing using faecal culture (by the radiometric BACTEC method) and antibody ELISA (Institut Pourquier). The lambs were brought to and held within a control farm free from MAP infection for two weeks to acclimatise to the new environment. Negative infection status of the lambs was verified by antibody ELISA, IFNγ ELISA and faecal culture prior to experimental inoculation (Begg et al., 2010). All animals were managed similarly under conventional Australian sheep farming conditions in open paddocks; control animals were kept on pasture isolated from MAP-exposed animals. Fifty-seven 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 MAP S strain (n=37). MAP exposed groups received a total of 6.72×10^9 of a clonal isolate or 7.52×10^9 of a gut homogenate from a sheep with clinical Johne's disease (de Silva et al., 2010). For this study, data from animals exposed to the clonal isolate and gut homogenate were considered together as 'MAP exposed' animals.

2.2. Sampling

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.

2.2.1. Blood and serum

Blood was collected from the jugular vein into lithium heparin-coated tubes (BD Vacutainer®) for the IFNγ, IL-10 and Proliferation assays and into tubes without anticoagulant for the collection of serum at 4, 8 and 12 months p.i. For all three assays whole blood or cells were cultured with medium alone (RPMI 1640/10% foetal calf serum/Penicillin/Streptomycin/β-mercaptoethanol) or with added MAP antigen (316v, 10 µg/mL).

2.2.2. Faeces

Faecal pellets were collected from each animal at the same time as blood samples and stored at -80^{o}C until used for detection of viable MAP by culture or MAP DNA by PCR.

2.2.3. Tissues

Animals were euthansed (barbiturate i.v.) at 12 months p.i . 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^{o}C until processed) or fixed in formalin.

2.3. Laboratory testing

2.3.1. Blood and serum

2.3.1.1. IFNγ assay

The IFNγ assay was carried out using whole blood cultured with MAP-specific antigen (316v) for 48 hrs as previously described (Begg et al., 2009). Reagents for the in-house IFNγ ELISA were: capture antibody, IFN 6.19 (a generous gift from Dr Gregers Jungersen); detection antibody, CC302biotin (Serotec); conjugate, Streptavidin horseradish peroxidase (Vector Labs); substrate, 3,3´,5,5´-tetramethylbenzidine (TMB) (Pierce).

2.3.1.2. IL-10 assay

The IL-10 assay was carried out using peripheral blood mononuclear cells cultured with MAP-specific antigen (316v) as previously described (de Silva et al., 2011). Reagents for the IL-10 ELISA were: capture antibody, CC318 (Serotec); detection antibody, CC320biotin (Serotec); conjugate, streptavidin horseradish peroxidise (Vector Labs); substrate, 3,3´,5,5´-tetramethylbenzidine (TMB) (Pierce).

2.3.1.3. Antibody ELISA

Serum samples were tested for MAP-specific antibodies using a commercially available kit (Institut Pourquier) as described previously (Gumber et al., 2006). An S/P of 0.7 or greater was considered to be positive.

For the IFNγ, IL-10 and antibody ELISAs the S/P were calculated as:

$(OD_{sample}-OD_{negative\ control})/(OD_{positive\ control}-OD_{\ negative\ control})$

2.3.1.4. Proliferation assay

The flow cytometric Proliferation Assay was carried out using peripheral blood mononuclear cells and MAP-specific antigen (316v) as previously described (de Silva et al., 2010). Cells were labelled with the fluorescent tracking dye CFSE (carboxyfluorescein diacetate succinimidyl ester) prior to culture.

For the Proliferation Assay the Stimulation Index was calculated as:

% $CFSE^{dim}$ cells in the presence of MAP antigen/% $CFSE^{dim}$ cells in the presence of culture medium

2.4. Faecal culture and MAP DNA quantification

The presence of viable MAP in faecal samples was detected by radiometric BACTEC culture and growth confirmed by IS*900* and IS*1311* PCR (Whittington et al., 1998). For quantification, MAP DNA was extracted from faecal samples and was assessed by quantitative IS*900* PCR as described previously (Kawaji et al., 2007; Kawaji et al., 2011).

