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RESEARCH ARTICLE

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Lack of relationship between Visna/maedi infection and scrapie resistance genetic markers

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Abstract

The relationship between *Visna/maedi virus* (VMV) antibody status and scrapie genetic resistance of 10,611 Rasa Aragonesa sheep from 17 flocks in Aragón (Spain) was investigated. The fifteen most common *PRNP* gene haplotypes and genotypes were identified and the genotypes were classified into the corresponding scrapie risk groups (groups 1 to 5). ARQ (93.3%) and ARR (31.8%) were the most common haplotypes and ARQ/ARQ (56%) and ARR/ARQ (25.6%) were the most common genotypes. The frequencies of scrapie risk groups 1, 2, 3, 4 and 5 were 3.3%, 27.3%, 63.5%, 1.2% and 4.8%, respectively. Overall Visna/maedi seroprevalence was 53% and flock seroprevalence ranged between 21-86%. A random effects logistic regression model indicated that sheep VMV serological status (outcome variable) was not associated with any particular scrapie risk group. Instead, VMV seropositivity progressively increased with age, was significantly greater in females compared to males and varied between flocks. The absence of a relationship between VMV infection and scrapie genotypes is important for VMV control and specifically for sheep participating in an ELISA-based Visna/maedi control program.

Additional key words: sheep health; lentivirus; prion; genetic resistance; PRNP; ELISA.

Introduction

Scrapie and Visna/maedi (VM) are small ruminant infections that represent relevant chronic health problems in sheep production worldwide. Scrapie is believed to be caused by a misfolded isoform of the prion protein (PrPSc) that derives from the normal cellular isoform of the prion protein (PrPC) (Prusiner, 1982) whereas VM is caused by a lentivirus called *Visna/maedi virus* (VMV) (Gudnadóttir, 1974) that is similar to other lentiviruses such as the human immunodeficiency virus (Forsman & Weiss, 2008). Both chronic infections have a profound impact on sheep health and production and may represent a relevant economic problem. At the moment there is neither treatment nor vaccine for these two diseases and only different control and eradication systems have been implemented for both diseases.

Detection of VMV infection is performed mostly by serological screening using one of the currently-available ELISA tests based on recombinant proteins and/or peptides that overcome the low sensitivity and high work input of the classical agar gel immunodiffusion (AGID) serological test (Varea *et al*., 2004;

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Abbreviations used: PRNP (prion protein gene); PrPSc (prion protein scrapie); ROD (relative optical density); VM (Visna/maedi); VMV (*Visna/maedi virus*).

Brinkhof & van Maanen, 2007). An intensive regimen of testing at short intervals for VMV antibodies combined with strict management could result in eradication of the infection in a short period of time (Pérez *et al*., 2010).

There are five different scrapie risk groups (R) named R1 to R5, that depend on the genotype of the individual: R1 includes sheep with genotype ARR/ARR that is linked to the highest resistance to scrapie whereas R5 is associated to the highest susceptibility to scrapie and includes sheep with genotypes containing the VRQ haplotype in combination with AHQ, ARH, ARQ or VRQ haplotypes. Finally, R2, R3 and R4 include sheep with genotypes with other haplotype combinations that are linked to low, intermediate or high susceptibility to scrapie (Dawson *et al*., 1998). In 2002, it was decided to improve the European Union (EU) sheep population genetic resistance to scrapie by implementing a compulsory prion protein gene (*PRNP*)-based genetic selection in EU countries. All animals were genotyped and classified according to the abovementioned criteria and only R1 rams and R1, R2 and R3 ewes could be used for breeding purposes (OJ, 2001, 2003).

Genetic selection favouring scrapie resistance may have unsuspected effects in other genetic traits. However, none of the health traits studied so far has demonstrated relationship with *PRNP* genotypes (EFSA, 2006; Sweeney & Hanrahan, 2008). Among these studies, the resistance to infection by intestinal nematodes (Gruner *et al*., 2004) or by *Salmonella* spp. (Vitezica *et al*., 2007), the milk somatic cell count (de Vries *et al*., 2005; Álvarez *et al*., 2006), the specific immune system responses (Eaton *et al*., 2007) and the blood levels of VM provirus (Harrington *et al*., 2009) have been investigated.

In certain geographical areas, a temporal coincidence between the two control programs (against VMV and scrapie) has occurred but no scientific work has been performed on the possible interaction between them. The objective of this work was to assess the potential relationship between genetic susceptibility to scrapie and VMV infection. The study was conducted in more than ten thousand Rasa Aragonesa sheep in Aragón (Spain) that were enrolled in a VMV control program (Pérez *et al*., 2010). This work also offered the opportunity to describe the *PRNP* genotype frequencies in sheep from flocks enrolled in a scrapie genetic resistance selection breeding program.

