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Identification of the amino acids in the Major Histocompatibility Complex class II region of Scottish Blackface sheep that are associated with resistance to nematode infection

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ABSTRACT

Lambs with the Major Histocompatibility Complex *DRB1*1101* allele have been shown to produce fewer nematode eggs following natural and deliberate infection. These sheep also possess fewer adult *Teladorsagia circumcincta* than sheep with alternative alleles at the *DRB1* locus. However, it is unclear if this allele is responsible for the reduced egg counts or merely acts as a marker for a linked gene. This study defined the MHC haplotypes in a population of naturally infected Scottish Blackface sheep by PCR amplification and sequencing, and examined the associations between MHC haplotypes and faecal egg counts by generalised linear mixed modelling. The *DRB1*1101* allele occurred predominately on one haplotype and a comparison of haplotypes indicated that the causal mutation or mutations occurred in or around this locus. Additional comparisons with another resistant haplotype indicated that mutations in or around the *DQB2*GU191460* allele were also responsible for resistance to nematode infections. Further analyses identified six amino acid substitutions in the antigen binding site of *DRB1*1101* that were significantly associated with reductions in the numbers of adult *T. circumcincta*.

1. Introduction

There is increasing interest in breeding livestock for enhanced resistance to disease, especially nematode infection in sheep (Stear et al., 2007b; Pickering et al., 2015; Sweeney et al., 2016) due to the widespread evolution of drug resistance in parasite populations (Leathwick and Besier, 2014). There is considerable genetic variation among animals in resistance to nematode infection and much of this variation is under genetic control (Stear et al., 2009). There are three breeding strategies for exploiting genetic variation in resistance to disease: substitution of susceptible breeds by resistant breeds, cross-breeding with resistant animals and selective breeding within populations (Stear et al., 2007b). Selective breeding for resistance to nematode infection is being carried out in some commercial flocks in several countries including Australia, New Zealand and the UK.

A better understanding of the sources and magnitude of genetic variation would provide insight into the host-parasite relationship and the impact of control measures. For example, a recent mechanistic model of the interaction between sheep and the nematode *Teladorsagia circumcincta* relied heavily on our knowledge of genetic variation to provide reliable predictions of the response to infection (Prada Jimenez de Cisneros et al., 2014). In addition,

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enhanced knowledge of the sources of genetic variation would simplify the identification of resistant animals from resistant breeds and for cross-breeding or selective breeding.

Class II antigen-presenting loci of the Major Histocompatibility Complex (MHC) represent strong candidate genes for nematode resistance as they influence antibody specificity, and both IgA and IgE play important roles in resistance to nematode infection (Stear et al., 1995b; Lee et al., 2010; McRae et al., 2015). In sheep, the MHC region has been split by an inversion on chromosome 20 (Lee et al., 2012). The classical antigen presenting loci occur in the class IIa region closely linked to the class I region (Siva Subramaniam et al., 2015). The organisation of this part of the MHC in sheep has been reported (Gao et al., 2010) but this arrangement has been criticised and is inconsistent with a detailed reanalysis of the class I region (Siva Subramaniam et al., 2015). Sequencing of 160,000 bp from one class II haplotype (Herrmann-Hoesing et al., 2008) gave the order of loci as *DRB1 – DQA1 – DQB1 – DQA2 – DQB2*. The *DRA* and *DRB2* loci are assumed to lie closer to *DRB1* than to *DQB2*. Sheep do not possess equivalent loci to *HLA-DPA* and *HLA-DPB* (Scott et al., 1987).

Associations with nematode infection have been reported for at least two antigen-presenting class II loci: *DRB1* (Schwaiger et al., 1995; Sayers et al., 2005; Stear et al., 2005; Hassan et al., 2011) and *DQA2* (Hickford et al., 2011). In particular, the same class II allele (*DRB1*11:01*) has been associated with reduced faecal egg counts in Scottish Blackface (Stear et al., 2005), Suffolk (Sayers et al., 2005) and Texel sheep (Ali et al., 2019). However, it is not clear if these associations are direct effects or if the alleles are acting as markers of haplotypes and the causative locus lies elsewhere within the MHC.

The Scottish Blackface is one of the most numerous breeds of sheep in Europe and is found mainly in Scotland, Northern Ireland and Ireland. It is predominately kept in hilly and upland regions. It is relatively hardy and has been used for a number of studies on resistance to nematode infection (Abbott et al., 1985; Stear et al., 1995a, 2000; Wallace et al., 1996). This paper characterizes MHC class II region variation in a flock of Scottish Blackface sheep, confirms previous reports of associations with faecal egg count and the number of adult *T. circumcincta*, compares haplotypes to fine-map the causative mutations and identifies several polymorphisms within the antigen-binding site of the DRB1 protein.

