MHC class II *DRB1* gene polymorphism in the pathogenesis of Maedi–Visna and pulmonary adenocarcinoma viral diseases in sheep

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Abstract Ovine pulmonary adenocarcinoma (OPA) and Maedi–Visna (Maedi) are two chronic respiratory diseases of retroviral origin which occur worldwide. It is known that different host genetic factors influence the outcome of viral infections. To determine if variation in the *Mhc-DRB1* gene was associated with progression to these ovine diseases, sheep lungs with and without OPA and Maedi lesions were collected. A sequence-based method was applied and 40 different alleles were detected in the sample analysed. In the allele-by-allele association analysis, allele *DRB1**0325 had a significant association with susceptibility to Maedi ($P=0.045$). For OPA, *DRB1**0143 and *DRB1**0323 were significantly associated with susceptibility ($P=0.024$ and $P=0.029$), and allele *DRB1**0702 was significantly associ-ated with resistance ($P=0.012$). Based on these results, the *Mhc-DRB1* alleles were classified by effect in three categories—susceptible (S), resistant (R) and neutral (N)—and animals were reassigned the genotypes as S/S, S/R, S/N, R/R, R/N and N/N. In a second analysis, penalised logistic regression models including a flock effect were run. In Maedi, significant association was detected for the N/S heterozygote ($P=0.0007$), but not for the S/S homozygote, probably as a result of the low number of S/S animals. In OPA, association was detected for both the S/S and R/R homozygotes ($P=0.005$ and $P=0.047$). This allele grouping method may be applied in association studies with highly variable genes. This is the first study demon-strating significant associations between sheep *Mhc-DRB1* alleles and susceptibility to OPA and Maedi. Therefore, both diseases are suitable candidates for more comprehensive genetic studies.

Keywords Maedi–Visna · Ovine pulmonary adenocarcinoma · *Mhc-DRB1* gene · Disease association · Sheep

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Introduction

Ovine pulmonary adenocarcinoma (OPA) and Maedi–Visna (Maedi) are two important chronic respiratory diseases of retroviral origin which occur worldwide in sheep and can reach high incidence in some flocks. Maedi–Visna was the first lentivirus infection discovered and was also a model on which a new category of diseases was defined, the slow infections. Its aetiological agent, the Maedi–Visna virus (MVV), is classified as a lentivirus of the *Retroviridae* family. The organs targeted by MVV are the lungs, mammary glands, joints and central nervous system, where

through a local inflammatory process, it can lead to a wasting disease that can result in significant production losses and have major welfare implications (reviewed in Peterhans et al. 2004). Breed differences in susceptibility to MVV infection and disease as well as experiments using artificially created isogenic twin lambs support the notion that host genetic factors influence the outcome of MVV infection (Cutlip et al. 1986; Houwers et al. 1989; De la Concha-Bermejillo et al. 1995), although interpretation of breed susceptibility studies is complicated by variation in viral strains and host genetics (DeMartini et al. 2000).

OPA is a contagious tumour originating in type II pneumocytes and Clara cells after infection by Jaagsiekte sheep retrovirus (JSRV). OPA is the main and most frequent tumoural process in sheep, and it causes major losses wherever a flock is affected (De las Heras et al. 2003). Interestingly, OPA is similar to a peculiar form of human cancer—bronchioloalveolar carcinoma—with which it shares clinical, radiological and histopathological features (Palmarini and Fan 2001). In some cases, OPA and Maedi coexist and OPA seems to play a role in MVV infection, although no synergistic effect of the simultaneous infection or expression of concurrent lesions seems to occur (González et al. 1993).

As neither antiviral treatment nor vaccination is available for either disease, the control or eradication of Maedi and OPA is limited to the elimination of infected sheep and/or the artificial rearing of colostrum-deprived lambs, methods difficult to implement in large commercial flocks (Houwers 1990; Voigt et al. 2007; Reina et al. 2008; Grego et al. 2008; Cousens et al. 2009).

