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Differential expression of pattern recognition receptors in the three pathological forms of sheep paratuberculosis

Citation for published version:

Nalubamba, K, Smeed, J, Gossner, A, Watkins, C, Dalziel, R & Hopkins, J 2008, 'Differential expression of pattern recognition receptors in the three pathological forms of sheep paratuberculosis', *Microbes and Infection*, vol. 10, no. 6, pp. 598-604. <https://doi.org/10.1016/j.micinf.2008.02.005>

Digital Object Identifier (DOI):

[10.1016/j.micinf.2008.02.005](https://doi.org/10.1016/j.micinf.2008.02.005)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Microbes and Infection

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Elsevier Editorial System(tm) for Microbes and Infection
Manuscript Draft

Manuscript Number: MICINF-D-07-00363R1

Title: Differential expression of pattern recognition receptors in the three pathological forms of sheep paratuberculosis

Article Type: Original article

Keywords: pattern recognition receptors; paratuberculosis; sheep

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Abstract: Paratuberculosis is a chronic inflammatory disease of the gut caused by *Mycobacterium avium* subspecies paratuberculosis. Three forms have been described in sheep - paucibacillary, multibacillary and asymptomatic. The pauci- and multibacillary forms are characterized by type 1 and type 2 immune responses respectively; asymptomatic animals have no clinical signs or pathology. What determines this polarization is unknown, although pattern recognition receptors (PRR) have been implicated in other mycobacterial diseases. To investigate this in sheep paratuberculosis we used real-time RT-PCR to quantify the expression of fifteen PRR and adaptor genes from forty infected and nine control animals.

These data show that there is a relationship between the different pathological forms and PRR transcript profiles. Nine PRRs were up-regulated in asymptomatic animals; with TLR9 being significantly raised in relation to the other three groups.

Comparison of the three infected groups showed increases in many PRRs, with CARD15 and Dectin-2 being particularly high in both diseased groups. Significant differences between the pauci- and multibacillary animals included TLR2, CD14 and Dectin-1. Sequence analysis of TLR2 exon 2 and CARD15 exon 11 in the forty animals failed to identify any relationship between SNPs and pathological form.



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Ms. Ref. No.: MICINF-D-07-00363

Title: Differential expression of pattern recognition receptors in the three pathological forms of sheep paratuberculosis

Dear Géraldine,

Many thanks for the referee's comments they were helpful and informative. An excellent referee.

1. Comments in Discussion about link with infection... this shows that it is easy to get too close to the issue. The referee's comment is correct and I have included some discussion on this point (p 14), stating the obvious - as recommended by the referee.
2. The issue of high PRR and disease is also discussed (p 14) and I have added a comment about whether increases in PRRs are consequential on infection or causative.
3. The point about non-functional PRRs (or at least mutated) etc is included at the very end of the discussion (p 15).
4. I have removed the implication that tuberculosis has lepromatous pathology (para 1, p 13).
5. I have changed round the labelling of the Figures (i.e. disease vs control etc rather than control vs disease) as well as how this is referred to in the text etc. It is embarrassing that I let that through.
6. Table 3 has been renumbered Table 2.
7. Tetra arms... non-synonymous (another silly mistake by me that no one else picked up)

I hope that these alterations are fine and that this paper is now acceptable for publication.

Many thanks

A handwritten signature in black ink, appearing to read 'John Hopkins', written in a cursive style.

1 **Differential expression of pattern recognition receptors in the three**
2 **pathological forms of sheep paratuberculosis**

3

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11

12 **Short title:** PRRs in paratuberculosis

13

14

15 **Keywords:** pattern recognition receptors; paratuberculosis; sheep

16

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1 **Abstract**

2 Paratuberculosis is a chronic inflammatory disease of the gut caused by *Mycobacterium*
3 *avium* subspecies *paratuberculosis*. Three forms have been described in sheep –
4 paucibacillary, multibacillary and asymptomatic. The pauci- and multibacillary forms are
5 characterized by type 1 and type 2 immune responses respectively; asymptomatic animals
6 have no clinical signs or pathology. What determines this polarization is unknown, although
7 pattern recognition receptors (PRR) have been implicated in other mycobacterial diseases. To
8 investigate this in sheep paratuberculosis we used real-time RT-PCR to quantify the
9 expression of fifteen PRR and adaptor genes from forty infected and nine control animals.
10 These data show that there is a relationship between the different pathological forms and PRR
11 transcript profiles. Nine PRRs were up-regulated in asymptomatic animals; with TLR9 being
12 significantly raised in relation to the other three groups.
13 Comparison of the three infected groups showed increases in many PRRs, with CARD15 and
14 Dectin-2 being particularly high in both diseased groups. Significant differences between the
15 pauci- and multibacillary animals included TLR2, CD14 and Dectin-1. Sequence analysis of
16 *TLR2 exon 2* and *CARD15 exon 11* in the forty animals failed to identify any relationship
17 between SNPs and pathological form.