2.5. Tissue culture and histopathology

Tissue samples were cultured by the radiometric BACTEC method as described previously (Whittington et al., 1999). Growth index positive samples were confirmed by IS*900* PCR and restriction enzyme analysis (REA).

In addition, duplicate tissue sections of the cultured samples were fixed in formalin, sectioned at 5 µm, stained with haematoxylin and eosin or the Ziehl Neelsen stain and graded following Perez et al (Perez et al., 1996). The lesion type (3a and 3c, paucibacillary; 3b multibacillary) or absence of a lesion was also used to classify animals.

2.6. Disease classification

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 1[1]).

2.7. Data analysis

2.7.1. Explanatory variables

Faecal MAP 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.

2.7.2. Outcome variables

The following outcomes were defined for conducting statistical analyses:

(a) *Infected*: This was a binary outcome based on tissue culture results which classified all sheep into positive (infected) and negative (uninfected) groups. Sheep that had MAP cultured from at least one tissue section, using radiometric BACTEC culture, and growth confirmed by IS*900* and IS*1311* PCR were classified as having MAP infection (de Silva et al., 2010) (Table 1). Sheep were classified as uninfected if MAP was not detected in their tissues by culture.

(b) *Infectious:* A binary variable which classified a sheep with at least one faecal culture positive result at any time point as positive.

(c) *Multibacillary:* A binary variable representing whether or not a sheep had developed multibacillary lesions at the time of necropsy.

(d) *Resistant/recovered:* A binary variable representing sheep that were uninfected at the termination of the trial despite prior exposure to MAP. These animals did not shed MAP in

[1] All tables and figures are located at the end of this document.

their faeces at any of the sampling time points during the study and were uninfected (based on tissue culture at the time of necropsy).

(e) *Increasing disease severity-I:* Sheep were classified into four ordered groups depending on histopathology and infection status: (i) recovered/resistant sheep as described above, (ii) sheep with paucibacillary (Perez type 3a, 3c) lesions (infectious and non-infectious) or no lesions but infected, and (iii) sheep with multibacillary (Perez type 3b) lesions.

(f) *Increasing disease severity-II:* A second outcome variable was created using the same criteria as in (e) but after excluding the infected sheep without any lesions within the paucibacillary group.

2.7.3. Statistical modeling

All statistical analyses were conducted using the SAS statistical program (© 2002-2010 by SAS Institute Inc., Cary, NC, USA) unless indicated otherwise.

2.7.3.1. Descriptive analyses

Initially, descriptive analyses were conducted to make a preliminary evaluation of the association of explanatory variables with the outcome variables. This included calculation of summary statistics and creation of box-and-whisker plots for faecal MAP DNA, antibody, IFNγ, IL-10 and proliferation index measurements made at 4, 8 and 12 months p.i. – both overall as well as after classification by the outcome variables.

2.7.3.2. Univariable analyses

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). The descriptive and univariable analyses were facilitated by SAS UniLogistic macro (Dhand, 2010b).

2.7.3.3. Multivariable analyses

All the explanatory variables with some association with the outcome ($P < 0.20$) at the univariable level were then tested for collinearity and only one of a pair of highly collinear variables was retained for further analyses. Multivariable logistic regression analyses (binomial for binary outcomes and ordinal for ordinal outcomes) were then conducted to evaluate the association of explanatory variables after adjusting for each other. As the interest was in the selection of a best subset of variables from the earliest period p.i. for predicting the outcomes (to enable early discrimination of future diseased and non-diseased individuals), variables representing measurements at 4 months p.i. were first included in multivariable models followed by those from 8 and 12 months. Variables from the 8 and 12 month time points were only retained if their inclusion did not result in exclusion of the variables representing measurements made at 4 months p.i. Variables with $P < 0.05$ were retained in multivariable analyses. Finally, first order interactions were tested and retained if significant ($P < 0.05$). Multivariable analyses were performed using SAS MultiLogistic macro (Dhand, 2010a).

