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Valerie Hughes

Susan Denham

J. P. Bannantine

Francesca Chianini

Karen Kerr

See next page for additional authors

Authors

Valerie Hughes, Susan Denham, J. P. Bannantine, Francesca Chianini, Karen Kerr, Linda May, Joyce McLuckie, Mintu Nath, and Karen Stevenson

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Short communication

Interferon gamma responses to proteome-determined specific recombinant proteins: Potential as diagnostic markers for ovine Johne's disease

Valerie Hughes[a,*], Susan Denham[a], John P. Bannantine[b], Francesca Chianini[a], Karen Kerr[a], Linda May[a], Joyce McLuckie[a], Mintu Nath[c], Karen Stevenson[a]

[a] Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ, United Kingdom
[b] National Animal Disease Center, USDA-Agricultural Research Service, Ames, IA, USA
[c] Biomathematics & Statistics Scotland, James Clerk Maxwell Building, The King's Building, Edinburgh EH9 3JZ, United Kingdom

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ABSTRACT

Johne's disease (JD), or paratuberculosis is a fatal enteritis of animals caused by infection with *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). There may be a long subclinical phase with no signs of clinical disease.

Diagnosis of JD is problematic and no test can reliably detect sub-clinical disease. Th1 responses to *Map* are believed to be activated first with a later switch to Th2 responses and progression to clinical disease. Detection of a cell-mediated response, indicated by interferon gamma (IFN-γ) produced in response to mycobacterial antigens, may give an early indication of infection. Crude extracts of *Map* (PPDj) have been used to detect the cell-mediated response, but more specific, quantifiable antigens would improve the test.

Thirty *Map*-specific proteins were screened for their ability to raise a cell-mediated response in subclinically infected sheep. Four proteins were selected and tested using blood from subclinical animals and controls from a JD-free flock. Three proteins elicited IFN-γ levels which were higher in the subclinical group than in the control group, two were statistically significant. Thus these proteins have the ability to discriminate groups of infected and uninfected animals and may have use in diagnosis of JD.

1. Introduction

Johne's disease (JD), or paratuberculosis, is a fatal, chronic granulomatous enteritis of animals caused by *Mycobacterium avium* subspecies *paratuberculosis* (*Map*), characterised by severe emaciation, poor body condition and in some species diarrhoea. The disease is mainly spread through ingestion of contaminated faecal material and following infection, a long incubation period may ensue during which there are no signs of clinical disease. These subclinical animals may go undetected and infect their herd-mates before clinical symptoms become apparent (Sweeney et al., 1992; Chiodini, 1996; Toman et al., 2003).

The diagnosis of JD is problematic and no test can reliably detect subclinical disease. Subclinical animals are particularly difficult to diagnose, they may be shedding small numbers of *Map* in their faeces intermittently and levels of circulating antibodies against *Map* may be low and not detectable by ELISA tests. PCR detection or bacteriological culture of faeces may not be sufficiently sensitive to detect low levels of organisms or may miss intermittent shedders. Thus control programmes are hampered by the inability to detect subclinical animals which fall below the radar of currently available tests.

Abbreviations: JD, Johne's disease; IGRA, interferon gamma release assay.

* Corresponding author. Tel.: +44 (0)131 445 5111; fax: +44 (0)131 445 6111.
E-mail address: Val.Hughes@moredun.ac.uk (V. Hughes).

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Early detection of *Map* infection is desirable to lessen the risks of transmission. The classical switch profile has been described for the immunological response of animals to *Map* infection (Chiodini et al., 1984; Stabel, 2000, 2006), in which Th1 responses are the first to be activated. Thus detection of a cell-mediated immune response by measuring the levels of interferon gamma (IFN-γ) produced in response to mycobacterial antigens (often PPD) (Wood et al., 1989) may give an early indication of subclinical animals and may occur before faecal shedding or antibodies directed against *Map* can be detected (Benedixen, 1978; Chiodini, 1996). A drawback to the IFN-γ test, which is the requirement for fresh blood samples, has recently been overcome by the addition of preservatives (IL-7, IL-12 and anti-IL-10), which allows a lengthened time period between collection and assay (Plain et al., 2012; Mikkelsen et al., 2012).