1 **1. Introduction**

2 The primary sensing of conserved microbial structures, known as pathogen-associated
3 molecular patterns, is achieved by germline-encoded receptors - the pattern recognition
4 receptors or PRRs. PRR association with microbial ligands alerts the innate immune system to
5 the presence of infection and triggers host defence mechanisms [1] including the activation of
6 the adaptive immune response. There are two broad groupings of PRRs. Firstly, the Toll-like
7 receptors (TLRs) and NACHT-LRR proteins including NOD1 and NOD2 (CARD15) [2];
8 engagement of which leads to intracellular signalling and the synthesis of effector molecules
9 [3]. Secondly, lectins that bind pathogens through recognition of carbohydrate moieties and
10 function through complement fixation, opsonization and/or cell activation [4].

11 Thirteen TLRs have so far been reported in vertebrate species although only ten are
12 present in humans, cattle and sheep [2,5,6]. The engagement of each TLR initiates an
13 intracellular signalling cascade through a series of adapter molecules, the most common being
14 MyD88 [7]. Each TLR has its own array of ligands, although their repertoires are expanded
15 by heterodimerization [4] and association with non-TLR molecules like CD14. The calcium-
16 dependent C-type lectins are central to several physiological processes, including antigen
17 uptake [8]. Two major C-type lectins are Dectin-1 and Dectin-2 that have specificity for
18 fungal β -glucans and are expressed preferentially on macrophage and dendritic cell (DC)
19 lineage cells [9,10] and engagement with ligands triggers phagocytosis and cell activation.

20 Paratuberculosis (Johne's disease) is a common intestinal disease of ruminants caused
21 by *Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*). Infection of
22 sheep can give rise to three different forms of disease with only about 30% of animals in an
23 infected flock becoming clinically affected. The majority are infected but asymptomatic
24 [10,11]. The remaining clinically-affected sheep show two distinct forms of the disease: the
25 paucibacillary form with very few bacteria and a T cell infiltration into the gut; and the

1 multibacillary form characterized by a high level of bacterial infection and a macrophage and
2 B cell infiltration. Both the pauci- and multibacillary forms are equally fatal but there is no
3 evidence that the asymptomatic animals ever succumb to disease [10,12].

4 Sheep with paucibacillary disease have strong cell-mediated immunity (CMI), high
5 levels of IFN γ and IL-12p40 and low levels of antibody [10,12], a pattern similar to
6 tuberculoid leprosy [13]. In contrast, multibacillary cases have a similar pathology to
7 lepromatous leprosy with high antibody and weak CMI. The asymptomatic animals are
8 positive for bacterial growth, IS900 and specific antibody and express reduced levels of IL-18
9 when compared to uninfected controls [10,14].

10 As with human leprosy [15] it seems clear that the polarization of the immune
11 response is critical to the clinical outcome of the paratuberculosis infection [10]. The
12 intestinal tissue damage that results from a Th1 response (paucibacillary disease) is
13 fundamentally different to that caused by a Th2 response [10], which leads to multibacillary
14 disease and dissemination of infection. It is becoming clear that distinct antigen-presenting
15 cell subsets play a crucial role in the polarization of immune responses through the
16 differential expression of IL-10 and IL-12 [16,17]. These subsets also show differential
17 expression of PRRs [6,18] and differential PRR activation can tailor the response [19]. The
18 critical importance of PRRs to the health of an animal is illustrated by the numerous examples
19 of the outcome of infection being influenced by quantitative expression of individual PRRs
20 [20] and where PRR polymorphisms are associated with disease susceptibility [21]. Indeed,
21 there is growing evidence for the linkage of *TLR2 exon 2* and *CARD15 exon 11* mutations and
22 susceptibility to human mycobacterial diseases [22-24].

23 This study tested the hypothesis that expression levels of a panel of fifteen PRR genes would
24 be different at the site of infection in the three forms of sheep paratuberculosis, and that these
25 differences could relate to the observed pathologies. This study also analyses the

1 asymptomatic form of sheep paratuberculosis in relation to uninfected control animals.
2 Furthermore, a preliminary sequence analysis of the sheep *TLR2 exon 2* and *CARD15 exon 11*
3 was performed to investigate if the genotype is linked to paratuberculosis pathology.