Material and methods

Selected flocks and animals

Flock selection criteria were based on: i) having at least 20% VM seroprevalence as determined in a previous study (Pérez *et al*., 2010), ii) being a fully scrapie-genotyped flock, and iii) having birth dates and sex records for the studied sheep. A total of 17 Rasa Aragonesa flocks distributed in Aragón (Fig. 1), were selected. The total number of animals included in the study was 10,611 sheep and the minimum, maximum, median and interquartile range flock size were 241, 2234, 593 and 281-741 sheep, respectively. Seroprevalence for VM and *PRNP* genotype data were obtained between years 2004 and 2008.

Sheep PRNP genotypes and VMV serological antibody status

Individual *PRNP* genotype results for codons 136, 154 and 171 were used to classify sheep in the corresponding scrapie risk groups (R1-R5; Dawson *et al*., 1998) (Table 1). The individual sheep VMV ELISA antibody status was known from previous analysis performed in the frame of the control and eradication program supported by the Government of Aragón and published elsewhere (Pérez *et al*., 2010). The test used in the previous study was the highly sensitive and specific recombinant protein-based ELISA "ELITEST™" (Hyphen Biomed, France; Saman *et al*., 1999). Sample optical density (OD) values were divided by the ELISA plate cut-off value to obtain the relative OD (ROD), according to the manufacturer's instructions.

Statistical analysis

Yates-corrected chi-square test and analysis of variance were used to compare proportions and means, respectively, using Epi Info 6.0 (CDC, Atlanta, USA). The Glimmix macro in the SAS program (SAS Inst., Cary, NC, USA) was employed to carry out random effects logistic regression analysis for investigating the relationship between sheep VMV serological status and scrapie susceptibility-related *PRNP* haplotypes, risk groups and genotypes (present in at least 50 sheep), taking into account age, gender and flock of

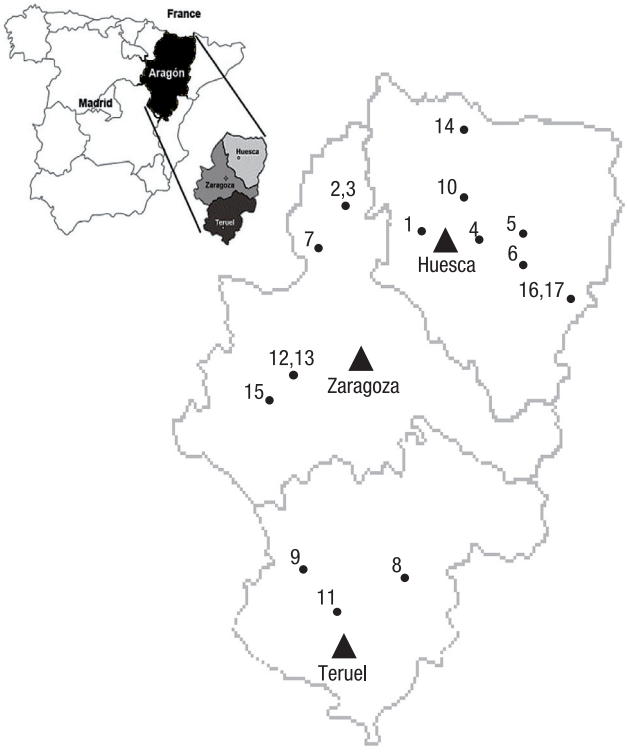

Figure 1. Spatial distribution of flocks in Aragón (Spain) participating in the study of the relationship between *Visna/maedi virus* (VMV) serological status and scrapie *PRNP* resistance/susceptibility (n = 17). ▲ Geographic location of capital provinces in Aragón (Spain).

origin. In logistic models, VMV status was the dependent variable, gender, age and one of the previous *PRNP* data were included as independent fixed categorical variables, and finally flock of origin was included as a random independent variable. Values *p* were from likelihood-ratio chi-square test (SAS Inst.) and alpha was 5% (two sided).

Results

PRNP haplotype and genotype frequencies in sheep and flocks

Table 1 summarizes the percentages of sheep carrying the different PRNP haloptypes and genotypes. ARQ followed by ARR were the most common haplotypes, present in 93.3% and 31.8% of sheep, respectively, and genotypes were dominated by ARQ/ARQ and ARR/ARQ present in 56% and 25.6% of sheep, respectively. The frequencies of scrapie risk groups R1, R2, R3, R4 and R5 were 3.3%, 27.3%, 63.5%, 1.2% and 4.8%, respectively.

The ARQ haplotype frequency was high in all flocks and ranged between 83% and 96%. In contrast, frequencies of ARR haplotype and the ARQ/ARQ and ARR/ARQ genotypes were more variable and ranged between 23 and 56%, 32 and 67% and 19 and 40%, respectively ($p < 0.05$, χ^2 test) (Table 1). Flock frequency of the ARQ/VRQ genotype, the most common genotype associated with high susceptibility to scrapie present in this study, ranged between 1 and 9% ($p < 0.05$, χ^2 test) (Table 1).