The major histocompatibility complex (MHC) genomic region contains a diverse array of genes which are crucial for the initiation of the adaptive immune response. The class II antigens, encoded by MHC class II genes, bind processed peptides form extracellular antigens and present them to epitope specific CD4+ T lymphocytes. Genetic disease resistance linked to MHC class II molecules has been reported for some ruminant retroviral infections, including caprine arthritis encephalitis (Ruff et al. 1993) and enzootic bovine leucosis (García-Briones et al. 2000).

Previous studies have pointed to the putative involvement of ovine MHC (Ovar from Ovis aries) class II antigens as a component of the MVV-Env receptor (Dalziel et al. 1991). In addition, Singh et al. (2006) described that the proliferative response of T cell lines from persistently infected sheep was restricted to MHC class II DR and was therefore due to CD4+ T lymphocytes. Very recently, Herrmann-Hoesing et al. (2008) have detected that Ovar-DRB1 alleles contribute as a host genetic factor that controls provirus levels.

Although the immune response to JSRV and JSRV-induced tumourigenesis is poorly understood, as no JSRV-specific circulating antibodies or T cells have been detected to date (Summers et al. 2002), MHC class II-positive cells have been found intratumourally, peritumourally and in the surrounding alveolar lumina (Summers et al. 2005). Moreover, association analyses between the Ovar-DRB1 gene and the development of tumours in sheep after experimental infection with bovine leukaemia virus (BLV; Nagaoka et al. 1999) indicate that the differences in immune response were due to differences in DRB1 alleles and reflected the risk of BLV-induced leukaemogenesis.

The Latxa sheep is excellent material for the study of the genetic basis of infectious disease, due to available information on the prevalence and dynamics of many such diseases in this breed (González et al. 1993; Berriatua et al. 2003; Leginagoikoa et al. 2006) and because it has already been the subject of several studies on the MHC and its polymorphism (Jugo and Vicario 2000, 2001; Arrieta-Aguirre et al. 2006).

This study has two objectives:

1. To characterise the allele diversity of the Mhc-DRB1 gene in the Latxa population by means of sequence-based typing (SBT).
2. To perform association analyses in order to determine whether different alleles in the DRB1 gene are associated with resistance or susceptibility to OPA and MV.

Materials and methods

Animals and samples

All the animals included in this study were adult Latxa sheep. The samples were obtained from two experimental flocks under long-term monitorisation located in the province of Araba and several flocks from the province of Gipuzkoa from which animals were sampled at culling (Table 1). Both provinces belong to the Basque Autonomous Community in the north of Spain.

The lungs were macroscopically evaluated, and to confirm the presence of lesions characteristic of Maedi

Table 1 Sources of samples

Flocks	Animals	Controls	Maedi	OPA
Araba Exp-A	32	10	3	19
Araba Exp-B	52	43	4	5
Gipuzkoa	81	35	26	20
Total	165	88	33	44

and OPA, tissue samples were fixed in 10% buffered formalin, processed for paraffin inclusion, sectioned at 4 to 5 μm and stained with hematoxylin and eosin for light microscopy examination.

Of the 156 animals selected for this study, 32 had lesions of Maedi, 44 had lesions of OPA and 88 had no pulmonary lesions of Maedi and OPA (see Table 1).

Mhc-DRB1 typing

DNA was extracted from lung samples by standard phenol–chloroform extraction. The protocols and locus-specific primers used in this study for sequence-based typing of Ovar-DRB1 alleles were based on our previous study (Arrieta-Aguirre et al. 2006). This is a high-resolution Ovar typing method using PCR amplification of genomic DNA, followed by direct sequencing of the second exon of the Mhc-DRB1 gene. The 234-bp amplicons were purified using a QIAquick PCR Purification Kit (QIAGEN) and sequenced using fluorescence-labelled dideoxynucleotide terminator chemistry. The analysis was carried out on a 3100 DNA sequencer using ABI Sequencing Analysis Software 5.2.