2. Materials and methods

2.1. Sheep

Five consecutive cohorts of 200 lambs were studied to give a total of 1000 animals, although a small number (24) of lambs were not studied for various reasons, mainly missing at muster. They came from a commercial flock maintained in the Strathclyde region near Lochwinnoch, Scotland, UK. The flock was a purebred Scottish Blackface flock and the owners purchased rams privately or at sales to avoid inbreeding. The lambs were the offspring of 39 sires and 496 dams. They were born from 1992 to 1996. The flock has been described previously (Strain et al., 2002) and used to explore the relationship of serologically defined class I and sequence defined *DRB1* molecules with nematode infection (Schwaiger et al., 1995; Stear et al., 1996, 2005). Blood samples were collected by jugular venepuncture into a vacutainer tube (Becton-Dickinson) containing EDTA as an anticoagulant. Buffy coat and plasma were separated by centrifugation at 1200 *g* for 20 min and stored at −20 °C until required. Genomic DNA was extracted from the buffy coat using QIAamp DNA Blood Maxi Kits (Qiagen) following the manufacturer's instructions.

2.2. Amplification and sequencing

The methods used to amplify and sequence PCR products were similar to those previously described by us (Ali et al., 2017). Ovar *DRB1* primers ERB3 and SRB3 (Table 1) (Konnai et al., 2003) were used to amplify the exon 2 region of the *DRB1* locus. Primers *DRB1* 275 and *DRB1* 268 (Table 1) located within intron 1 and intron 2, respectively, were used to obtain complete exon 2 sequences (Ballingall et al., 2008). Each PCR was carried out in a final volume of 20 µl containing 50 ng of genomic DNA, 0.5 µM of each primer, 250 µM dNTP (Invitrogen), 1 unit Taq DNA polymerase (Qiagen), 1× reaction buffer (supplied) and 3.375 mM $MgCl_2$. Amplification was carried out in a Duocycler Thermocycler (VWR International Ltd., UK) and for *DRB1* 275 and *DRB1* 268 consisted of denaturation at 95 °C for 2 min, followed by 32 cycles of 95 °C for 60 s, 60 °C for 60 s and 72 °C for 60 s; this was followed by an extension step at 72 °C for 5 min. For primer pair ERB3 and SRB3, denaturation at 94 °C for 2 min was followed by 32 cycles

Table 1
The primers used to amplify Major Histocompatibility Complex class II genes in Scottish Blackface sheep.

Primer	Sequence	Target	Size (bp)
DRB1			
275 (F)	ATTAGCCTCTCCCCAGGAGTC	Intron 1	368
268 (R)	CACACACACACTGCTCCACA	Intron 2	
ERB3 (F)	CTCTCTCTGCAGCACATTTCCT	Exon 2	276
SRB3 (R)	CGCTGCACAGTGAAACTC	Exon 2	
DQA1			
NikDQA1 (F)	ACTGGCCACAAATGAAGCCCACAA	Intron1	525
NikDQA1 (R)	AGAAGGCAGAAGATGAGGGTTCAG	Intron 2	
LN827891 (F)	CCCTGACTCAGCTGACCACA	Exon2 and flanking intron 1	268
LN827891 (R)	AACACTTACTGTTGGTAGCAGCA	Exon2 and flanking intron 2	
DQA2			
DQA2s-up (F)	ACTACCAATCTCATGGTCCCTCT	Exon 2	241
DQA2s-dn (R)	GGAGTAGAATGGTGGACACTTACC	Exon 2	
DQB1			
JM05	TCTCCCCGCAGAGGATTTCGTG	Exon2 and flanking intron 1	278
JM06	CTCGCCGCTGCCAGGTGAAGG	Exon2 and flanking intron 2	
DQB2			
JM05	TCTCCCCGCAGAGGATTTCGTG	Exon2 and flanking intron 1	277
JM07	GCCGCTGCAAGGAGGTGATGAG	Exon2 and flanking intron 1	

of 94 °C for 30 s, 61 °C for 30 s and 72 °C for 30 s with a final extension step at 72 °C for 5 min.

Ovar *DQA1* Primers NikDQA1F and NikDQA1R (Table 1) were designed to amplify the exon 2 region of the *DQA1* locus (Ali et al., 2017). Each PCR was carried out in a final volume of 20 µl containing 50 ng of genomic DNA, 0.5 µM of each primer, 250 µM dNTP (Invitrogen), 1 unit Taq DNA polymerase (Qiagen) and 1× reaction buffer containing 1.5 mM $MgCl_2$ (supplied). Amplification for all primer pairs was denaturation at 95 °C for 2 min followed by 30 cycles of 95 °C for 30 s, 62.2 °C for 30 s and 72 °C for 60 s with a final extension step at 72 °C for 5 min.

Ovar *DQA2* Primers DQA2s-up and DQA2s-dn (Hickford et al., 2004) were used to amplify the exon 2 region of the *DQA2* locus. Each PCR was carried out in a final volume of 20 µl containing 50 ng of genomic DNA, 0.25 µM of each primer, 250 µM dNTP (Invitrogen), 1 unit Taq DNA polymerase (Qiagen) and 1× reaction buffer containing 1.5 mM $MgCl_2$ (supplied). Amplification consisted of denaturation at 94 °C for 2 min followed by 33 cycles of 94 °C for 30 s, 63 °C for 30 s and 72 °C for 45 s with a final extension step at 72 °C for 5 min.