Model fit for binomial logistic regression models was evaluated using the Hosmer-Lemeshow Goodness-of-Fit Test. The assumption of proportional hazard for ordinal models was evaluated using Score test in the SAS Logistic procedure.

2.7.3.4. Calculation of diagnostic sensitivity and specificity

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 were calculated and reported.

3. Results

3.1. Status of exposed and unexposed animals

Non-exposed control sheep were included in the trial to ensure that the parameters measured were different to the MAP-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 MAP 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 MAP during the study period. Data from the control group were not used for further analysis.

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. For the MAP-exposed sheep, disease outcome was classified based on tissue and faecal culture and histopathology results (Table 1). To determine if a sheep was infected we cultured tissue samples from six intestinal sites – including the sites most commonly associated with JD lesions in sheep the ileo-caecal region (Perez et al 1996) – and two non-intestinal sites and their associated lymph nodes i.e. a total of 12 tissue samples. These sections were also assessed for the presence of histological lesions. While this was an extensive set of samples from each animal it is still possible that we may have missed the only site of infection in some sheep which may have resulted in misclassification of these sheep as uninfected. Faecal shedding of MAP was not detected in any of the MAP-exposed uninfected sheep at any of the time points sampled during the study. 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.

3.2. Descriptive results

Summary statistics for the explanatory variables for the MAP-exposed sheep over time are shown in Table 2. Overall, serum anti-MAP antibodies and faecal MAP DNA increased with time while IFNγ and PBMC proliferation peaked at 8 months p.i.

3.3. Logistic regression results

Six logistic regression models were built for as many outcome variables. Results of univariable models (only with P-values <0.05) are shown in Table 3. Variables representing faecal MAP DNA (log 10) at 4, 8 and 12 months had significant associations with most outcomes. The odds ratios for these variables – representing the change in the odds of the outcome for one unit increase in the log 10 MAP DNA content (Table 3) – were >1 for all outcomes except the outcome 'Resistant/recovered'. It suggests that as faecal MAP DNA content increased, odds of a sheep to be tissue culture positive, faecal culture positive or being multibacillary increased but odds of a sheep to be disease-free (resistant/recovered) decreased.

The assumption of linearity (on logit scale) was evaluated in the final models. Although not perfect, the association was found to be approximately linear for most of the explanatory variables which is considered to be adequate (Hosmer et al., 2005). Results of final multivariable logistic regression models are presented in Figure 1 as odds ratios and 95% confidence intervals. The six disease outcomes shown on the vertical axis in Figure 1are described in Section 2.7.2 above and in Table 1. 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 but reduced the likelihood to be resistant/recovered (free from disease at 12 months p.i.).

A lower MAP-specific IFNγ response at 4 months p.i. significantly predicted three outcomes: positive faecal culture (infectious), multibacillary disease (severe pathology) and increasing disease severity-I (Fig. 1). Interestingly, the results indicated that 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 1). Similarly, the IL-10 response at 4 months was significant in three models and had strong associations with tissue culture, being resistant and increasing disease severity (Fig. 1). The results suggest that lambs with a higher specific IL-10 response at 4 months p.i. are less likely to be infected (tissue culture positive), are more likely to be disease-free (resistant/recovered) and are less likely to have severe disease at 12 months p.i. 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.

3.4. Diagnostic sensitivity and specificity

Based on ROC curves (Fig 2), 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 MAP 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 4). 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 MAP 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 MAP DNA was high or when IFNγ was low. For this combination, sensitivity ranged from 58-75% and specificity ranged from 67-83%.

4. Discussion

While lambs are more susceptible to MAP infection than older animals, sheep of all ages exposed to MAP may also become infected (McGregor et al., 2012). 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 the host response contains a wealth of information regarding the eventual disease outcome.

The most striking observation was that the IFNγ response is a marker of exposure to MAP but is not a marker of infection. This has previously been noted by Jungersen et al in relation to bovine paratuberculosis (Jungersen et al., 2012). We are able to expand on this and report that 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 MAP in their faeces), more likely to be truly infected (i.e. have viable MAP 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.