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2. Materials and Methods

2.1. Animals and Tissues

Animals with the clinical disease and asymptomatic animals were out bred sheep with naturally acquired *M. paratuberculosis* infection. Individual sheep were selected based on clinical symptoms of paratuberculosis. All sheep were euthanized and diagnosis was confirmed by histopathology of the terminal ileum and IS900 real-time PCR in ileum and mesenteric lymph node as previously described [10]. Paucibacillary animals (n=12) had T cell infiltration and few bacteria as assessed by Ziehl-Neelsen stain (ZN); multibacillary sheep (n=16) had a macrophage and B cell infiltration and were ZN+ [10]. Sheep from the same flocks with no clinical signs of Johne's disease, but were positive for IS900 were considered to be asymptomatic (n=12). All control sheep tested negative for IS900 (n=9). Terminal ileum blocks (~0.5 g) were placed in five volumes of *RNAlater* (Ambion, Huntingdon, UK), which were then incubated overnight at 4 °C and then stored at -80 °C.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from tissues using the RNeasy Mini kit (Qiagen, Crawley, UK); tissue samples were finely chopped and homogenized in 350 µl of lysis buffer. Each sample was diluted with 550 µl of nuclease-free water and digested with 10 µl proteinase K at 20 mg/ml (Sigma-Aldrich, Poole, UK.) for 15 min at 55 °C. Genomic DNA was sheared using a 20-g needle. Homogenates were microfuged and RNA purified using Qiagen mini spin columns. RNA samples from the same biopsy were pooled, volumes adjusted to a total volume of 100 µl in nuclease-free water, purified using Qiagen RNA mini spin columns and eluted in 30 µl of nuclease-free water. Total RNA was quantified by spectrophotometry. RNA quality and integrity was confirmed using a RNA LabChip on an Agilent® 2100 bioanalyzer; all samples had an RNA integrity number >7.

1 For cDNA synthesis, 2.5 µg of total RNA from each tissue sample was mixed with 0.5
2 µg Oligo(dT)₁₅ primer, 5 µl of M-MuLV RT 5x reaction buffer, 1 µl of dNTPs mix (10 mM),
3 1 µl M-MuLV RNaseH⁻ reverse transcriptase (Promega, Southampton, UK) and nuclease free
4 water up to 25 µl. The reaction was incubated at 40 °C for 10 min, 42 °C for 50 min and
5 inactivated at 70 °C for 15 min. The cDNA was diluted four-fold in nuclease free water and
6 stored at -20 °C until used.

7

8 2.3. *Quantitative real-time PCR for sheep PRRs*

9 Two-step, quantitative real-time RT-PCR (qPCR) was carried out using a Rotor-
10 Gene™ 3000 (Corbett Life Science, Sydney, Australia) using primers for sheep PRRs exactly
11 as previously described [6]. Standard curves for each PRR were generated using 10-fold serial
12 dilution series of linearized plasmid DNA templates. The correlation coefficient was between
13 0.9 to 0.99 with a slope value of the standard curves in the range of -3.33 +/- 0.3 and the PCR
14 efficiency of >90% calculated from slope. Quantitative real-time PCRs were carried out in a
15 final volume of 20 µl containing 2 µl of template cDNA and 18 µl of qPCR master mix
16 containing the primers, 200 µM dNTPs, 1.5 mM MgCl₂, 0.7 µl SYBR Green I (1/1000
17 dilution) and 0.75 U Faststart® Taq (all Roche Diagnostics Ltd., Lewes, UK) per reaction
18 was made up in nuclease free water. Cycling conditions were; 94°C for 10 min, followed by
19 forty cycles of 94°C, 20 s; 62.5°C, 20 s; 72°C, 20 s and fluorescence signal acquisition. Melt
20 curve analysis with an initial 94°C for 20 s prior to a temperature gradient from 65-94 °C with
21 a heating rate of 0.3°C per second was performed after each qPCR run to assess the specificity
22 of amplification. Copy numbers were determined from the Ct values of each sample in
23 comparison to the copy number values assigned from the plasmid DNA standard using Rotor-
24 Gene analysis software (6.0.34). Data were normalized using β-actin and succinate
25 dehydrogenase (SDHA) housekeeping genes, a normalization factor, taking into account the

1 geometric means of both housekeeping genes, was calculated using geNORM plugin for
2 Excel [25]. One way analysis of variance and Tukey's multiple comparison tests were used
3 for the pair-wise comparisons of the normalized data.