Ovar DQB Primers JM05 and JM06 (Feichtlbauer-Huber et al., 2000) (Table 1) were used to amplify the exon 2 region of the putative *DQB1* locus (Feichtlbauer-Huber et al., 2000). Each PCR was carried out in a final volume of 20 µl containing 50 ng of genomic DNA, 0.5 µM of each primer, 250 µM dNTP (Invitrogen), 1 unit Taq DNA polymerase (Qiagen), 1× reaction buffer (supplied) and 3.375 mM $MgCl_2$. Amplification with primer pair JM05 and JM06 consisted of denaturation at 94 °C for 7 min followed by 33 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s followed by an extension step at 72 °C for 5 min.

Ovar DQB Primers JM05 and JM07 (Table 1) were used to amplify the exon 2 region of the putative *DQB2* locus. In addition, a modified version of JM05 (JM05mjs; Table 1) was combined with JM07 to amplify alleles AJ238935 and AJ238946. Each PCR was carried out in a final volume of 20 µl containing 50 ng of genomic DNA, 0.5 µM of each primer, 250 µM dNTP (Invitrogen), 1 unit Taq DNA polymerase (Qiagen), 1× reaction buffer (supplied) and 3.375 mM $MgCl_2$. Amplification consisted of denaturation at 94 °C for 7 min followed by 33 cycles of 94 °C for 30 s, 65 °C for 30 s and 72 °C for 45 s followed by an extension step at 72 °C for 5 min.

PCR products were visualised by electrophoresis in 1.5% w/v Seakem LE agarose (BioWhittaker Molecular Applications, Rockland, ME, USA) gels using 1× TBE buffer containing 0.1 µg/ml of ethidium bromide. PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen) following the manufacturer's instructions and sequenced in both directions using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Life Technologies). The reactions were run on an ABI 3130.

2.3. Sequence matching

DNA sequences were examined with CLC Genomics Workbench software v7.0 (CLC Bio, Qiagen) and allocated to one or two alleles by searching using the BLAST algorithm and manual matching. Sequence matching and alignments were made with CLC Genomics software.

2.4. Cloning

Cloning was used to identify all new alleles and to resolve unclear sequences. Purified PCR products were cloned into a pCR®4-TOPO® plasmid vector (Invitrogen) and then transformed into One Shot Top10 chemically competent *Escherichia coli* as per the manufacturer's instructions (Invitrogen). Clones were grown on agar plates under 50 µg/ml of ampicillin selection. Sixteen independent clones of each target sequence were picked and sequenced with the M13 forward primer as per the manufacturer's instructions.

2.5. Sequence analysis

Similarity matrices were created using Clustal Omega on the EBI website (http://www.ebi.ac.uk/Tools/msa/clustalo/). Sequences were named after the DNA sequence, whether DNA or protein sequences were being examined. This was to avoid confusion. Structures were modelled in Phyre2 (Kelley et al., 2015) and further examined, and specific amino acids indicated with EZmol (Reynolds et al., 2018).

2.6. Allele nomenclature and assignment of sequences to loci

DRB sequences behaved as the products of a single locus and were named following the nomenclature recommended by the Immunopolymorphism database (IPD) hosted by the European Bioinformatics Institute (UK; https://www.ebi.ac.uk/ipd/) (Maccari et al., 2018). Two nomenclatures have been used to label DQ sequences as alleles (Hickford et al., 2007; Ballingall et al., 2018). However, these nomenclatures conflict. In modern nomenclature, allele *DQA2*01:01:01* (Hickford et al., 2007) is different from allele *DQA2*01:01:01* (Ballingall et al., 2018). Therefore to minimise confusion, their accession numbers have been used to refer to all DQ sequences. Supplementary Table S1 provides a list of alternative names and identical sequences for each of the alleles found in this study.

The assignment of DQA sequences to loci followed previous results (Hickford et al., 2004, 2007; Zhou and Hickford, 2004). Some sequences share similarities with products of both *DQA1* and *DQA2* loci and have been called DQA2-like; these sequences were discovered in animals that lacked a detectable DQA1 sequence (Hickford et al., 2004). The DQA2-like sequences were assumed to be DQA1 genes that arose by intragenic recombination but three haplotypes in Scottish Blackface sheep possessed both *DQA1* and DQA2-like sequences. The three DQA2-like sequences were assigned to the DQA3 locus.

The assignment of DQB sequences to loci is controversial. The DQ primers amplify the products of more than one locus (Atlija et al., 2015). Therefore the sequences could not be reliably assigned to loci on the basis of the primers used to amplify each sequence. Classically, alleles have been assigned to allelic series by minimising the number of recombination events needed to produce observed patterns of inheritance and by ensuring that no haplotype possessed more than one member of an allelic series. Each allelic series was assumed to represent an independent locus. Some authors have attempted to assign sequences to loci on the basis of sequence similarity (Ballingall et al., 2018). Sequences in the same sequence group are assumed to belong to the same locus. Unfortunately, several haplotypes contain more than one sequence from the same group (Ballingall et al., 2018), probably because gene conversion is known to occur in the MHC (Hickford et al., 2004) and it creates similarity among alleles at different loci (Chen et al., 2007). Consequently, sequence similarity is not a reliable criterion for assigning sequences to loci. We have used classical methods to assign sequences to loci. When there is only one product detected on a haplotype, the assignment to a locus can be arbitrary without additional information. These haplotype products have been labelled DQBN for not known.