Another intriguing finding of this study is the importance of the antigen-specific IL-10 response soon after exposure on future disease outcome. An IL-10 response at 4 months p.i. was 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 MAP (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 MAP 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 MAP-exposed sheep (Begg et al., 2011). 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. Clearly there is much to be discovered about the role of IL-10 in the systemic host response to mycobacterial diseases.

Anti-MAP antibody levels at 12 months p.i. were also significant in univariable analyses. At this time point, with one unit increase in antibody S/P ratio, the odds of an animal to be infected increases 7.4 times, to be infectious increases 3.2 times, to be multibacillary increases 4 times and to be resistant increases 0.1 times. However, antibody results were not significant at earlier time points and by this time (i.e. 12 months p.i.) the sheep had already started to show signs of clinical disease.

Some sheep are able to recover from natural MAP infection (Dennis et al., 2011). Similarly we also found in this study that about 30% of MAP-exposed sheep had either resisted or recovered from infection. The absence of histological lesions or viable MAP 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 MAP exposure had occurred as the lambs were dosed orally three times

and all had an antigen-specific IFNγ response. In addition, there were two MAP-exposed animals that were infected but did not have any histological lesions. Since it was not clear where these animals fit within the paratuberculosis disease spectrum, when grouping for the outcome variable for increasing disease severity (for statistical analysis), two different schemes were created. These animals were included with the paucibacillary group in Increasing Disease Severity-I and excluded from analysis for Increasing Disease Severity-II. The results for both disease severity outcomes supported results for the other outcome variables.

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 MAP in their faeces and contaminate the environment. Based on the tests used in this study, faecal MAP 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, will be valuable in limiting the spread of paratuberculosis.

In conclusion, we have shown that early host responses in chronic MAP infection can be predictive of disease outcomes. This brings us closer towards understanding the nature of protective immunity in paratuberculosis and will aid in the development of effective vaccines.

Acknowledgements

This work was supported by Meat and Livestock Australia and by Cattle Council of Australia, Sheepmeat Council of Australia and WoolProducers Australia through Animal Health Australia. The funding bodies were not involved in the design or conduct of experiments or in manuscript preparation. The authors thank the Farm Animal and Veterinary Public Health Laboratory Manager Ms. Anna Waldron; Ms. Nicole Carter and Ms. Ann-Michele Whittington for laboratory support; Mr. Craig Kristo and Mr. Nobel Toribio for animal husbandry support.

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Tables and Figures

Table 1: Disease outcome of experimental exposure of sheep to MAP

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 MAP
Tissue culture							
(Infected)	Negative	12	32				
	Positive	26	68	2-6	3a, 3c, 3b	3-6	
Faecal culture							
(Infectious)	Negative	21	57	0-4			
	Positive	16	43	3-6	3a, 3c, 3b	5-6	
Multibacillary							
	No	27	71	0-4			
	Yes	11	29	4-6	3b	6	Detected in hepatic tissue
Resistant/Recovered							
	No	26	68				
	Yes	12	32	0	None, except for two with 3a lesion with no AFB	0-1	None
Increasing disease severity-I							
	Recovered/resistant	12	32				
	Paucibacillary lesions*	15	39	2-4	3a, 3c	3-6	
	Multibacillary lesions	11	29				
Increasing disease severity-II							
	Recovered/resistant	12	34				
	Paucibacillary lesions	12	34				
	Multibacillary lesions	11	31				

*Included infected animals with no lesions

Table 2: Summary of results for explanatory variables

Explanatory variables	Time post infection	N	Minimum	Lower Quartile	Median	Upper Quartile	Maximum
Antibody (S/P)							
	4 months	38	0.002	0.01	0.01	0.02	0.242
	8 months	37	0.003	0.03	0.27	0.67	2.566
	12 months	34	-0.001	0.11	0.59	1.30	2.569
IFNγ (S/P)							
	4 months	38	0.029	0.38	0.67	1.14	1.499
	8 months	37	-0.014	0.62	0.95	1.14	1.326
	12 months	37	0.005	0.10	0.32	0.72	1.177
Faecal MAP DNA (log10)							
	4 months	38	-5.00	-5.00	-5.00	-2.42	-1.09
	8 months	38	-2.90	-2.48	-2.23	-0.88	2.34
	12 months	37	-5.00	-2.01	-1.09	1.71	3.33
Proliferation (SI)							
	4 months	38	1.2	2.9	4.1	7.9	30.4
	8 months	36	0.7	5.6	11.0	17.0	37.0
	12 months	37	0.4	2.4	6.1	8.8	87.0
IL-10 (S/P)							
	4 months	37	0.000	0.02	0.10	0.24	0.494
	8 months	33	0.000	0.01	0.03	0.19	0.598
	12 months	32	0.005	0.05	0.11	0.37	0.754