PPDj is a crude extract of *Map* and contains many antigenic components shared with the closely related environmental organism *M. avium* subspecies *avium* and with other pathogenic mycobacteria including *M. bovis*. Thus use of PPDj has issues for quality control and specificity. Substitution of PPD by specific recombinant proteins would greatly enhance the confidence in this test to diagnose *Map* infection and allow definition and quantification of antigen in the assay. Recently, *Map* recombinant proteins have been used to improve the specificity of the response in cattle (Mikkelsen et al., 2011). Here we describe a study to determine the efficacy of different *Map* recombinant proteins to elicit a Th1 response in naturally exposed sheep obtained from a flock with a history of JD and identify three recombinant proteins, which may have the ability to detect early *Map* infection when incorporated into an interferon gamma release assay (IGRA).

2. Materials and methods

2.1. Selection of subclinically infected animals

Subclinically infected animals were selected from two farms. Farm A had a high prevalence of ovine JD, with 27 clinical cases diagnosed between 2002 and 2008. Of these, 22 were classified as paucibacillary and the remainder as multibacillary disease following histopathological examination. Farm B had a low prevalence of JD with only one clinical case confirmed by tissue culture over the same period. The flocks on the farms were screened using the Bovigam™ (Celtic Diagnostics Ltd, Dublin, Ireland) IGRA. Whole blood was stimulated with PPDj and incubated for 24 h and the IGRA was performed. Animals producing more than 400 pg/ml of IFN-γ were considered responders and subclinically infected. These animals did not show any clinical signs of JD and were transported to the Moredun Research Institute where they were housed until the end of the study.

2.2. Selection of control animals

Control animals were sourced from Farm C with no history of clinical JD. Animals were negative in the following tests: Bovigam™ IGRA, JD serum ELISA and faecal culture.

2.3. Blood collection from sheep

Blood was collected by venipuncture in vacutainer tubes (those for IGRA contained preservative-free heparin). Experimental procedures were assessed and approved by an Experiments and Ethics Committee and authorised under the Animals (Scientific Procedures) Act 1986.

2.4. Preparation of PBMCs

PBMCs were prepared from buffey coat of heparinised blood using lymphoprep (Nycomed). Residual erythrocytes were removed by hypotonic lysis. PBMCs were suspended in RPMI complete (RPMI 1640 [84%, v/v], foetal bovine serum [10%, v/v], glutamine [1 mM], penicillin [100 U/ml], streptomycin [100 μg/ml], gentamycin [100 μg/ml], β-mercapto ethanol [50 μM], sodium bicarbonate [0.08%, w/v], HEPES [25 mM]), counted and adjusted to 2×10^6 cells per ml.

2.5. Incubation of blood with antigen

Whole blood (750 μl) or 500 μl of PBMCs, were incubated with either PPDa (purchased from AHVLA Weybridge), PPDj or purified recombinant protein added in a volume of 50 μl to a final concentration of 2.5, 2.5 or 10 μg/ml, respectively. ConA or SEB (final concentrations of 5 or 1 μg/ml, respectively) were used as stimulation controls, media controls (either RPMI or PBS) were also included. Stimulated blood was incubated in 5% (v/v) CO_2 for 24 h, centrifuged (500 g, 5 min at room temperature) and approx 500 μl supernatant removed and stored at −70 °C until required.

2.6. Bovigam™ IFN-γ release assay

The IGRAs were performed as described by the manufacturer. Ovine IFN-γ standards ranging from 0 to 5000 pg/ml were assayed on each plate analysed. This data enabled calculation of the amount of IFN-γ released during the stimulation. Alternatively, ODs were calibrated according to the OD-values of the positive and negative controls on the respective plates as described previously (Jungersen et al., 2002).