VM seroprevalence and mean ROD in seropositive sheep according to scrapie susceptibility-related PRNP haplotypes and genotypes

The overall VM seroprevalence in studied sheep was 53% and flock seroprevalence varied between 21-86% with a median and interquartile range of 50% and 33-68%, respectively. Table 2 shows the percentage of seropositive sheep (seroprevalence) and the natural logarithm of the mean ROD +1 according to specific *PRNP* haplotypes, genotypes and scrapie risk groups. Haplotype-specific seroprevalence ranged between 5 and 60%, genotype-specific seroprevalence was 44-100% and scrapie risk group-specific seroprevalence ranged between 47 and 55% ($p < 0.05$). Seroprevalence was significantly higher in scrapie risk group 3 compared to groups 1 and 2 ($p < 0.05$) and it was similar between other groups ($p > 0.05$). Excluding genotypes with less than 100 sheep, VM seroprevalence ranged between 47% in sheep with the ARR/ARR genotype and 62% in ARR/AHQ sheep ($p < 0.05$).

Relationship between VM seroprevalence, PRNP scrapie susceptibility and resistance genotype risk group and other independent variables

VM seroprevalence increased with age from 17% (n = 1314) among one year-old sheep to 78% among 5 year-old sheep (n = 1952) and it was 39% (n = 177) in rams compared to 54% (n = 9825) in ewes ($p < 0.05$). Random effects logistic regression analysis indicated that VM serostatus was independent of the scrapie risk groups based on *PRNP* haplotypes or the nine geno-

Table 1. Scrapie susceptibility-related *PRNP* haplotypes, genotypes and scrapie risk groups (R)[1] frequencies in sheep from 17 Spanish Rasa Aragonesa flocks (n=10,611 sheep)

Variable	R	Sheep (%)	Flocks				
			Minimum	25%	Median	75%	Maximum
Haplotype							
ARR[2]	—	31.8	23	26	33	40	56
AHQ	—	6.8	3	6	7	10	13
ARH	—	2.8	0	1	2	4	14
ARQ	—	93.3	83	91	93	95	96
VRQ	—	5.9	1	5	6	7	12
Genotype							
ARR/ARR	1	3.3	1	2	4	4	11
ARR/AHQ	2	1.2	0	1	1	2	2
ARR/ARH	2	0.5	0	0	0	1	3
ARR/ARQ[2]	2	25.6	19	22	27	30	40
AHQ/AHQ	3	0.1	0	0	0	0	1
AHQ/ARH	3	0.1	0	0	0	0	1
AHQ/ARQ	3	5.2	2	4	5	8	9
ARH/ARH	3	0.0	0	0	0	0	0
ARH/ARQ	3	2.1	0	1	2	3	10
ARQ/ARQ[2]	3	56.0	32	46	53	63	67
ARR/VRQ	4	1.2	0	1	1	2	3
AHQ/VRQ	5	0.2	0	0	0	0	1
ARH/VRQ	5	0.1	0	0	0	0	0
ARQ/VRQ[2]	5	4.4	1	4	4	5	9
VRQ/VRQ	5	0.1	0	0	0	0	1

[1] Dawson *et al.* (1998). [2] Differences in haplotype and genotype frequency were significant ($p < 0.05$).

types present in at least 50 sheep and confirmed its positive association with age and gender ($p < 0.05$) (Table 3).

Discussion

Results from the present study suggest that antibody levels due to VMV infection are not associated with genetic resistance to scrapie. Hence, the ongoing genetic selection for resistance to scrapie program is not expected to have a direct effect on the control and eradication of VMV infection based on antibody detection. As in the present study, Harrington *et al*. (2009) did not find an association between scrapie and VM showing that VMV proviral levels in blood monocytes in infected sheep were not affected by the presence of arginine (R) at codon 171. The results of our work have an added value as they have a broader application because conclusions are based on more than 10,000 sheep from different flocks. The relationship between both diseases has been demonstrated at the pathological level in several independent previous reports in both sheep and goats (Ligios *et al*., 2005; González *et al*., 2010; Salazar *et al*., 2010). Indeed, VMV-related lymphoproliferative pneumonia or mastitis favour the accumulation of PrP^{Sc} in the target organ. However, Lacroux *et al*. (2008) pointed out that accumulation of PrP^{Sc} in the mammary gland is unnecessary for excretion of the scrapie protein in milk or for transmission of infection by this route. Actually, both diseases can be transmitted separately through milk, independently of VM/scrapie coexistence and regardless of any associated mastitis (González *et al*., 2010). To the best of the authors' knowledge, this is the first report investigating the possible interaction between two independent but time-coincident control programs of two worldwide ovine diseases (scrapie and VM).

