Prion protein gene polymorphisms in healthy and scrapie-affected Spanish sheep

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The Rasa Aragonesa sheep is the second most important Spanish breed after the Merino breed. Reported here is the prion protein (PrP) haplotype frequency distribution for scrapie-related codons (136, 154 and 171) and a sequencing study of the complete PrP gene open reading frame for this breed and six other closely related breeds. The study includes four scrapie-affected sheep flocks belonging to Rasa Aragonesa and Rasa Navarra breeds. Thirty-eight scrapie-affected sheep, 502 healthy sheep from scrapie-affected flocks and 905 sheep from a breed survey were genotyped. The most frequent PrP haplotype in both scrapie and healthy flocks was ARQ, which was found at significantly higher frequency in scrapie-affected sheep. The susceptibility-associated VRQ haplotype was found at low frequencies in six out of eight breeds, but was not present in the 38 scrapie-affected sheep. The resistance-associated ARR haplotype was found in all breeds except one (Ojinegra) at frequencies $\geqslant$14 %. Fourteen amino acid polymorphisms were detected in these Spanish sheep, including the known amino acid substitutions at codons 112, 136, 141, 143, 154, 171 and 176, and new polymorphisms at codons 101 (Q→R), 151 (R→G), 151 (R→H), 172 (Y→D) and 175 (Q→E). Most of the novel polymorphic codons show frequencies lower than 5 %.

INTRODUCTION

Scrapie is a fatal, neurodegenerative disease that affects sheep and goats and belongs to the transmissible spongiform encephalopathies (TSEs) or prion diseases, which include bovine spongiform encephalopathy (BSE) in cattle and chronic wasting disease (CWD) in deer from the USA. Scrapie is a disorder characterized by the deposition of the prion protein (PrP^{sc};associated with TSE) in the central nervous system and lymphoreticular system. Characteristic clinical signs of the disease are behavioural disturbances, pruritus and increased difficulty in locomotion. Studies of naturally and experimentally scrapie-affected sheep have shown that genetic susceptibility to the disease is modulated by allelic variation in the PrP gene (for review see Hunter, 2000). Amino acid variations such as A136V, R154H, Q171R and Q171H have been shown to be of particular importance in scrapie resistance for many sheep breeds (Belt *et al.*, 1995; Clouscard *et al.*, 1995; Hunter *et al.*, 1996; Thorgeirsdottir *et al.*, 1999; Tranulis *et al.*, 1999). In addition, amino acid polymorphism at positions M112T, M137T, S138N, L141F, H143R, R151C, Q171K, N176K and R211Q has also been described (Laplanche *et al.*, 1993; Hunter *et al.*, 1996; Smits *et al.*, 1997; Stephens *et al.*, 1998; Thorgeirsdottir *et al.*, 1999; Vaccari *et al.*, 2001; Gombojav *et al.*, 2003; Guo *et al.*, 2003).

At present, little is known about PrP haplotype distribution in Spanish sheep; Hurtado *et al.* (2002) have described the haplotype frequencies observed for this gene in Latxa sheep. The Rasa Aragonesa breed is a white, polled, thin-tailed, medium-wool sheep (Mason, 1991). Its direct ancestor was *Ovis aries ligeriensis* and it remained as a native breed (without foreign interbreeding) until the early 19th century. During the last two centuries, the Rasa Aragonesa has been genetically mixed with other sheep, mainly Merino, and now forms a relatively heterogeneous group (Altarriba & Lamuela, 1983). It now represents the second most common Spanish breed, after the Merino sheep, at 16·2 % of all Spanish sheep.

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The first scrapie case detected in Spain was in 1987 (García de Jalón *et al.*, 1987) and by 2001 there has been an increase in the number of scrapie-affected sheep flocks. In this study, we have analysed four natural scrapie-affected sheep flocks, belonging to Rasa Aragonesa and Rasa Navarra breeds that were diagnosed in the Spanish National Reference Centre for TSE between 2001 and 2003.

The aim of this study was to determine the PrP polymorphisms in Spanish sheep, with special regard to scrapie incidence in the Rasa-related population. Genotypic frequencies observed in the scrapie-affected animals were compared with those detected in healthy sheep from these flocks. In addition, 16 healthy flocks belonging to Rasa Aragonesa, Ojinegra, Cartera, Maellana, Roya Bilbilitana, Ansotana and Churra Tensina breeds were also analysed in order to determine the genetic scrapie risk of these native populations. All these breeds are considered as derivative breeds from the ancient *O. aries ligeriensis* and are bred in the same geographical region, contributing to possible breed admixtures.