2.7. JD antibody ELISA test

Blood samples were commercially tested for serum antibody (Biobest Midlothian, UK) using the JD ELISA test (Institute Pourquier, Montpellier, France).

2.8. Preparation of recombinant proteins

Thirty recombinant *Map* proteins, previously identified as potentially *Map*-specific were tested. Maltose Binding Protein (MBP)-fusion recombinant proteins were cloned and purified (Hughes et al., 2008). Protein solutions were either diluted in RPMI complete for PBMC stimulation or PBS for whole blood stimulations.

2.9. Post mortem and histopathology

Animals were euthanized with an intravenous injection of 100 mg/kg pentobarbital. Samples of ileum, distal jejunum (DJ), mesenteric lymph node (MLN), ileo-caecal valve (ICV) and ileo-caecal lymph node (ICLN) were collected, and fixed in 10% buffered formalin saline to be processed for histology. Samples of MLN, terminal ileum (TI) and ICLN were removed for bacteriological culture. Microscopical examination was performed on paraffin-wax embedded, 5 μm-thick sections mounted on glass microscope slides and stained with haematoxylin and eosin (H&E) and Ziehl–Neelsen staining (ZN) for acid-fast bacteria. Animals were classified as paucibacillary or multibacillary according to the criteria described by Clarke and Little (1996).

2.10. Bacteriological culture from post mortem tissues

Tissue sections (either fresh or previously frozen at −80 °C) were trimmed of fat and connective tissue, homogenised, decontaminated and used to inoculate slopes of Middlebrook 7H11 supplemented with mycobactin J as described previously (Hughes et al., 2007). MLN and TI were submitted for bacteriological culture for all animals, ICLN was also submitted from W363, PC212, P7 and 4349.

2.11. Bioinformatics

Kyoto encyclopedia of genes and genomes (KEGG) was used to retrieve gene sequences. The SOSUI system for classification and secondary structure prediction was used to determine the likelihood of these proteins being membrane associated (Hirokawa et al., 1998).

2.12. Statistical analysis

Because of the small number of observations per animal and the presence of heterogeneity in variance of the status of animal, a linear model using generalised least-squares was fitted to the OD data. The infection status of animals, recombinant protein and the interaction of these factors were fitted as categorical fixed effects. The heterogeneity in variance associated with the status of animal was explicitly modelled. The estimate of the residual variance component was obtained by the Restricted Maximum Likelihood (REML) method (Pinheiro and Bates, 2000). To take into account the multiple comparisons of several proteins, the p-values estimated under the fitted model for each pairwise comparison were adjusted so that the overall false discovery rate was 5% (Benjamini and Hochberg, 1995). All statistical analyses were carried out using the R software version 2.13.1 (R Core Team, 2012).

3. Results and discussion

Naturally infected flocks were utilised to establish a panel of sheep identified by IGRA screening; selecting individuals with no clinical signs of disease but elevated IFN-γ responses to PPDj. The assumption was that the selected animals had been exposed to *Map* and were subclinically infected. Thirty recombinant proteins were tested for their ability to elicit IFN-γ production in PBMCs of nine subclinical sheep, results are presented in Table 1.

Animals showing production of IFN-γ in excess of 50 pg/ml were deemed to have responded to the antigen. Responses of animals to individual proteins varied considerably, no two animals responded to the same subset. Of the 30 recombinant *Map* proteins investigated, all were deemed to elicit a IFN-γ response in at least one animal. Some animals were particularly responsive to the protein antigens whilst others had limited responses. Four proteins (MAP1297, MAP1365, MAP3651c and MAP0268c), were selected for further investigation primarily on the basis of the number of animals exhibiting a response and its magnitude.