4

5 2.4. PCR and DNA sequencing

6 PCR amplification of the forty genomic DNA samples was performed with each of the
7 primers pairs; CARD15 For/Rev (For 5' - TCATTGGGAATCTCAGACAGG, Rev 5' -
8 GAACCAGATTCATCCCATGC, annealing temp 57°C), DNA1TLR2 For/Rev (For 5' -
9 TTTCTCATCTCCCAAATCTGC, Rev 5' - AATGGCCTTCTTGCAATGG; annealing temp
10 59°C) and DNA2TLR2 For/Rev (For 5' - TGTGGAGACGTTAACAATACGG, Rev 5' -
11 TCATCAAAGAGACGGAAATGG annealing temp 59°C) as follows: 100 ng DNA was
12 placed in a thin walled microfuge tube and 5 µl 10x PCR buffer (Promega); 1 µl dNTP mix
13 (Promega); 20 pmol of each primer and nuclease free water was added to a final volume of
14 49 µl. The PCR mixture was incubated at 95°C for 2 min prior to the addition of 1U of *Taq*
15 polymerase. Reactions were then cycled under the following conditions: thirty cycles of :
16 denaturing at 95°C, 5 s; annealing at the temperatures shown above; extension at 72°C, 120 s,
17 followed by a final extension at 72°C for 10 min. PCR amplicons were analysed by agarose
18 gel electrophoresis, purified using the QIAquick® system (Qiagen) and used as templates for
19 direct sequencing using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems,
20 Warrington, UK.) using both the forward primer and reverse primer. To minimize base calling
21 errors DNA sequence chromatograms were analysed using the Staden sequence assembly
22 package (<http://staden.sourceforge.net/>). Sequence from both strands was obtained from three
23 independent PCR reactions of all products. Contiguous regions were assembled for each
24 genomic DNA sample and the resultant sequences aligned for comparison and determining
25 possible single nucleotide polymorphisms (SNPs). Each of the SNPs determined from

1 sequence comparison were confirmed by manual inspection of the chromatographs.
2 Verification of the non-synonymous SNPs was obtained using Tetra-ARMS with primers
3 (Table 1) designed using the tetra-ARMS primer design program;
4 http://cedar.genetics.soton.ac.uk/public_html/primer1.html. The distribution of alleles, breeds
5 and genotypes was compared between the pathological groups using χ^2 -test.
6

1 3. Results

2 The expression levels of PRR transcripts in sheep ileum from the three groups of
3 paratuberculosis-infected sheep and uninfected controls is shown in Table 2 which shows
4 transcript copy number of all the PRRs in relation to the two housekeeping genes, β -actin and
5 SDHA. These data show that different PRRs are present at very different levels in ileal tissue,
6 varying from less than 100 copies (e.g. TLR7) to almost 300,000 copies (Dectin-2). They also
7 show that levels of individual PRR transcripts are highly variable between animals, even
8 within the same pathological group. Furthermore, it shows that individual PRRs are
9 differentially expressed in the distinct disease states.

10 This is more clearly shown in Figs. 1 and 2 where the results of only those PRRs that
11 show statistically significant ($p \leq 0.05$) fold change; data are compared in six pairs –
12 asymptomatic vs control, paucibacillary vs control and multibacillary vs control (Fig. 1);
13 paucibacillary vs asymptomatic, multibacillary vs asymptomatic and multibacillary vs
14 paucibacillary (Fig. 2). The expression levels of three transcripts, TLR1, TLR5 and TLR10
15 was relatively consistent in all the animals regardless of disease status and TLR7 transcripts
16 were marginally detectable in any sample group.

17 There were significant differences between the uninfected asymptomatic and control
18 samples (Fig. 1a), with increased levels of eight PRRs in the asymptomatic ileum. TLR2,
19 TLR3, TLR4, TLR8 and Dectin-1 were raised 2 – 5 fold over the controls, while 10 – 14 fold
20 increases were observed with TLR9, CD14 and Dectin-2. The level of the adapter MyD88
21 was also increased (~1.6 fold) although not at a significant level ($p=0.07$). Many of the PRR
22 transcript levels in both diseased forms were also raised. When the paucibacillary and control
23 animals were compared (Fig. 1b), there were 3 – 6 fold increases in the expression levels of
24 TLR2, TLR3, TLR4, TLR6, TLR8 and MyD88 and 8 - 16 fold changes of CARD15, CD14
25 and Dectin-1; Dectin-2 was shown to be raised >600 fold. A similar comparison between

1 multibacillary and control animals (Fig. 1c) showed an equivalent profile but with four
2 exceptions; TLR2 transcripts were raised 20 fold, TLR9 was raised 4 fold and the increase in
3 Dectin-2 was >1000 fold. TLR6 was also increased approximately two fold, but this was not
4 at a significant level (p=0.08).