2.7. Haplotype assignment and frequency determination

All offspring from the same sire were examined to determine which sequences were inherited from the sire. The sequences

inherited from the same parent were grouped to form haplotypes. A small proportion of individuals had missing or extra sequences and they were retested. Haplotype frequencies were determined by counting the number of individuals with each haplotype.

2.8. Parasitological measurements

Standard procedures were used to estimate faecal egg counts (Bishop et al., 1996), worm numbers (Stear et al., 1997, 1998) and worm lengths (Stear et al., 1997, 1998).

2.9. Statistical analysis of the associations between MHC polymorphism and resistance to nematode infection

General linear and generalised linear modelling were used to assess the association between faecal egg counts in September when the animals were on average 5 months of age (Stear et al., 2005). In the general linear model, the number of nematode eggs per gram of faeces was transformed to form the response variable. In the generalised linear model, the number of eggs per gram was divided by 50 to give the response variable and a negative binomial distribution with a log link was used to estimate the effect of MHC class II haplotypes. The model included the fixed effects of year and sex while sire and dam were fitted as random effects. MHC haplotypes were fitted as covariates. Haplotypes present in fewer than five animals were not included and the most common haplotype (*DRB1*05:01 DQA1*Z28418 DQB1*AJ238941 DQA2*AY312382 DQB2*GU191459*) was set to zero to avoid dependencies among the equations. The models test for the effects of allele substitution; they do not examine non-additive interactions among and between alleles and loci. When a large number of comparisons are made there is a possibility that low probabilities will arise by chance alone. To avoid this, we focused on the haplotypes carrying the alleles (*DRB1*11:01* and *DRB1*03:05)* previously reported to be associated with faecal egg counts and with the number of adult *T. circumcincta* (Schwaiger et al., 1995; Sayers et al., 2005; Stear et al., 2005; Hassan et al., 2011). As the alleles have been associated with reduced faecal egg counts, one-tailed tests were used. $P < 0.05$ was considered significant.

3. Results

3.1. Definition of haplotypes

The MHC class II sequences in this flock of Scottish Blackface sheep formed 33 haplotypes (Table 2). All haplotypes had one *DRB1* sequence but the number of DQA and DQB sequences varied from one to three. The 1952 haplotypes from 976 lambs contained 18 *DRB1* alleles, eight *DQA1* alleles, 12 *DQB1* alleles, nine *DQA2* alleles, 17 *DQB2* alleles, three *DQA3* alleles and one *DQB3* allele. In addition, four DQB alleles could not be reliably assigned to a locus because they were the only DQB allele detected on a haplotype.

3.2. Haplotype frequency

The haplotype frequencies varied from 0.05% (one occurrence) to 14.65% (286/1952 haplotypes). There were 80 animals that had two identical haplotypes and were consequently MHC homozygous. The number of homozygotes ranged from 26 for haplotype *DRB1*05:01 DQA1*Z28418 DQB1*AJ238941 DQA2*AY312382 DQB2*GU191459* to only one for haplotype *DRB1*04:04 DQA1*NULL DQB1*GU191455 DQA2*NULL DQB2*LT882586 DQA3*AY312396 DQB3* LT882589*.

Table 2
Composition and frequency of the 33 Major Histocompatibility Complex class II haplotypes in a flock of Scottish Blackface sheep.