Firstly, sequencing for typing was performed for a single strand in the reverse direction. Sequences were viewed using Bioedit software v.7.0.5.3 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Data were aligned to a reference sequence, and base mismatches were identified manually. The sequences were then subjected to computer analysis using the Haplofinder software (Miltiadou et al. 2003) and the script written in PHP language called AurkiSek (Arrieta-Aguirre et al. 2006; http://www.ehu.es/bjugo/aurkisek), which assigns the Ovar allele names to the respective heterozygous sequences. When genotypes were not assigned, sequences were sequenced again in both directions and the computer analysis was repeated. The library used for allele assignment contained 94 different allele sequences (reviewed by Konnai et al. 2003 and new sequences in Arrieta-Aguirre et al. 2006). The nomenclature used to name the alleles was therefore as in those papers.

Statistical analysis

The allelic and genotypic frequencies were estimated with GENEPOP (Raymond and Rousset 1995; http://www.genepop.com). In order to evaluate the typing method, we compared the DRB1 gene diversity between breeds by means of the rarefaction method implemented in the CONTRIB v.1.02 program (Petit et al. 1998). This method corrects the variation in sample size, and the comparison is made by allelic richness, which is independent of sample size.

In a first step, the allele frequencies amongst groups were compared using the Fisher exact test. In addition, in order to assess allelic differences for the locus as a whole, the likelihood ratio test for the $k \times 2$ unordered table of allele frequencies (where k is the number of alleles) was performed for the alleles with at least four observations, using the Monte Carlo estimation of P value with 10,000 tables sampled, for Maedi and OPA separately.

Secondly, a logistic regression model (Hosmer and Lemeshow 2000) for allelic comparisons was applied separately for each disease. As numbers of diseased and clinically normal sheep were not evenly distributed across flocks, a flock effect was included in the statistical models. The odds ratio (OR) was used as a measure of association between presence of each allele and disease. P values were calculated for the test of significance of each OR (the null hypothesis was that the OR was 1, which means no association, whereas the alternative was other than 1).

At last, based on these analyses, alleles were categorised in the following ways:

1. DRB1 alleles were classified as neutral (N), susceptible (S) or resistant (R) based on their OR and the P value: Alleles with $P<0.30$ were classified as either R or S depending on whether the OR was smaller or greater than 1, respectively, and the remaining alleles were classified as N (Traul et al. 2007). Alleles carried by less than four animals were also assigned to the neutral category.
2. Animals were assigned to six genotype classes based on the classification of their two alleles: neutral/neutral (N/N), neutral/resistant (N/R), neutral/susceptible (N/S) resistant/susceptible (R/S), resistant/resistant (R/R) and susceptible/susceptible (S/S).
3. Finally, due to the uneven sample distribution, a maximum likelihood estimation was not possible, and therefore, a penalised logistic regression model (Firth 1993; Heinze and Ploner 2003) was run with flock and genotype categories as the explanatory variables and presence or absence of lesions as the response variable (Hosmer and Lemeshow 2000). Flock×genotype class interaction term was tested for significance with the logistic models. However, this term was not significant and was therefore removed from the final overall models for both diseases. Moreover, a 2×6 exact test for case/control and genotype was performed, adjusted by flock.

Basic statistical analyses were performed using SAS software v.8.2 (SAS Institute, Cary, NC, USA) and STATXACT v 3.1 (Cytel Software Corporation, Cambridge, MA, USA). Penalised logistic regressions were performed using the logistf package v. 1.05 (Heinze and Ploner 2003) in the R software v.2.5. (http://www.r-project.org). Results with P values<0.05 were considered statistically significant.