The likelihood of the selected proteins being membrane associated was determined using the SOSUI system, only MAP0268c was indicated as membrane-associated, and is a hypothetical protein with a thiopurine S-methyltransferase motif. The other proteins were predicted as soluble and annotated as phosphoribosyl isomerise (MAP1297), ornithine carbamoyltransferase (MAP1365) both involved in amino acid biosynthesis, and FADE3_2 (MAP3651c) central to terpenoid and geraniol metabolism. To assess the diagnostic potential of the selected proteins, IFN-γ responses of subclinical and uninfected animals were compared. Six subclinical animals from Farm A (P7, P33, W363, W395, PC212, 0269) and four control animals (724A, 329A, 307A, and 463A) selected at random from Farm C were used in this study. Whole blood was used rather than PBMCs for the IGRA as this would be the sample of choice for diagnostic testing. IGRAs on whole blood were carried out in duplicate with the selected proteins, MBP, and PPDa and data were analysed (Fig. 1A). The mean OD value for each blood sample tested was calibrated and corrected for the contribution of MBP to the stimulation. The data showed heterogeneity in variance for the status of infection and it was noted that the OD value of one of the animals in the infected group stimulated with MAP0268c was an order of magnitude higher than the other comparable readings and considered an outlier.

Statistical analysis of data following exclusion of the outlier (observation on animal W363 for MAP0268c) showed that the interaction effect of treatment group and protein was statistically significant ($p = 0.022$) which indicated that there was evidence of genuine differences in mean responses between the control and subclinical animal groups for some of the proteins. For all proteins, mean levels were higher in the subclinical group and the means were markedly different for MAP0268c, MAP1365 and MAP3651c. For the recombinant protein MAP3651c, the mean OD value (±standard error of mean) of the infected group (0.198 ± 0.035) was statistically significantly (false discovery rate-adjusted $p = 0.010$) higher than the mean OD value of the control group (0.015 ± 0.043). Additionally, for the protein MAP0268c, there was weak evidence (FDR-adjusted $p = 0.051$) that the mean OD value of the infected group (0.051 ± 0.017) was higher than the mean OD value of the control group (-0.009 ± 0.019). The

Table 1
IFN-γ responses to recombinant *Map* antigens in subclinical animals.

	P7	P15	P33	W363	PC212	W395	O269	1707N	1585N	Number of responders
MAP0068	×	×	×	✓	×	✓	×	×	×	2
MAP0139c	×	×	×	×/✓	✓	×	×	×	×/✓	3
MAP0268c	×	×	✓	✓	✓	×	✓	✓	×/✓	6
MAP0334	×/✓	ND	✓	✓	✓	✓	✓	×	×	6
MAP1012c	×	×	×	✓	×/✓	×	×/✓	✓	×/✓	5
MAP1160c	×/✓	×	×	×/✓	✓	×	×	×	×	3
MAP1293	×	×	✓	✓	✓	×	×	×	×	3
MAP1297	×	ND	✓	✓	✓	✓	ND	✓	✓	6
MAP1365	✓	ND	✓	✓	✓	✓	ND	×	×	5
MAP1564c	×	×	✓	✓	×/✓	✓	×	×	×	4
MAP1754c	×	×	×	✓	×	×/✓	×	×	×	2
MAP2541c	×	×	×	✓	×/✓	×	×/✓	×/✓	×	4
MAP2685	×	ND	✓	✓	✓	✓	ND	×	×	4
MAP2872c	×	×	×	✓	×	×	×/✓	×	×/✓	3
MAP2878c	×	ND	✓	✓	×/✓	✓	✓	×	×	5
MAP3175	×	ND	✓	✓	✓	✓	ND	✓	×	5
MAP3205	×	×	×	×	×/✓	×	×	×	×	1
MAP3385	×	×	×/✓	✓	×/✓	×/✓	×	×	×	4
MAP3457	×	×	×	✓	×	×/✓	×	×	×	2
MAP3491	×	×	×/✓	✓	×	×/✓	×	×/✓	×	4
MAP3540c	×	×	×	✓	×	×	×	×/✓	×	2
MAP3567	×	×	✓	✓	×/✓	×	×	×/✓	×	4
MAP3627	×	×	×	×/✓	×/✓	×	×	×	×	2
MAP3651c	×	×	×/✓	✓	×/✓	✓	×/✓	✓	×/✓	7
MAP3692c	×	ND	×	✓	✓	✓	×	✓	×	4
MAP3841	×	×	×	×/✓	×	×	×	×	×	1
MAP3857	×	ND	✓	✓	✓	✓	ND	×	×	4
MAP3932c	×	ND	✓	×/✓	×	×	×	×	×	2
MAP4147	×	×	×/✓	✓	×/✓	×	×/✓	×/✓	×	5
MAP4233	×	×	×	✓	×	×	×/✓	×/✓	×	3