DRB1	*DQA1*	*DQB1*	*DQA2*	*DQB2*	*DQA3*	*DQB3*	Frequency
*DRB1*09:01*	*DQA1*LN827891*	*DQB1*LN868261*	*DQA2*AY312388*	*DQB2*AJ238939*			5.69
*DRB1*04:04*	*DQA1*NULL*	*DQB1*GU191455*	*DQA2*NULL*	*DQB2*LT882586*	*DQA3*AY312396*	*DQB3*LT882589*	1.69
*DRB1*04:04*	*DQA1*NULL*	*DQB1*GU191455*	*DQA2*NULL*	*DQB2*AJ238940*	*DQA3*AY312396*	*DQB3*NULL*	0.10
*DRB1*04:02*	*DQA1*HE574810*	*DQB1*NULL*	*DQA2*AY312377*	*DQB2*LT882587*			0.31
*DRB1*04:02*	*DQA1*NULL*	*DQB1*NULL*	*DQA2*NULL*	*DQB2*LT882586*	*DQA3*AY312396*	*DQB3*LT882589*	0.77
*DRB1*05:01*	*LN827895*	*DQB1*KR048654*	*DQA2*AY312377*	*DQB2*GU191454*			4.35
*DRB1*05:01*	*DQA1*Z28418*	*DQB1*AJ238941*	*DQA2*AY312382*	*DQB2*GU191459*			14.65
*DRB1*08:02*	*DQA1*NULL*	*DQB1*AJ238936*	*DQA2*AY312378*	*DQB2*AJ238944*	*DQA3*AY312385*	*DQB3*NULL*	4.71
*DRB1*03:05*	*DQA1*LN827890*	*DQB1*LT882585*	*DQA2*AY312387*	*DQB2*GU191453*			0.72
*DRB1*03:05*	*DQA1*LN827890*	*DQB1*LT882585*	*DQA2*AY312387*	*DQB2*GU191454*			0.05
*DRB1*03:05*	*DQA1*LN827890*	*DQB1*LT882585*	*DQA2*AY312387*	*DQB2*GU191459*			0.10
*DRB1*03:05*	*DQA1*LN827890*	*DQB1*LT882585*	*DQA2*AY312387*	*DQB2*GU191460*			1.90
*DRB1*11:01*	*DQA1*NULL*	*DQB1*NULL*	*DQA2*AY312375*	*DQB2*AJ238946*	*DQA3*AY312394*	*DQB3*NULL*	12.91
*DRB1*11:01*	*DQA1*NULL*	*DQB1N*LT882588*	*DQA2*AY312375*	*DQBN2*NULL*	*DQA3*AY312394*	*DQB3*NULL*	0.10
*DRB1*03:04*	*DQA1*AY265308*	*DQB1*U07032*	*DQA2*NULL*	*DQB2*LN811403*	*DQA3*AY312385*	*DQB3*NULL*	0.31
*DRB1*03:04*	*DQA1*AY265308*	*DQB1*LT882584*	*DQA2*AY312390*	*DQB2*LT882587*			0.20
*DRB1*01:02*	*DQA1*M33304*	*DQB1*HQ728667*	*DQA2*AY312377*	*DQB2*AJ238937*	*DQA3*AY312396*	*DQB3*NULL*	0.82
*DRB1*01:01*	*DQA1*NULL*	*DQBN1*LN810546*	*DQA2*NULL*	*DQBN2*NULL*	*DQA3*AY312394*	*DQB3*NULL*	0.46
*DRB1*01:01*	*DQA1*NULL*	*DQBN1*AJ238938*	*DQA2*AY312375*	*DQBN2*NULL*	*DQA3*AY312394*	*DQB3*NULL*	4.25
*DRB1*01:01*	*DQA1*NULL*	*DQB1*NULL*	*DQA2*AY312375*	*DQB2*U07033*	*DQA3*AY312394*	*DQB3*NULL*	5.28
*DRB1*01:01*	*DQA1*NULL*	*DQB1*NULL*	*DQA2*AY312375*	*DQB2*AJ238935*	*DQA3*AY312394*	*DQB3*NULL*	13.01
*DRB1*01:01*	*DQA1*NULL*	*DQBN1*U07030*	*DQA2*AY312375*	*DQBN2*NULL*	*DQA3*AY312394*	*DQB3*NULL*	0.15
*DRB1*01:01*	*DQA1*NULL*	*DQB1*NULL*	*DQA2*AY312375*	*DQB2*AJ238946*	*DQA3*AY312394*	*DQB3*NULL*	1.64
*DRB1*01:01*	*DQA1*Z28418*	*DQB1*AJ238941*	*DQA2*AY312382*	*DQB2*GU191459*	*DQA3*AY312394*	*DQB3*NULL*	6.40
*DRB1*03:02*	*DQA1*LN736359*	*DQB1*AJ238933*	*DQA2*AY312389*	*DQB2*AJ238945*			7.94
*DRB1*10:01*	*DQA1*M33304*	*DQB1*HQ728667*	*DQA2*AY312377*	*DQB2*AJ238937*			5.43
*DRB1*03:01*	*DQA1*LN827891*	*DQB1*AJ238942*	*DQA2*AY312381*	*DQB2*AJ238931*			3.28
*DRB1*07:01*	*DQA1*NULL*	*DQB1*NULL*	*DQA2*AY312375*	*DQB2*AJ238940*	*DQA3*AY312394*	*DQB3*NULL*	1.54
*DRB1*07:01*	*DQA1*NULL*	*DQB1*NULL*	*DQA2*AY312375*	*DQB2*LT882587*	*DQA3*AY312394*	*DQB3*NULL*	0.10
*DRB1*03:08*	*DQA1*LN827890*	*DQB1*LT882585*	*DQA2*AY312387*	*DQB2*GU191453*			0.10
*DRB1*13:01*	*DQA1*NULL*	*DQB1*NULL*	*DQA2*NULL*	*DQB2*AJ238940*	*DQA3*AY312396*	*DQB3*NULL*	0.46
*DRB1*19:01*	*DQA1*M33304*	*DQB1*GU191455*	*DQA2*AY312389*	*DQBN2*NULL*			0.41
DRB1:08:01	*DQA1*LN827891*	*DQB1*Z28424*	*DQA2*AY312381*	*DQB2*GU191459*			0.10

3.3. Haplotype associations with reduced faecal egg counts

The putative resistance allele *DRB1*11:01* was predominantly found on one haplotype (*DRB1*11:01 DQA1*NULL DQB1*NULL DQA2*AY312375 DQB2*AJ238946 DQA3*AY312394 DQB3*NULL*) at a frequency of 12.91%. General linear mixed modelling and generalised linear mixed modelling both showed that this haplotype was associated with reduced faecal egg counts ($P < 0.05$ and $P < 0.05$). Another haplotype (*DRB1*01:01 DQA1*NULL DQB1*NULL DQA2*AY312375 DQB2*AJ238946 DQA3*AY312394 DQB3*NULL*) was identical except for the *DRB1* allele. This haplotype was not associated with reduced egg counts ($P = 0.273$) but it was quite rare at 1.64% (32 lambs). The comparison of the two haplotypes suggests that one or more causative mutations on the resistant haplotype are likely to lie in or close to the *DRB1* locus.