5 The comparison of PRR transcripts between the three infected groups of sheep is
6 shown in Fig. 2. When the two diseased forms were compared with the asymptomatic samples
7 only MyD88, CARD15 and Dectin-2 were significantly up-regulated in paucibacillary ileum,
8 (Fig. 2a), while TLR9 was significantly reduced (0.12 fold change). More differences were
9 observed with the multibacillary sheep; with less than 4 fold increases with TLR2, TLR4,
10 TLR8, MyD88 and CD14, an approximate 6 fold increase in CARD15 and Dectin-1 and a
11 >80 fold rise in Dectin-2. The levels of TLR9 transcripts were also significantly reduced (0.27
12 fold). The comparison of the two disease forms highlighted a 2–4 fold increase of TLR2,
13 CD14, Dectin-1 and Dectin-2 in the multibacillary tissues.

14 PCR amplification of the forty genomic DNA samples were performed with each of the
15 primers pairs; DNA1TLR2 For/Rev, DNA2TLR2 For/Rev and CARD15 For/Rev. Primers
16 were designed to result in mutually overlapping fragments within the TLR2 gene primer set to
17 facilitate sequence assembly. Analysis of the 40 sequences identified seven single nucleotide
18 polymorphisms (SNPs). Table 3 shows the location and distribution of these SNPs in the
19 pathological types. Two non-synonymous mutations were identified, A182C (numbered as
20 accession number AM117123) results in an arginine to alanine substitution and the T1516C
21 mutation results in a leucine to phenylalanine substitution. The other five mutations were
22 silent. The distribution of all the SNPs identified in this study was almost equally distributed
23 between the three pathological forms of the disease and not significantly skewed towards any
24 one group (χ^2 , $p \geq 0.92$ for all comparisons). The animals tested were of a variety of breeds and
25 crosses, but Table 4 shows that there was no discernable, significant relationship between

- 1 pathology, breed and genotype (χ^2 , $p \geq 0.79$ for all comparisons). No SNPs were identified
- 2 after the analysis of *CARD15 exon 11* sequences from the same forty animals.
- 3

1 **4. Discussion**

2 The three major mycobacterial diseases, tuberculosis, leprosy and paratuberculosis
3 share many similarities despite the fact that they affect different organ systems. All three are
4 caused by related facultative intracellular pathogens that mainly target macrophage
5 populations; the majority of infected individuals control the infection and never show signs of
6 clinical disease; and with leprosy and paratuberculosis, infection can give rise to one of two
7 distinct forms of clinical disease.

8 It is clear that the two forms of clinical disease are manifestations of differential
9 polarization of the immune response to the pathogen. The tuberculoid or paucibacillary form
10 is mediated by a type 1 response where bacterial growth within infected macrophages is
11 controlled by $\text{IFN}\gamma$ and $\text{TNF}\alpha$ [10]. In tuberculosis and leprosy this results in a self-limiting
12 infection although in sheep paratuberculosis it is an end-stage disease [13]; the difference is
13 probably due to the organ system that is affected. The lepromatous or multibacillary form is
14 mediated by a type 2 response, where there is little $\text{IFN}\gamma$ and therefore less control of
15 intracellular bacterial growth; consequently it is the form of disease that is mainly responsible
16 for the transmission of infection [11]. Polarization of the immune response is, therefore
17 critical to the outcome of infection and it is becoming increasingly clear that innate receptor
18 engagement and signalling can have a profound influence on this [21].

19 The mechanism of disease resistance in the asymptomatic cases is obscure. In
20 paratuberculosis, animals are infected (as assessed by IS900 PCR) but there is no evidence of
21 bacterial growth or immunopathology and the tissue histology is normal [10]. It is likely
22 therefore that innate mechanisms play a central role in the control of infection. In addition,
23 the epidemiology of these diseases suggests a genetic susceptibility and polymorphisms in
24 PRRs have been linked both to polarization of the immune response and to susceptibility to
25 tuberculosis and leprosy [22].