Table 3: Univariable results

Outcome Variable	Explanatory Variables	Odds ratios	LCL	UCL	P-value
Tissue culture (Infected)					
	Faecal MAP DNA (log10) at 4 months p.i.	1.7	1.0	3.4	0.050
	Faecal MAP DNA (log10) at 8 months p.i.	3.5	1.3	20.5	0.004
	Faecal MAP DNA (log10) at 12 months p.i.	1.5	1.1	2.4	0.007
	Antibody (S/P) at 12 months p.i.	7.4	1.8	56.1	0.004
Faecal culture (Infectious)					
	Faecal MAP DNA (log10) at 4 months p.i.	1.8	1.1	3.1	0.029
	Faecal MAP DNA (log10) at 8 months p.i.	42.2	4.7	-	<0.001
	Faecal MAP DNA (log10) at 12 months p.i.	2.6	1.6	5.3	<0.001
	Antibody (S/P) at 12 months p.i.	3.2	1.1	11.0	0.028
	Proliferation (SI) at 8 months p.i.	0.9	0.8	1.0	0.014
	IFNγ (S/P) at 4 months p.i.	0.1	0.01	0.7	0.015
Multibacillary					
	Faecal MAP DNA (log10) at 8 months p.i.	8.2	2.8	55.5	<0.001
	Faecal MAP DNA (log10) at 12 months p.i.	13.5	3.2	859.2	<0.001
	Antibody (S/P) at 12 months p.i.	4.0	1.3	15.7	0.015
	Proliferation (SI) at 8 months p.i.	0.9	0.8	1.0	0.030
	Proliferation (SI) at 12 months p.i.	0.5	0.3	0.8	<.0001
	IFNγ (S/P) at 12 months p.i.	0.02	-	0.4	0.005
	IL-10 (S/P) at 12 months p.i.	0.01	-	0.7	0.035
Resistant/Recovered					
	Faecal MAP DNA (log10) at 4 months p.i.	0.6	0.3	1.0	0.050
	Faecal MAP DNA (log10) at 8 months p.i.	0.3	0.05	0.8	0.004
	Faecal MAP DNA (log10) at 12 months p.i.	0.6	0.4	0.9	0.007
	Antibody (S/P) at 12 months p.i.	0.1	0.02	0.6	0.004
Increasing disease severity-I					
	Faecal MAP DNA (log10) at 4 months p.i.	1.7	1.1	2.8	0.026
	Faecal MAP DNA (log10) at 8 months p.i.	5.9	2.5	20.5	<0.001
	Faecal MAP DNA (log10) at 12 months p.i.	2.4	1.6	3.9	<0.001
	Antibody (S/P) at 12 months p.i.	5.3	1.9	18.2	0.001
Increasing disease severity-II					
	Faecal MAP DNA (log10) at 4 months p.i.	1.7	1.1	2.9	0.028
	Faecal MAP DNA (log10) at 8 months p.i.	6.0	2.5	21.4	<0.001
	Faecal MAP DNA (log10) at 12 months p.i.	2.3	1.6	3.8	<0.001
	Antibody (S/P) at 12 months p.i.	5.0	1.8	17.2	0.002

[a] LCL: Lower 95% confidence limit; [b]UCL: Upper 95% confidence limit

Table 4: 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

Figure 1. 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.