The statistical modelling examined 242 lambs with the resistant haplotype (*DRB1*11:01 DQA1*NULL DQB1*NULL DQA2*AY312375 DQB2*AJ238946 DQA3*AY312394 DQB3*NULL);* 14 of these lambs were homozygotes. For lambs with zero, one or two copies of the resistant haplotype, the least squares mean and standard errors of the log transformed egg counts were 1.65 ± 0.5, 1.48 ± 0.09 and 1.79 ± 0.32. These results indicate that animals that are heterozygous for the resistant haplotype had the lowest egg count. Homozygotes for the resistant haplotype were more susceptible than heterozygotes.

The other putative resistance allele (*DRB1*03:05*) was found on four haplotypes which differed in the *DQB2* allele. The frequencies of these haplotypes were 0.72, 0.05, 0.10 and 1.90%. Only two of these four haplotypes occurred in sufficient lambs for analysis. The haplotype with GU191453 ($P < 0.05$ and $P < 0.01$) was associated with reduced egg counts but not the haplotype with GU191460 ($P = 0.288$ and $P = 0.215$). This result suggests that on this resistant haplotype, the *DQB2* locus is more likely to contain the causative mutations than the *DRB1* locus.

The expected number of animals with both resistance alleles (*DRB1*1101* and *DQB2*GU191453*) was less than two. There were no lambs that were heterozygous for both alleles and no analysis of the joint effect of both alleles was possible.

3.4. Associations of haplotypes with reduced numbers of adult T. circumcincta

In addition to the association with faecal egg counts, general linear mixed models were used to examine the association of the two resistant haplotypes with the number of adult *T. circumcincta* and mean worm length. Haplotype *DRB1*11:01 DQA1*NULL DQB1*NULL DQA2*AY312375 DQB2*AJ238946 DQA3*AY312394 DQB3*NULL* was associated with a reduced number of adult *T. circumcincta*; the estimate and its standard error were −0.24 ± 0.09 ($P = 0.005$). However, there was no association with reduced worm length ($P = 0.598$). The other resistant haplotype (*DRB1*03:05 DQA1*LN827890 DQB1*LT882585 DQA2*AY312387 DQB2*GU191453*) was not associated with reduced worm length ($P = 0.390$) or reduced worm number ($P = 0.869$).

3.5. Associations of specific amino acids with resistance to nematode infection

The potential influence of single amino acid sites was investigated at the polymorphic sites in exon 2. Fig. 1 shows the exon 2 sequences of the 18 *DRB1* alleles in the Blackface flock. Of the 89 amino acid sites in exon 2, 28 were polymorphic. Each polymorphism was examined in a separate generalised linear mixed model against the number of adult *T. circumcincta.* Six of the polymorphic amino acid sites were associated with the number of worms (Table 3). In these analyses, the most common amino acid was set to zero and the one letter code for this amino acid is presented to the right of the site position. Five substitutions – Y27H, D52S, K66A, D71N, Y73V – indicated that the protein encoded by the *DRB1*11:01* allele possessed the amino acid associated with reduced worm numbers. One substitution, P51R, indicated that exchanging the proline encoded by *DRB1*11:01* for an arginine would reduce worm numbers even further. Interestingly, all of the amino acids associated with reduced worm numbers were found in multiple alleles and were not unique to either of the resistant alleles.

3.6. Structural modelling of the protein encoded by DRB1

Structural modelling with EZmol (Fig. 2) indicated that the polymorphism at site 27 occurred in the Beta-pleated sheet that forms the floor of the antigen binding site. The polymorphisms at sites 51, 52, 66, 71 and 73 were present in the alpha helices that line the side of the antigen binding site.

4. Discussion

This study has defined the haplotypes in a flock of Scottish Blackface sheep, examined the associations with faecal egg counts and worm numbers, indicated that the causative mutations are not in the same position in different haplotypes and, finally, identified the amino acid sites that show the strongest associations with reduced numbers of adult *T. circumcincta.*

Detecting the alleles, assigning them to loci and identifying the haplotypes was relatively straightforward but time-consuming.