1 Despite the fact that sheep are unrelated, of different breeds and are infected naturally
2 rather than experimentally, the variation in PRR transcript expression levels is generally much
3 less within each group than between the groups. Furthermore, the data show unambiguously
4 that there is a relationship between the different pathological forms of sheep paratuberculosis
5 and PRR transcript profiles within the target tissue. The most obvious conclusion from these
6 data is that in most cases high levels of PRRs are linked to either infection or disease. Our
7 results suggest that this is a consequence of infection rather than causative. Firstly, the degree
8 of variation of expression in the control group, which may succumb to any of the three
9 pathologies if infected, is relatively low. Second, the ileum tissue in each form of the disease
10 is comprised of different cell populations [10,11]. The paucibacillary lesions are largely made
11 up of lymphocytes, eosinophils and multinucleate giant cells with few macrophages [10]
12 while multibacillary tissue contains large numbers of epithelioid macrophages. In sheep, as in
13 other species, these cell types express a different spectrum of PRRs [6].

14 These do not provide an explanation for all the observed changes. Firstly, the cellular
15 composition of the asymptomatic and control ileum seems to be identical [10] but there are
16 significant differences in their PRR transcript expression profiles; of particular note is the >10
17 fold increase in TLR9 in asymptomatic tissue. This high level of TLR9 is highlighted by the
18 fact that it is also ~8 fold higher than in paucibacillary tissue and ~4 fold higher than in
19 multibacillary animals. This receptor for bacterial DNA has been implicated in responses to
20 *Mycobacterium tuberculosis* and seems particularly associated with the control of IL-12
21 production by dendritic cells and therefore in the development of type 1 responses [26].
22 Secondly, the PRR profiles of the paucibacillary vs control and multibacillary vs control
23 sheep are similar despite the fact that the tissues contain different cell populations.

24 A direct comparison between the three infected groups shows hugely increased levels
25 of Dectin-1 and Dectin-2. These C-type lectins are known to be important in anti-fungal

1 responses and their role in mycobacterial pathology is open to speculation although a recent
2 report links Dectin-1 to enhanced IL-12p40 production by splenic dendritic cells [27]. A
3 significantly raised level of TLR2 is a specific feature of multibacillary disease and
4 macrophage TLR2 interaction with mycobacterial lipoarabinomannans seems to drive type 2
5 responses [19,28], which are contraindicated in mycobacterial diseases. The role of TLR2 in
6 mycobacterial pathogenesis is further emphasized by the fact that several *TLR2 exon 2*
7 polymorphisms, possibly resulting in alterations of function, are linked to disease
8 susceptibility in both tuberculosis and leprosy [22,29]. *CARD15 (NOD2)* is significantly
9 raised in both pathological forms and polymorphisms of this gene are similarly linked to
10 mycobacterial susceptibility and inflammatory bowel diseases [30]. The sequence analysis of
11 the *TLR2 exon 2* and *CARD15 exon 11* of the forty sheep in this study shows no relationship
12 between any SNP in these gene segments and the different pathological forms of
13 paratuberculosis. However, it is possible that polymorphisms outside these regions have
14 adverse effects on PRR function and therefore affect sheep paratuberculosis pathology.

15
16
17

1 **Acknowledgements**

2 The work was funded by Association of Commonwealth Universities Academic Staff
3 Scholarship to KSN and by BBSRC Grant 15/S13964. JAS is funded by BBSRC/Genesis-
4 Faraday CASE studentship and sponsored by Moredun Scientific Ltd. Midlothian, UK. Our
5 thanks to Mr Juan Escala, Managing Director, Moredun Scientific Ltd. for general discussion
6 of the project.

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13

1 *Legends of Figures*

2 Fig. 1. Statistically significant changes in genes between the three IS900+ groups and the
3 uninfected control group.

4 (a) comparison of asymptomatic and control; (b) comparison of paucibacillary and control; (c)
5 comparison of multibacillary and control. Results are given as significant ($p \leq 0.05$) fold-
6 changes of mean copy-numbers relative to the mean copy-numbers of the comparative group.

7

8

9 Fig. 2. Statistically significant changes in genes between the three IS900+ groups.

10 (a) Comparison of paucibacillary and asymptomatic; (b) comparison of multibacillary and
11 asymptomatic; (c) comparison of multibacillary and paucibacillary. Results are given as
12 significant ($p \leq 0.05$) fold-changes of mean copy-numbers relative to the mean copy-numbers
13 of the comparative group.