× < 50 pg
×/✓ > 50 pg<100pg IFN-γ
✓ > 100pg IFN-γ
ND not determined
Proteins which were selected for further investigation are highlighted.

difference of the mean OD values of infected and control groups for MAP1365 was short of statistical significance ($p = 0.16$) and that for MAP1297 protein was also not statistically significant.

Inclusion of the outlying data point vastly increased the mean OD for the infected animal group but also increased the observed variation and the observed effect ceased to be statistically significant. Exclusion of the data point is not unreasonable given that technical errors in plate manipulation can lead to spurious high OD readings.

During the period of recombinant antigen testing, responses of animals to PPDj were also monitored and the results are presented in Fig. 1B. All animals (except P15) exhibited consistently high levels of IFN-γ production in response to PPDj, although a degree of fluctuation was observed. Despite this fluctuation, most IGRA results were in excess of the arbitrary 400 pg/ml cut off we had applied in selecting the animals for investigation. Animal P15 however, demonstrated the classical IFN-gamma response pattern in JD (Stabel, 2000), in that it responded highly to PPDj in the subclinical stage (day 1-levels in excess of 500 pg ml^{-1}), but the response was lost rapidly after transport to Moredun (0–5 pg ml^{-1}) where it rapidly succumbed to JD 30 days after purchase.

Subclinical animals were monitored (up to 4 years) for IFN-γ responses to PPDj and ELISA tests were performed periodically for detection of *Map* antibodies. Over the course of the experiment, four animals were found to have a positive JD antibody ELISA test (P15, PC212, P7 and 4349). Results of the repeated ELISA tests can be found in supplementary Table S1.