Results

Mhc-DRB1 SBT typing and allelic distribution

The SBT typing procedure enabled us to detect 40 different alleles in 156 animals (see Table 2), half of which had been detected in our previous studies (Arrieta-Aguirre et al. 2006; Jugo and Vicario 2000). The alleles with the highest frequencies were those previously identified in Latxa sheep (Table 2). The most frequent alleles were DRB1*0104, DRB1*0608 and DRB1*0702, with frequencies of 0.103, 0.106 and 0.115, respectively. The alleles identified for the first time all had frequencies of less than 0.019. Eight alleles were found exclusively in healthy individuals (DRB1*0101, DRB1*0103, DRB1*0115, DRB1*0404, DRB1*0601, DRB1*1101, Z92731, Z92727), three alleles (DRB1*0132, DRB1*0202 and DRB1*0405) were only found in Maedi individuals and two alleles (DRB1*0114 and DRB1*0414) were exclusive to the OPA group.

In the Fisher comparison test between controls and diseased individuals (Table 2), no alleles reached significance in Maedi, and P values of two alleles (DRB1*0323, P=0.019; and DRB1*0702, P=0.003) were significant in OPA. Considering the locus as a whole, a significant result was obtained in the $k \times 2$ likelihood ratio test for OPA (P= 0.021), but not for Maedi (P=0.545).

The Latxa breed seems to be very variable at this locus so in order to compare this breed with others typed by similar methods, we applied the rarefaction method to the data of the next breeds: Suffolk with 28 alleles in 71 animals (Konnai et al. 2003), and Polypay, Columbia and Rambouillet with 17, 26 and 21 alleles in 126, 129 and 128 animals, respectively (Herrmann-Hoesing et al. 2008). The allelic richness ranged from 11.29 in Columbia to 21.49 in Latxa.

Analysis of associations between Ovar-DRB1 alleles and Maedi and OPA status

Allele associations and categorisation of alleles by effect

To study the association between the presence of disease and DRB1 alleles, logistic regression tests including a flock effect were run separately for each disease (Table 3). For Maedi, allele DRB1*0325 was significantly associated with susceptibility (OR=5.354, P=0.045). In addition, another two alleles, DRB1*0117 and DRB1*0353, were classified as S (P<0.3, OR<1). Three alleles (DRB1*0115, DRB1*0116 and DRB1*0416) were classified as R (P<0.3, OR<1).

For OPA, alleles DRB1*0143 and DRB1*0323 (OR= 4.321, P=0.024 and OR=3.602, P=0.029, respectively) were statistically associated with susceptibility, whereas allele DRB1*0702 was associated with resistance (OR=

0.181, P=0.012). Moreover, two other alleles (DRB1*03032 and DRB1*0321) were classified as S, and DRB1*0608 was also considered R.

Association analyses based on grouped alleles

After reclassifying the sheep as N/N, N/R, N/S, R/S, R/R or S/S (Table 4) based on their DRB1 genotype and the alleles' category, interestingly in OPA, eight of the nine animals with the susceptible homozygote genotype (S/S) were affected, and the nine animals with resistant homozygote genotype were healthy. For Maedi, this genotype distribution was not as clear.

Finally, a penalised logistic regression adjusted by flock was applied to measure the association between genotype and disease (see also Table 4). The logistic regression model had a likelihood ratio test of 32.79 (P<0.001) and 44.34 (P<0.001), and the Hosmer and Lemeshow goodness of fit statistic was 3.06 (P=0.8017) and 3.92 (P=0.7887) for Maedi and OPA, respectively. All these values indicate that the data fit the models well.

For Maedi, the N/S category, with alleles DRB1*0117, DRB1*0201, DRB1*0323, DRB1*0325 and DRB1*0353 in heterozygosity, was associated with clinical Maedi (OR= 6.838, P=0.0007). The S/S genotype was also associated with the disease, but it did not reach significance.

For OPA, the S/S category, with alleles DRB1*0143, DRB1*03032, DRB1*0321 or DRB1*0323 in homozygosity or combined, was associated with clinical OPA (OR= 11.411, P=0.005). Moreover, the R/R category, with alleles DRB1*0608 and DRB1*0702, in homozygosity or combined, was resistant to the disease (OR=0.090, P=0.047). The results of the overall test of significance of genotype adjusted by flock based in the 2×6 exact test were significant for both diseases (P=0.0058 for Maedi and P= 0.0023 for OPA).