14

Table 1
Tetra-ARMS primers for TLR2 exon 2 SNPs and expected allele specific products

SNP	Primer sequence 5' → 3'	Allele	Product Size (bp)
A ¹⁸² C	GTTGTATGTGCCAAAGAGTTTAAAGT	Outer Forward	194
	TAAGTATGATGATTAATTTCACTGATGGA	Outer Reverse	
	GCAAATTAGTATCTCTCAGTTCTAAATGAT	A	143
	ATTCTTATAGATATTGTAAGTTCCTTGGC	C	110
T ¹⁵¹⁶ C	TGGTACATGAAGATGATGTGGGCCT	Outer Forward	351
	GCAGCATCGTTGTTCTCATCAAAGAGA	Outer Reverse	
	GGAGCTGGAGCACTTCAACCCTCACT	T	214
	AAGTCTCGCTTATGAAGACACAGCTTCAG	C	192

Table 2
 Pattern Recognition Receptor transcript expression levels in terminal ileum from
 paratuberculosis-infected and uninfected control sheep

	Control	Asymptomatic	Paucibacillary	Multibacillary
TLR1	2100 ± 1440 ^a	3274 ± 2137	4274 ± 2520	2541 ± 1905
TLR2	1430 ± 464	7427 ± 2575	6921 ± 3641	26414 ± 9625
TLR3	816 ± 410	2591 ± 1311	3777 ± 947	2593 ± 1055
TLR4	1910 ± 657	5289 ± 1791	5779 ± 2480	11138 ± 3709
TLR5	3302 ± 1227	5769 ± 3804	4784 ± 3467	2775 ± 1504
TLR6	2125 ± 810	5528 ± 2669	6242 ± 2987	4604 ± 2348
TLR7	32 ± 52	70 ± 74	26 ± 17	55 ± 44
TLR8	2613 ± 880	9335 ± 3723	11401 ± 5078	20240 ± 6973
TLR9	112 ± 106	1577 ± 702	193 ± 108	425 ± 206
TLR10	2842 ± 2105	3903 ± 2826	2623 ± 1683	1837 ± 1682
MyD88	3676 ± 1886	5784 ± 2234	12077 ± 3169	10672 ± 2422
CARD15	321 ± 196	840 ± 672	5340 ± 2729	5831 ± 3867
CD14	302 ± 114	2989 ± 979	2832 ± 840	5400 ± 1304
Dectin 1	2598 ± 2745	10793 ± 5258	18182 ± 6626	64189 ± 40253
Dectin 2	255 ± 200	3392 ± 2147	155099 ± 54571	295486 ± 68896

^aCopy number ± SD, normalized to SDHA and β actin

Bold figures, significant difference in comparison with Controls (p ≤ 0.05)

Table 3
 SNP analysis of *TLR2* exon 2 from sheep paratuberculosis cases

TLR2 polymorphism		Frequency		
Genotype	Phenotype	Asymptomatic	Paucibacillary	Multibacillary
A ¹⁸² C	Asp → Ala	2/12	3/12	3/16
C ¹²⁴⁵ G	Silent	5/12	3/12	4/16
T ¹²⁵⁷ G	Silent	3/12	2/12	1/16
T ¹⁵¹⁶ C	Leu → Phe	4/12	4/12	5/16
T ¹⁵⁴⁵ C	Silent	3/12	3/12	2/16
T ¹⁵⁶³ C	Silent	7/12	4/12	3/16
C ¹⁷⁴⁰ T	Silent	6/12	4/12	3/16

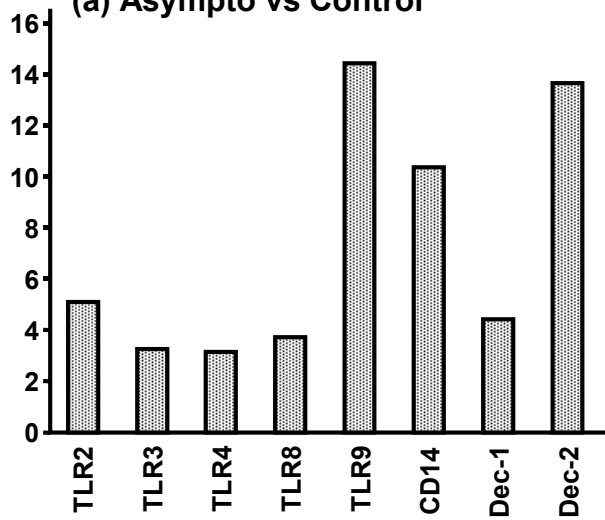
Nucleotide numbering based on submitted sequence of ovine TLR2,
 Genbank Accession Number AM117123