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DRB1*01:01 HFLEYTKKECRFSNGTERVRFLDRYFHNGEETLRFDSDWGEYRAVAELGRPDAKYWNSQKDFLERARAAVDTYCRHNYGVIESFTVQRR 89
DRB1*01:02 ........................................................................................G...S.... 89
DRB1*03:01 ..........................YA..........................EI...R.TE..............S.... 89
DRB1*03:02 ......................Y....YV..........................EI...R.TE..............S.... 89
DRB1*03:04 ......................Y....YA.....................RS.E.....EI...R.TE..............S.... 89
DRB1*03:05 ......................Y....YA.....................RS.E.....EI..QT..E...........G...S.... 89
DRB1*03:08 ......................Y....YA.....................RS.E.....EI...K..............G........ 89
DRB1*10:01 .....STS..H.F.........Y....YV..........................EI...R.TE..............S.... 89
DRB1*05:01 .....A.S....F.........E...Y.............................EL...K..N...........G........ 89
DRB1*11:01 .....STS..H.F.........Y.............................EI...K..............G...S.... 89
DRB1*04:02 .....H.S..........Y....Y....YV...N.....................RS.E.........QT..E..........S.... 89
DRB1*04:04 .....H.S..........Y....Y....YV...N....F................RS.E.........K..E..........S.... 89
DRB1*08:01 .....H.S..H.F.....................FV.....F.....RS.E.....EL...R.TE..........F...S.... 89
DRB1*08:02 .....A.S..H.F.....Y....YV.....F.....RS.E.....EL...R.TE..........F...S.... 89
DRB1*19:01 .....A.S..H.F.....Y....YV.....F.....RS.E.....QT..E..........G........ 89
DRB1*07:01 .........H.F.....L.E...Y....YV.....F.....................SR.T..........G........ 89
DRB1*13:01 .....S.S..H.F.....YT....NV.....F...T.....EQ.....SR.T..........F...S.... 89
DRB1*09:01 .....YRS..H.F.....L.E.....FA.....F...T.....A.EQ.....NI..QK..E.N.V.....F...A.... 89
```

Fig. 1. The amino acid sequences of the 18 *DRB1* alleles found in 976 lambs from a flock of Scottish Blackface sheep.

Table 3
Associations of amino acid substitutions with numbers of adult *Teladorsagia circumcincta* following natural infection.

Substitution	Effect	Standard Error	Probability
T6S	−0.12	0.07	0.098
T6A	−0.07	0.06	0.264
T6H	−0.29	0.15	0.054
T6Y	+0.17	0.11	0.134
K7R	−0.08	0.07	0.271
K7T	+0.22	0.11	0.050
K8S	+0.10	0.05	0.057
R11H	−0.10	0.06	0.085
S13F	−0.04	0.05	0.423
D21F	+0.04	0.07	0.529
D21Y	−0.25	0.15	0.100
D23E	+0.11	0.06	0.052
F26Y	+0.01	0.05	0.775
Y27H	+0.15	0.05	0.006[b]
Y27T	−0.23	0.31	0.445
L27A	+0.04	0.08	0.661
L27V	−0.08	0.06	0.212
S37N	−0.53	0.29	0.075
Y46F	−0.03	0.09	0.741
P51R	−0.22	0.08	0.004[b]
D52A	+0.18	0.11	0.114
D52S	−0.20	0.08	0.009[b]
K54E	−0.11	0.07	0.120
Y55Q	+0.18	0.11	0.090
E61D	+0.39	0.26	0.234
E61N	−0.05	1.14	0.971
I62F	+0.08	0.06	0.198
I62L	+0.02	0.07	0.776
R65Q	+0.07	0.09	0.423
R65S	+0.01	0.16	0.944
K66A	+0.16	0.07	0.015[a]
K66R	+0.04	0.07	0.562
K66T	−0.07	0.12	0.571
A68T	−0.05	0.06	0.447
A69E	−0.03	0.06	0.569
A69N	+0.04	0.07	0.554
D71N	+0.23	0.11	0.038[a]
Y73V	+0.24	0.11	0.038[a]
I81F	−0.05	0.08	0.569
I81G	−0.06	0.05	0.266
T85A	−0.39	0.24	0.108
T85S	−0.06	0.11	0.571

[a] $P < 0.05$.
[b] $P < 0.01$.

Fig. 2. A molecular model of *DRB1*1101* exon 2. Alpha helices form the sides of the antigen binding site while beta pleated strands form the base. The DRB1 molecule has additional domains encoded by additional exons and the DRA molecule also contributes to the binding site. The amino acids encoded by *DRB1*1101* exon 2 that are associated with resistance to nematode infection are indicated in yellow (light grey) and occur at positions 27, 51, 52, 66, 71 and 73. The start of the polypeptide encoded by exon 2 is at the lower centre of the figure. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Pedigree information greatly simplified the identification of haplotypes. The fact that nearly all haplotypes were detected independently in different animals greatly increases confidence in haplotype assignment. The numbers of alleles, loci and haplotypes in this population are minimum estimates because additional sequences that were not amplified by the primers used could also exist. Complete sequencing of the MHC could reveal additional sequences but this is not currently feasible for studies of disease associations. We did not attempt an exhaustive study of additional primers because testing 1000 lambs with each set of primers is not a trivial exercise. An alternative approach is to test a smaller number of animals or shorter sequences and impute the missing information. However, accurate imputation requires considerable confidence that all the existing haplotypes are known and this information was not available until this study.

The MHC of sheep is clearly very diverse with a large number of alleles at different loci and a large number of polymorphic sites within exon 2 of both DQA and DQB loci. The assessment of diversity will depend upon the parameter used for measurement. The number of protein-coding alleles would suggest that DQB loci are more diverse than DQA loci. However, the proportion of identical sites in exon 2 suggests that the DQA loci may be more diverse than the DQB loci. A composite measure of diversity has been developed (Leinster and Cobbold, 2012) and used to study MHC diversity in cattle (Codner et al., 2012).

There was no observed recombination between DQA1 and DQA2 in 510 New Zealand lambs (Hickford et al., 2007). Similarly, we did not observe any recombination in our 976 Scottish Blackface lambs or in 235 Texel lambs (Ali et al., 2017). Although not all lambs would be informative, these studies clearly indicate recombination between these loci is quite rare. The sheep class II region is relatively short (Herrmann-Hoesing et al., 2008) and this could explain the low recombination frequency. However, many haplotypes appear to have been generated by historical recombination (Hickford et al., 2007). In our Scottish Blackface flock, several haplotypes appear to have arisen by recombination e.g. there were four haplotypes with the *DRB1*03:05* allele that differed only in the allele present at the *DQB2* locus.