Discussion

The introduction of direct nucleotide sequencing for the DRB1 exon 2 genotyping in sheep has added considerable precision to the process (Sayers et al. 2005; Arrieta-Aguirre et al. 2006). The SBT based on direct sequencing and bioinformatical allele assignation has many advantages such as higher precision, increased resolution and accuracy of typing. However, it is usually based on the premise that all alleles in the population under study are known. In the present study, only three animals could not be assigned a genotype, presumably due to the presence of previously undetected alleles, so the applied methodology seems quite reliable. Furthermore, various ambiguities have been described when two or more allele combinations have

Table 2 *Mhc-DRB1* gene allele frequencies in controls and diseased animals

Allele	IPD–MHC Database nomenclature	Previous work[a]	This work (n=165)[b]	Controls (n=88)	Maedi (n=33)	P	OPA (n=44)	P
*DRB1*0101*	–	–	0.003	0.006	0.000	–	0.000	–
*DRB1*0103*	*DRB1*0102*	–	0.006	0.011	0.000	–	0.000	–
*DRB1*0104*	*DRB1*0103*	0.201	0.103	0.119	0.076	0.334	0.091	0.369
*DRB1*0109*	*DRB1*1601*	–	0.012	0.017	0.000	–	0.011	1.000
*DRB1*0114*	*DRB1*0303*	–	0.006	0.000	0.000	–	0.023	–
*DRB1*0115*	*DRB1*0308*	–	0.012	0.023	0.000	–	0.000	–
*DRB1*0116*	*DRB1*0304*	0.015	0.021	0.028	0.015	1.000	0.011	1.000
*DRB1*0117*	–	0.108	0.061	0.051	0.076	0.749	0.068	0.571
*DRB1*0132*	–	–	0.003	0.000	0.015	–	0.000	–
*DRB1*0141*	*DRB1*0301*	–	0.018	0.017	0.000	–	0.034	0.333
*DRB1*0142*	–	0.005	0.006	0.006	0.000	–	0.011	–
*DRB1*0143*	–	0.044	0.058	0.045	0.015	0.442	0.114	0.057
*DRB1*0201*	*DRB1*1001*	–	0.009	0.011	0.015	–	0.000	–
*DRB1*0202*	–	–	0.003	0.000	0.015	–	0.000	–
*DRB1*0203*	*DRB1*1101*	–	0.009	0.000	0.015	–	0.023	–
*DRB1*03011*	*DRB1*0801*	–	0.009	0.006	0.015	–	0.011	–
*DRB1*03032*	*DRB1*1502*	0.069	0.015	0.017	0.000	–	0.023	1.000
*DRB1*0308*	–	–	0.009	0.000	0.015	–	0.023	–
*DRB1*0321*	–	0.098	0.039	0.017	0.03	0.613	0.091	0.059
*DRB1*0323*	*DRB1*0402*	0.005	0.082	0.051	0.091	0.203	0.136	0.019*
*DRB1*0325*	–	0.015	0.03	0.023	0.076	0.061	0.011	0.664
*DRB1*0326*	*DRB1*0804*	0.005	0.012	0.011	0.03	0.299	0.000	–
*DRB1*03411*	*DRB1*1201*	0.005	0.015	0.017	0.015	1.000	0.011	1.000
*DRB1*0353*	–	0.029	0.012	0.006	0.045	0.061	0.000	–
*DRB1*0404*	*DRB1*2001*	–	0.003	0.006	0.000	–	0.000	–
*DRB1*0405*	–	–	0.003	0.000	0.015	–	0.000	–
*DRB1*0411*	–	–	0.018	0.023	0.015	1.000	0.011	0.664
*DRB1*0412*	*DRB1*0501*	–	0.018	0.017	0.03	0.613	0.011	1.000
*DRB1*0414*	–	–	0.003	0.000	0.000	–	0.011	–
*DRB1*0416*	–	0.020	0.055	0.051	0.045	0.725	0.068	1.000
*DRB1*0601*	–	–	0.006	0.011	0.000	–	0.000	–
*DRB1*0605*	*DRB1*1801*	0.069	0.073	0.08	0.061	1.000	0.068	1.000
*DRB1*0607*	–	0.010	0.015	0.017	0.000	_	0.023	1.000
*DRB1*0608*	–	0.132	0.106	0.136	0.091	0.475	0.057	0.070
*DRB1*0702*	*DRB1*1301*	0.103	0.115	0.148	0.136	1.000	0.034	0.003**
*DRB1*0801*	*DRB1*0901*	–	0.012	0.006	0.03	–	0.011	–
*DRB1*1101*	–	–	0.003	0.006	0.000	–	0.000	–
*DRB1*1301*	*DRB1*2101*	–	0.009	0.006	0.015	–	0.011	–
Z92731	–	–	0.003	0.006	0.000	–	0.000	–
Z92727	–	–	0.003	0.006	0.000	–	0.000	–