The frequency of *PRNP* haplotypes in the present study do not differ significantly from that described by Acín *et al*. (2004) in a study in 300 Rasa Aragonesa sheep prior to the start of the selection scheme; ARQ/ARQ (R3) and ARR/ARQ (R2) genotypes accounted for 51% and 21% of the genotypes, respec-

Table 2. Visna/maedi seroprevalence in seropositive sheep according to *PRNP* scrapie susceptibility haplotypes, genotypes and scrapie risk groups (R)[1] in a study in 10,611 Rasa Aragonesa sheep in Spain

Variable	No. of sheep	% VMV seropositive	No. of seropositive sheep
Haplotype			
ARR	3373	52	1753
AHQ	720	60	435
ARH	294	57	167
ARQ	9898	54	5323
VRQ	626	51	322
Genotype			
ARR/ARR	347	47	163
ARR/AHQ	125	62	78
ARR/ARH	57	51	29
ARR/ARQ	2720	52	1417
AHQ/AHQ	13	85	11
AHQ/ARH	9	86	8
AHQ/ARQ	552	59	325
ARH/ARH	1	100	1
ARH/ARQ	218	57	125
ARQ/ARQ	5943	54	3221
ARR/VRQ	124	53	66
AHQ/VRQ	21	62	13
ARH/VRQ	9	44	4
ARQ/VRQ	465	51	235
VRQ/VRQ	7	57	4
Risk groups[2]			
1	347	47	163
2	2902	53	1524
3	6736	55	3691
4	124	53	66
5	502	51	256

[1] Dawson *et al.* (1998). [2] Seroprevalence higher in scrapie risk group 3 compared to groups 1 and 2 ($p < 0.05$).

Table 3. Estimates from the random effects logistic regression model investigating the relationship between the *Visna/maedi virus* (VMV) serological status and the scrapie risk group (R) adjusted for gender, age and flock (n = 10,611)

Variable	Odds ratio	95% CI[1]	*p*
Fixed effects			
Gender			
Female	1.96	1.36, 2.82	0.0003
Male	1.00	—	
Age (years)			
1	0.12	0.09, 0.14	<0.0001
2	0.27	0.22, 0.32	<0.0001
3	0.37	0.32, 0.43	<0.0001
4	0.46	0.39, 0.55	<0.0001
5	0.84	0.77, 1.00	0.0522
>5	1.00		
R			
1	0.84	0.64, 1.10	0.2049
2	1.03	0.92, 1.15	0.5733
4	0.92	0.60, 1.41	0.6958
5	0.85	0.67, 1.06	0.1469
3	1.00	1.00	
Random variable	**Estimator**	**Standard error**	***p***
Flock	0.98	0.36	0.0034
Residual	0.99	0.01	<0.0001

[1] CI: confidence interval.

tively in that study and for 56% and 25%, respectively, in the present work. The frequencies of the VRQ (greatest susceptibility) and ARR (lowest susceptibility) haplotypes were similar. Differences in the genotype frequencies between these studies could be due to the small sample size in the study by Acín *et al*. (2004) and may also reflect variability in implementing the scrapie selection criteria in the flocks studied. Moreover, variability of certain characters or genes may not be easily comparable between populations (Huby *et al*., 2003). The distribution of the PRNP genotypes (or haplotypes) varies between the different Aragon sheep breeds and therefore, we can only compare our data with those reported for the Rasa Aragonesa breed (Acín *et al*., 2004).

Sheep in this study were part of a larger VM seroprevalence and flock risk factor investigation (Pérez *et al*., 2010). The sheep-level analysis performed here indicated that seropositivity gradually increased with age, was greater in females compared to males and differed between flocks. The age-specific seroprevalence pattern is compatible with VMV infection risk being dependent of cumulative horizontal exposure to the virus as shown in several sheep production systems (Berriatua *et al*., 2003; Leginagoikoa *et al*., 2006). Gender differences in VM seroprevalence are probably also the result of differential VMV exposure of rams and ewes, which are managed separately for long periods, rather than to intrinsic sex-associated differences in VMV susceptibility. Similarly, flock differences could relate to factors affecting VMV transmission. After taking into account age, sex and flock in the

logistic model VMV was not related to scrapie risk groups indicating the need to carry out multivariable analysis to reduce the chances of confounded associations between these two diseases.

In summary, the present study suggests that VM seroprevalence is independent of the scrapie genotype and thus individual resistance. This is important from an economical and sanitary point of view given the efforts to select for scrapie resistant genotypes and the similar interest to eliminate VMV from sheep populations. However, future studies should be carried out to further investigate the genetic basis of VM susceptibility and resistance as this would allow a more thorough investigation of its possible interaction with scrapie and other diseases.

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