Table 4 Pathology, breed and genotype of sheep

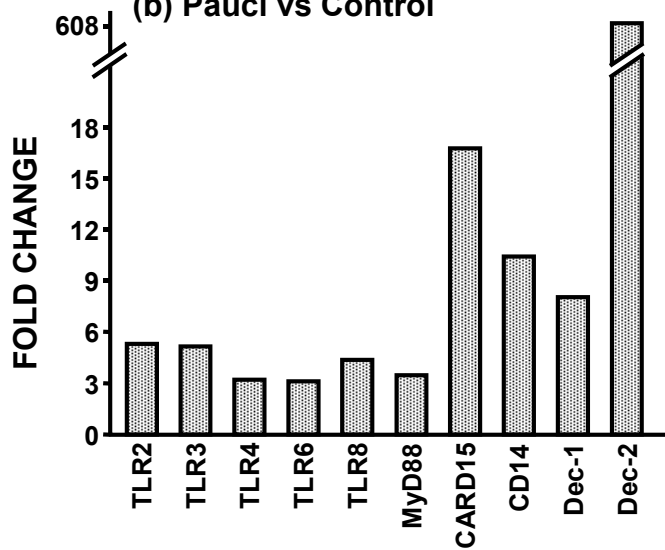
Pathology type	Sheep Breed	position 182	position 1516
Multi	Blackface x Bleu du Maine	A	G
Multi	Blackface x Bleu du Maine	A	T
Multi	Blackface	C	T
Multi	Blackface	A	G
Multi	Blackface	A	T
Multi	Blackface	A	T
Multi	Blackface	A	G
Multi	Blackface	A	T
Multi	Blackface	A	T
Multi	Blackface	A	T
Multi	Texel	C	G
Multi	Texel	A	T
Multi	Greyface	C	T
Multi	Greyface	A	T
Multi	Greyface	A	G
Multi	Greyface	A	T
Pauci	Blackface x Bleu du Maine	A	G
Pauci	Blackface x Bleu du Maine	C	T
Pauci	Blackface x Bleu du Maine	A	T
Pauci	Blackface x Bleu du Maine x Lley n x Roussin	C	T
Pauci	Blackface x Bleu du Maine	A	G
Pauci	Bleu du Maine	A	G
Pauci	Lley n x Roussin	A	T
Pauci	Blackface	A	T
Pauci	Blackface	A	T
Pauci	Texel	A	T
Pauci	Texel	C	T
Pauci	Greyface	A	G
Asympto	Blackface x Bleu du Maine	C	G
Asympto	Blackface x Bleu du Maine	A	T
Asympto	Blackface x Bleu du Maine	A	T
Asympto	Blackface x Bleu du Maine	A	G
Asympto	Blackface	A	G
Asympto	Blackface	A	T
Asympto	Blackface	A	T
Asympto	Texel	A	T
Asympto	Greyface	C	T
Asympto	Greyface	A	T
Asympto	Greyface	A	T
Asympto	Greyface	A	G

Figure

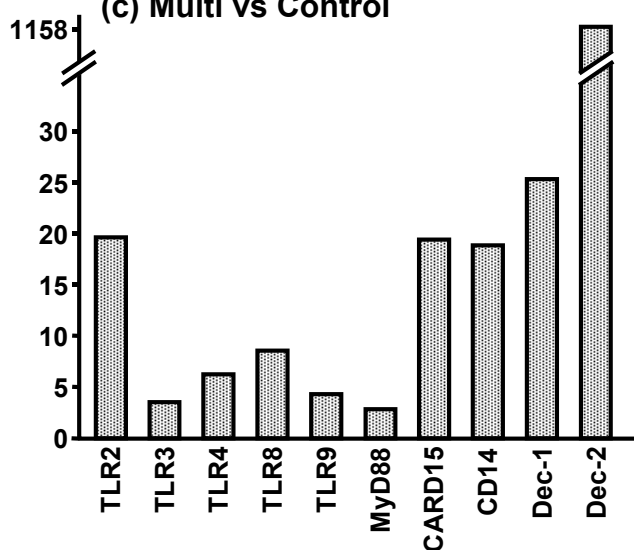
(a) Asympto vs Control



(b) Pauci vs Control



(c) Multi vs Control



Figure