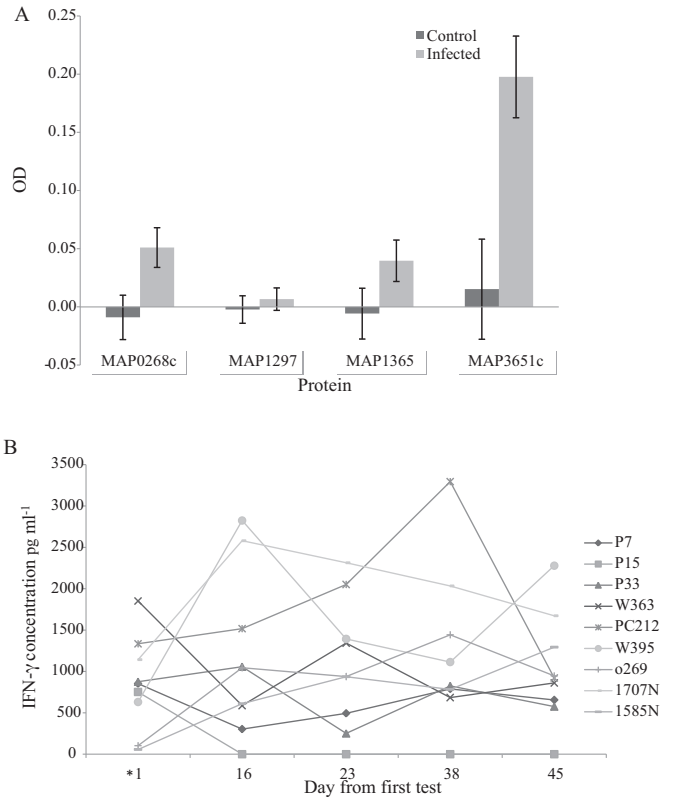

Fig. 1. (A) Mean OD values of infected and non-infected animals generated in Bovigam™ IFN-γ release assays. Whole blood was stimulated with four recombinant antigens (MAP268c, MAP1297, MAP1365 and MAP3651c). The infected group comprised six animals sourced from a paratuberculosis-infected flock the control group comprised four animals sourced from a flock with no history of paratuberculosis. The data was calibrated and corrected for the contribution of MBP and medium to the stimulation. The error bar indicates the standard error of mean. (B) Temporal changes in IFN-γ production of whole blood from nine subclinical sheep in response to PPDj. OD values were generated in Bovigam™ IFN-γ release assays. Nine animals (P7, p15, P33, W363, PC2121, W395, O269, 1707N, 1585N) sourced from paratuberculosis-infected farms were monitored periodically. The data was corrected for the contribution of medium to the stimulation.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2013.06.015.

At the end of the study period, subclinical animals were subjected to pathological and bacteriological investigations to determine their infection or disease status with respect to *Map*. A summary of this information including breed and age is given in Table 2.

Histological examination of lymph nodes and intestine samples from eight subclinical sheep was carried out. Three had lesions previously described as paucibacillary (PC212, P7, 4349) and samples of their ileum and lymph nodes showed a severe infiltrate of small lymphocytes and multifocal small granulomata, with multinucleated giant cells. In animals where macroscopic nodules were observed, some of these granulomata were encapsulated and showed evidence of calcification (Fig. 2B). ZN staining of these samples did not show any acid-fast organisms. Macroscopic observations of the gastrointestinal tract showed enlarged lymph nodes in two animals (PC212 and 4349) and widespread formation of small, round, 1–2 mm diameter nodules on the surface of the small intestine and lymph nodes of two sheep (P7 and 4349) (Fig. 2A). One had lesions consistent with multibacillary JD (P15) (Fig. 2C and D), changes in the ileum were characterised by sheets of closely packed macrophages with abundant, weakly eosinophilic cytoplasm, mainly seen in the mucosa. A milder similar infiltrate was observed in the lamina propria, submucosa and in the lymph nodes. ZN staining demonstrated a large number of acid-fast rods in the cytoplasm of macrophages (Fig. 2D). Two further animals (0269 and W395) had an inflammatory infiltrate characterised by large amounts of eosinophils, some neutrophils and small mineralised abscesses. The samples from the remaining two animals (W363 and P33) were within normal histological limits (Table 2). The significance of the small, mineralised nodules observed macroscopically on the surface and within the tissues was difficult to determine. Similar lesions were reported previously in JD, but were attributed to parasitic infection (Pérez et al., 1996). However, the cases described here had low parasitic loads and an absence of parasitic debris. Mineralisation of lymph node lesions has been described previously in JD-affected wild ruminants (Williams et al., 1983; de Lisle et al., 1993) so it is not implausible that the mineralised nodules observed in this study were pathological lesions of JD.

Animal PC212 was one of the higher producers of PPDj-stimulated IFN-γ and had concomitant high levels

Table 2
Summary of the post mortem pathological and bacteriological observations in the subclinical animals.