P: uncorrected two-tailed *P* value (Fisher's exact test)

*$P<0.05$; **$P<0.01$

[a] Arrieta-Aguirre et al. (2006)

[b] Pooled data for comparison with previous work

Table 3 Binary logistic regression analysis for Maedi and OPA adjusted by flock and classification of alleles according to their OR and P values

Allele	Maedi				OPA			
	OR	95% CI	P	Classication[b]	OR	95% CI	P	Classification[b]
DRB1*0101	–	–	–	N	–	–	–	N
DRB1*0103	–	–	–	N	–	–	–	N
DRB1*0104	0.920	0.284–2.983	0.890	N	0.953	0.331–2.743	0.930	N
DRB1*0109	–[a]	–	–	N	0.499	0.404–5.871	0.580	N
DRB1*0114	–	–	–	–	–	–	–	N
DRB1*0115	0.175	0.001–1.925	0.174	R	–[a]	–	–	N
DRB1*0116	0.299	0.032–2.798	0.289	R	0.385	0.041–3.605	0.403	N
DRB1*0117	3.059	0.677–13.82	0.146	S	1.147	0.284–4.638	0.848	N
DRB1*0132	–	–	–	N	–	–	–	–
DRB1*0141	–	–	–	N	1.016	0.144–7.180	0.988	N
DRB1*0142	–	–	–	N	–	–	–	N
DRB1*0143	0.335	0.037–3.068	0.333	N	4.321	1.217–15.338	0.024*	S
DRB1*0201	6.833	0.473–98.809	0.159	S	–	–	–	N
DRB1*0202	–	–	–	N	–	–	–	–
DRB1*0203	–	–	–	N	–	–	–	N
DRB1*03011	–	–	–	N	–	–	–	N
DRB1*03032	–	–	–	N	3.122	0.411–23.699	0.271	S
DRB1*0308	–	–	–	N	–	–	–	N
DRB1*0321	1.061	0.162–6.944	0.951	N	3.262	0.718–14.828	0.126	S
DRB1*0323	2.277	0.646–8.022	0.200	S	3.602	1.144–11.343	0.029*	S
DRB1*0325	5.354	1.04–27.553	0.045*	S	0.877	0.610–12.667	0.924	N
DRB1*0326	2.833	0.243–33.053	0.406	N	–		–	N
DRB1*03411	0.427	0.042–4.354	0.472	N	0.390	0.036–4.205	0.437	N
DRB1*0353	4.435	0.434–45.317	0.209	S	–	–	–	N
DRB1*0404	–	–	–	N	–	–	–	N
DRB1*0405	–	–	–	N	–	–	–	–
DRB1*0411	0.492	0.049–4.92	0.546	N	0.366	0.035–3.810	0.401	N
DRB1*0412	0.889	0.138–5.743	0.902	N	0.561	0.054–5.789	0.628	N
DRB1*0414	–	–	–	–	–	–	–	N
DRB1*0416	0.372	0.071–1.949	0.242	R	0.926	0.263–3.261	0.905	N
DRB1*0601	–	–	–	N	–	–	–	N
DRB1*0605	1.524	0.390–5.956	0.545	N	–	–	–	N
DRB1*0607	–	–	–	N	1.715	0.268–17.464	0.649	N
DRB1*0608	1.097	0.350–3.436	0.874	N	0.402	0.122–1.322	0.134	R
DRB1*0702	0.865	0.332–2.251	0.766	N	0.181	0.048–0.689	0.012*	R
DRB1*0801	–	–	–	N	–	–	–	N
DRB1*1101	–	–	–	N	–	–	–	N
DRB1*1301	–	–	–	N	–	–	–	N
Z92731	–	–	–	N	–	–	–	N
Z92727	–	–	–	N	–	–	–	N