The analysis of associations between the MHC and disease in livestock poses opportunities and challenges (Stear et al., 2007a). The relatively large number of protein-coding alleles at each locus means that large sample sizes are needed to achieve reliable results (Stear et al., 2007a). Possibly, the failure to detect an effect on worm number or worm length for one haplotype was a consequence of the rarity of this haplotype (0.72%). As the number of haplotypes is similar to the number of alleles at the more polymorphic loci, sample sizes designed for specific loci may yield useful information on haplotype associations. General and generalised linear models are valuable for the analysis of data in livestock because they allow the influence of factors such as year, sex, sire and dam to be taken into account.

Studies by us and others have shown that the *DRB1*11:01* allele is associated with reduced egg counts in Scottish Blackface (Schwaiger et al., 1995; Stear et al., 2005) as well as Texel (Ali et al., 2019) and Suffolk sheep (Sayers et al., 2005). In addition, the same allele is associated with reduced numbers of adult *T. circumcincta* following both deliberate and natural infection (Stear, M. J., Abuargob, O., Ben Othman, M., Bishop, S.C., 2006. Major genes and resistance to nematode infection in naturally infected Scottish Blackface lambs. 8th World Congress on Genetics Applied to Livestock Production. 13–18 August, Belo Horizonte, Brazil.; Hassan et al., 2011). Together these observations provide some of the

strongest support in any species for an association between the MHC and a disease (nematode infection) that is sufficiently important to provide selection pressure on the MHC. This study has confirmed these associations but many of the animals in this study formed part of the previous work on Scottish Blackface sheep.

The definition of haplotypes that include DRB, DQA and DQB loci, and their role in sheep disease, is novel. These studies indicate that both *DRB1* and *DQB2* loci are likely to play a role in nematode infection. Previous studies on MHC associations with disease have assumed that a single locus is responsible (Lechler and Warrens, 2000). This may be true for autoimmune and microbial diseases where the recognition of a single antigen is likely to have a strong influence on pathology or protection. However, complex metazoan parasites such as nematodes present multiple antigens to the immune system and protection involves the recognition of multiple antigens (Murphy et al., 2010). With multiple antigens, a role for multiple MHC loci is more plausible.

The resistant haplotype *DRB1*11:01 DQA1*NULL DQB1*NULL DQA2*AY312375 DQB2*AJ238946 DQA3*AY312394 DQB3*NULL* was associated with reduced numbers of adult worms but homozygotes for this haplotype had higher worm counts than heterozygotes. These results are consistent with our understanding of the protective immune response against natural, predominantly *T. circumcincta,* infection. The control of worm number is strongly associated with IgE-mediated mast cell degranulation (Miller, 1984; Stear et al., 1995b). During natural infection, the recognition of multiple molecules is necessary for effective protection (Murphy et al., 2010) and no single molecule seems able to generate effective control of worm numbers on its own. Presumably, the MHC class II molecules on the resistant haplotypes allow recognition of a more protective set of parasite molecules than the MHC class II molecules on the less resistant haplotypes. A more protective set of molecules could be generated if the resistant haplotypes recognise one or more molecules that are not commonly recognised by most of the other haplotypes because heterozygotes would recognise a wider set of parasite molecules. Animals that are homozygous for MHC class II molecules would recognise fewer parasite molecules and consequently would be more susceptible to infection. This explanation needs to be tested but experimental verification would appear feasible.

The MHC has a reasonably strong effect on resistance to nematode infection. Selective breeding for disease resistance will need to be managed carefully if we are to generate and maintain maximum disease resistance. Simply selecting resistant haplotypes is insufficient as this would lead to reduced heterozygosity. One option would be to favour resistant haplotypes and to weight haplotypes by their frequency because rare haplotypes produce more heterozygotes.

This set of analyses also examined individual amino acids and identified six amino acid substitutions that are associated with reduced worm numbers. These amino acids occur on the same proteins and they are not independent of each other. The amino acids associated with resistance all occurred on several alleles and were not confined to the resistant alleles (Fig. 1). This result suggests that increased resistance is a consequence of multiple amino acids but more work is needed to confirm this. Possibly, the combination of specific amino acids is needed to create a suitable binding site that can present parasite peptides to activate host responses.

In conclusion, a definition of MHC class II sequences in nearly 1000 lambs from a flock of Scottish Blackface sheep identified 18 DRB sequences, 20 DQA sequences and 36 DQB sequences. The number of DQA and DQB loci on each haplotype varied from one to three. Most haplotypes contained two DQA and two DQB sequences but there was insufficient information to assign all alleles to DQ loci. These *DRB1,* DQA and DQB sequences were arranged into just 33 haplotypes. A comparison of haplotype associations indicated that both *DRB1* and *DQB2* were associated with reduced faecal egg counts but only *DRB1* was associated with reduced worm numbers. Six polymorphic amino acid sites in the molecule encoded by DRB1 were associated with differences in worm numbers.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2019.05.003.

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