	W363♀	PC212♀	P7♀	4349♀[b]	0269♀	W395♀	P33♀	P15[a]♀
General condition	Good	Good, but thin	Good, but moderately thin	Good	Good	Good	Good	Scouring and very thin
FEC	0	0	0	0	0	0	0	High (6750/g)
Breed	Bleu du Maine X	Bleu du Maine X	Bleu du Maine X	Bleu du Maine X	Bleu du Maine	Bleu du Maine	Bleu du Maine X	Lleyn X
Age (yrs) at PM/death	8	8	7	6	7	6	5	3.5
Gross pathology	GI tract, ICLN & MLN normal	GI tract normal Enlarged ICLN & MLN	Wide spread cysts/nodules on surface of GI tract, ICLN & MLN and liver	Some cysts/nodules on surface of GI tract, ICLN, MLN & liver MLN enlarged with single cyst	GI tract, ICLN & MLN appeared normal	GI tract, ICLN & MLN appeared normal	GI tract, ICLN & MLN appeared normal	GI tract appeared normal, enlarged MLN and ICLN
Histopathology	No significant lesions in the intestine or lymph nodes.	Inflammatory infiltrate of IM, DJ, ICV and liver (eosinophils and giant cells) multifocal small granulomata, encapsulated with calcification	Inflammatory infiltrate of IM, DJ, ICV, LN and liver (eosinophils and giant cells) multifocal small granulomata, encapsulated with calcification	Inflammatory infiltrate J and DJ LN eosinophills and giant cells IM and ICV normal	Inflammatory infiltrate in IM & ISM mainly eosinophil, some neutrophil. Old abscesses in LN	Inflammatory infiltrate in IM & ISM mainly eosinophil, some neutrophil. Old abscess in LN	No significant changes in the IM or LN.	Inflammatory infiltrate (severe) of IM & ISM (macrophage, some eosinophil) LN moderate numbers macrophage
Map isolated from tissues	No	+TI, ICLN	+TI, MLN, ICLN	+TI, MLN	No	No	No	ND[c]
Diagnosis	Normal	Paucibacillary	Paucibacillary	Paucibacillary	Parasitic infection	Parasitic infection	Normal	Multibacillary

FEC, faecal egg count; Strongylata, Nematodirus and Coccidia were identified in faecal material; TI, terminal Ileum; IM, ileal mucosa; ISM, ileal submuscosa; LN, lymph node; DJ, distal jejunum; ICLN, ileo-caecal lymph node; ICV, ileo-caecal valve; MLN, mesenteric lymph node.

[a] Died.

[b] Animal recruited late to study and not used for screening recombinant antigens.

[c] Not done, however,large numbers of acid fast bacilli were noted in IM, ISM and LN and faeces was culture positive for Map.