R resistant, S susceptible, N neutral

*$P < 0.05$

[a] Uncalculated due to sample distribution

[b] Alleles with P values <0.3 were classified as either R or S based on OR<1 or OR>1, and the remaining alleles were classified as N

Table 4 Penalised logistic regression analysis of genotype effects in Maedi and OPA adjusted by flock

Disease	Genotype	Animals (healthy/diseased)	Coefficient	SE	Chi	P	OR	95% CI
Maedi	N/N	69 (56/13)	–	–	–	–	–	–
	N/R	13 (11/2)	−0.773	0.814	1.045	0.307	0.462	0.079–1.949
	N/S	28 (14/14)	1.922	0.618	11.512	0.0007***	6.838	2.199–25.06
	S/R	1 (1/0)	−0.509	2.337	0.099	0.752	0.601	0.004–12.175
	R/R	2 (1/1)	0.079	1.246	0.005	0.943	1.082	0.091–9.095
	S/S	8 (5/3)	1.147	0.896	1.761	0.184	3.147	0.565–17.613
OPA	N/N	47 (31/16)	–	–	–	–	–	–
	N/R	30 (26/4)	−0.852	0.649	1.899	0.168	0.427	0.111–1.416
	N/S	27 (15/12)	0.686	0.562	1.557	0.212	1.986	0.676–6.025
	S/R	10 (6/4)	0.568	0.796	0.538	0.463	1.765	0.374–8.065
	R/R	9 (9/0)	−2.412	1.682	3.939	0.047*	0.090	0.001–0.975
	S/S	9 (1/8)	2.435	1.045	8.029	0.005**	11.411	2.004–124.306

The N/N genotype was used as reference

*$P<0.05$; **$P<0.01$; ***$P<0.001$

SE standard error, *Chi* test of significance, *%95 CI* confidence intervals for the OR

identical heterozygous sequences (Voorter et al. 2007). In human leukocyte antigen class I typing, it has been estimated that ambiguities due to different allele combinations with identical heterozygous sequences are present in between 7% and 13% of typings (Voorter et al. 2007). In Latxa sheep, it is possible to identify most of the allelic combinations by sequencing a single strand (reverse) only, except for the alleles *DRB1*0116–DRB1*0117* and *DRB1*03032–DRB1*0325*, for which both strands need to be typed (Arrieta-Aguirre et al. 2006).

The sample of Latxa sheep examined in this study exhibited very high variation at the *DRB1* locus (40 alleles in 156 animals; Ho=0.896; He=0.937). The variability is twice as high as that detected in our previous study (Arrieta-Aguirre et al. 2006) probably because of the employed typing method and the number of flocks included, limited to one flock in the previous study. The correction by rarefaction methods shows that the difference in allele number in different breeds is mainly due to the differences in sample size, as all other studies analysed a single flock. Lastly, only eight alleles were breed specific (20%), so it seems that as the sample sizes increases, more alleles are common to different breeds. This result reinforces our previous population analyses results (Jugo and Vicario 2000; Arrieta-Aguirre et al. 2006).