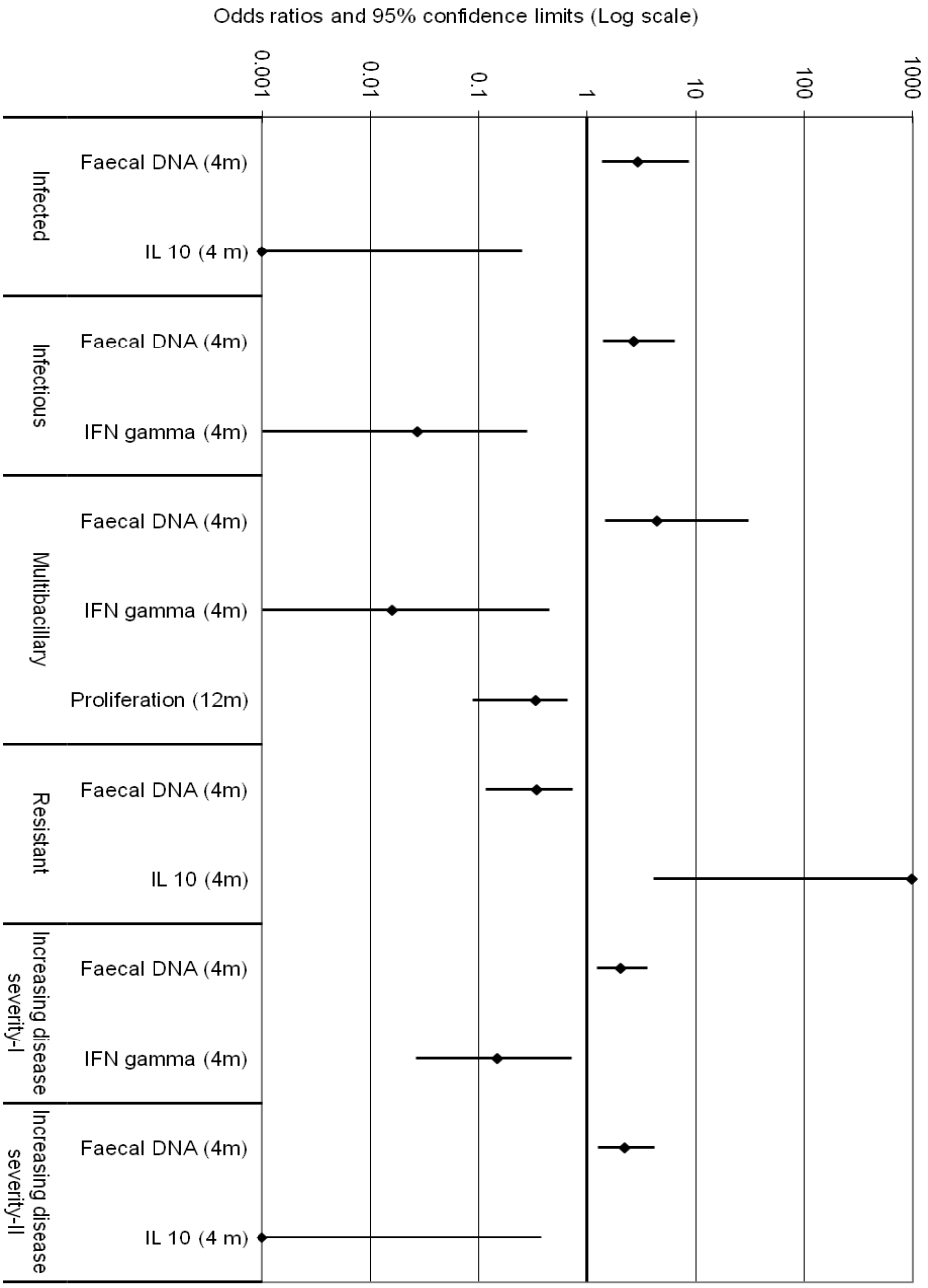

Figure 2. ROC curves based on logistic regression models for the following outcomes: (a) Tissue culture; (b) Faecal culture; (c) Multibacillary; (d) Resistance. Areas of the ROC curve based on each individual explanatory variable as well as the overall model are shown in each panel.