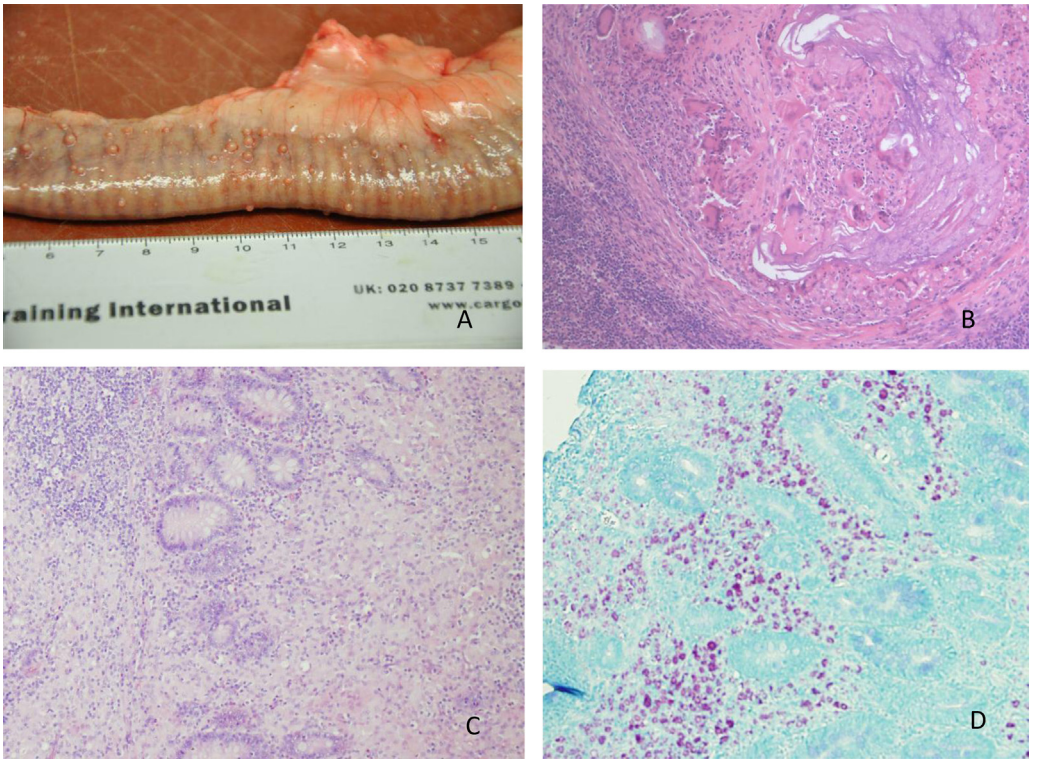

Fig. 2. (A) Ileum of sheep P7 showing macroscopical widespread 1–2 mm diameter nodules and microscopical encapsulated granuloma (B) with numerous multinucleated giant cells and calcification (H&E, original magnification ×200). (C) Multibacillary paratuberculosis (P15): very large number of macrophages with abundant cytoplasm infiltrating ileal mucosa (H&E, original magnification ×200) and (D) stained with ZN (original magnification ×200).

of antibody. This case shows that high levels of antibody can exist alongside a well developed Th1 response, which goes against the dogma of the switch hypothesis (Chiodini et al., 1984; Stabel, 2000, 2006) and supports the temporally coincident antibody/IFN-γ responses for some animals with JD as previously reported (Begg et al., 2011).

The ability of these proteins to discriminate between subclinical and control animals indicates their potential as diagnostic tools in IGRAs for the detection of subclinical infections in sheep and their identification is a step towards a better diagnostic tool for JD. The mean OD values of all antigens were lower than that obtained for PPDj stimulation of the infected group (0.7) which indicates that the complex antigenic mix of PPDj was a more powerful stimulant of IFN-γ production than any of the recombinant antigens. However, a mixture of the three antigens in a single IGRA increased the magnitude of response and was effectively the sum of the individual parts (authors unpublished observations). This indicates that cocktails of recombinant proteins maybe necessary to develop future sensitive diagnostic tests. Although specificity of these proteins is implied, an empirical approach to test the specificity of an IGRA incorporating these proteins is still required.

The application of the IFN-γ test for detection of individual animals likely to progress to clinical disease is still debatable. Global differences of IFN-γ response to PPD in exposed and non-exposed populations was clearly demonstrated (Jungersen et al., 2012), but its ability to predict shedding or clinical disease in individual animals was not (Huda et al., 2004; Mikkelsen et al., 2009). In our study, four out of eight sheep with elevated IFN-γ release (in excess of 400 pg/ml) either had a positive diagnosis of paratuberculosis as defined by the serum antibody ELISA or progressed to it within the time scale of the experiment (2–3 years). This high proportion of IGRA test positive animals which progressed to diagnosis by the classical serum ELISA test appears to be at odds with previous findings (Huda et al., 2004). The likelihood of an exposed animal (as defined by IFN-γ response) developing detectable infection (either faecal shedding or a positive serum ELISA test) and the impact of external factors is yet to be defined and crucial for determining the efficacy of the IGRA to act as a prognostic for JD.

A major drawback in control and eradication of JD is the lack of tools to identify early-stage infections. Generation of tools to monitor exposure of individual animals to *Map* may provide a starting point to understand disease progression and in ovine cases, may have applications in indicating animals which should be removed from flocks to control the spread of disease.

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