The sample size required to test whether there is an association between a DNA variant and a disorder is a key consideration. Using twice or three times more controls than cases can be helpful where the number of cases is limited due to a disease being rare (Daly and Day 2001). However, it is generally not advisable to increase this ratio beyond 4:1 because there is only a small increase in

statistical power (Gail et al. 1976). Up to twice as many control animals as diseased animals were selected for both diseases in this work.

In addition, farm animal populations are often stratified by farm and breed (Stear et al. 2007). In this sense, all the sheep in this study were from the same Latxa breed, and a flock effect was included in all the statistical models. In fact, some of the disease-associated alleles, such as *DRB1*0325* for Maedi and *DRB1*0143* for OPA, became significant after applying this correction in the logistic regression model. Therefore, the inclusion of flock effect is advisable in farm animal association studies as farms are epidemiological units where it is very likely that individuals are exposed to the same levels of infectious pressure under similar environmental factors.

Previous studies in Latxa sheep breed indicate that there is a major inheritable component of disease resistance in relation to seroconversion for Maedi; offspring born to dams that remained seronegative throughout their lives were significantly less likely to seroconvert (Berriatua et al. 2003). Moreover, Herrmann-Hoesing et al. (2008) detected that the presence of some alleles was significantly associated with lower provirus levels. In relation to the development of the disease, we detected that allele *DRB1*0325* was classified as a susceptible independently of the sample origin. Unfortunately, the resistance alleles of the work by Herrmann-Hoesing et al. (2008) were not present in our sample, so we could not analyse their effect.

For OPA, the frequent *DRB1*0702* allele (Arrieta-Aguirre et al. 2006; this study) shows association with resistance. Nagaoka et al. (1999) investigated the association between the *Ovar-DRB1* gene and development of

tumours in sheep after experimental infection with BLV. It appears that susceptibility to tumour development may be determined to some extent by polymorphic residues binding to antigenic peptides directly within the binding cleft of the ovine DR molecules: Alleles encoding the Arg-Lys (RK) motif at positions β70–71 might protect against development of tumours, and alleles encoding the Ser-Arg (SR) motif might be positively related to susceptibility. Interestingly, for OPA, the resistant allele *DRB1*0702* (*P*=0.012) encodes the motif SR in the same positions whilst the susceptible allele *DRB1*0143* (*P*=0.024) encodes the motif RK. Therefore, these motifs seem to play a key role in resistance/susceptibility to the development of tumours, but in a different way in OPA than in BLV.

It has been suggested that a strategy that groups alleles in functional categories may be more successful when searching for disease associations than conventional strategies based on studying different alleles (Gibert et al. 2003). Thus, the statistical analysis applied in this work was adapted to the characteristics of our highly diverse sample. *DRB1* alleles were functionally categorised and animals pooled based on their new genotype, according to the work of Traul et al. (2007) in bison. In this study, Maedi results have not been totally clarifying as the heterozygous genotype N/S formed by a neutral allele and a susceptible allele presented a highly significant sensitive effect associated with disease (*P*=0.0003), but the S/S genotype does not reach the statistical significance. This result can be explained taking into account the low number of diseased animals present in the experimental flocks and their heterogeneous distribution. However, in OPA, the grouping of S alleles raised the significance of the susceptible homozygous genotype (*P*=0.005) whilst the resistant homozygote maintained also a statistically significant association (*P*=0.047). This allele grouping method might be applied in association studies where highly variable genes are going to be studied. However, the grouping makes it difficult to discern individual effects of each allele.

With the recent development of extensive high-throughput genomic tools, research efforts have increasingly turned to quantifying the genetic control of host–pathogen interaction (Davies et al. 2009). Within the framework required to determine when breeding for disease resistance is appropriate, this work reports within-breed genetic variation to virus susceptibility/resistance for two respiratory diseases in sheep. In combination with the information about breed differences (Cutlip et al. 1986), both diseases seem to be suitable candidates for more comprehensive genetic studies.

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