METHODS

Sheep

Natural scrapie-affected sheep flocks (case–control study). Animals with clinical signs of scrapie were detected in four different flocks belonging to Rasa Navarra (1) and Rasa Aragonesa (3) breeds and the disease was confirmed in 23 animals by the World Organization for Animal Health (OIE)-designated diagnostic techniques of Western blotting and immunohistochemistry. In addition to affected animals, a representative number of blood samples was obtained from each scrapie-affected sheep flock (random sampling system, higher than 50 % of the flock in all cases) before slaughter: 37 sheep from the Rasa Navarra scrapie-affected sheep flock (RNS), 246 from the first Rasa Aragonesa scrapie-affected sheep flocks (RAS1), 64 from the second (RAS2) and 155 from the third (RAS3).

Scrapie-affected sheep. The total number of scrapie-affected sheep samples was 38. Twenty-three were from the flocks of the case–control study and 15 were from additional archival material (paraffin-embedded tissue) of scrapie cases diagnosed in the Spanish National Reference Centre for TSE.

Native healthy flocks. Blood samples from native breeds ($n=905$) were obtained from 16 healthy flocks that belong to Rasa Aragonesa ($n=296$; 4 flocks), Ojinegra ($n=182$; 4 flocks), Cartera ($n=136$; 2 flocks), Maellana ($n=115$; 2 flocks), Roya Bilbilitana ($n=96$; 2 flocks), Churra Tensina ($n=32$; 2 flocks) and Ansotana ($n=48$; 1 flock) breeds. All reproductive rams were sampled from each flock, except from the Ansotana flock, where only the ewes were available. As this breed is endangered, we obtained the samples from a conservation flock.

DNA extraction

Blood. Genomic DNA was extracted using the GFX genomic blood DNA purification kit (Amersham Pharmacia Biotech).

Paraffin-embedded brain. Sections of 10 μm were placed in a sterile 1·5 ml tube and 1 ml xylene was added and vortexed vigorously to remove the paraffin. The samples were incubated in a shaker for 15 min at room temperature and the tissue was pelleted by centrifugation at 13 250 ***g*** for 5 min, followed by the removal of the supernatant. These steps were repeated once. The pellet was washed in 1 ml 100 % ethanol and spun at 13 250 ***g*** for 5 min. The supernatant was removed and the tissue was dried for 15 min at 60 °C. Tissues were immersed in a solution of 180 μl ATL buffer (Qiagen) and 20 μl proteinase K (200 μg ml^{-1}; Qiagen). After vortexing vigorously for 1 min, the sections were incubated overnight at 56 °C. After overnight digestion, DNA was purified using a standard phenol/chloroform/isoamyl alcohol (25 : 24 : 1) treatment. The ethanol-precipitated DNA pellet was dissolved in 50 μl TE buffer (Qiagen).

PrP genotype analysis. Amino acids are described in the single letter code. The codon position is given after the letter; polymorphisms are shown for example as A136V. Haplotypes are presented as groups of amino acids in the order of their codons, whereby positions 136, 154 and 171 have no codon identification, whereas other dimorphisms are identified by a codon position following the letter (in superscript) i.e. A136-R154-R171 (ARR), A136-F141-R154-Q171 ($AF^{141}RQ$). Genotypes are presented as combinations of the haplotype codes as described above, i.e. ARR/$AF^{141}RQ$.

RFLP analysis. All blood and paraffin samples except for the RAS2 and RAS3 flocks were analysed using this methodology. Polymorphisms at codons 136 and 154 were detected by PCR-RFLP analysis using the restriction enzyme *Bsp*HI (New England Biolabs) as described by O'Doherty *et al.* (2000). Polymorphism at codon 171 was detected by two allele-specific PCR amplifications and further digestion with *Bsl*I for R171 and *Acc*I for H171 allele detection as described by Yuzbasiyan-Gurkan *et al.* (1999). The presence of Q171 was deduced indirectly from the absence of the expected restriction fragments. During the course of this study, it became apparent that novel polymorphisms at codon 171 (i.e. K171) or close to codon 171 (i.e. D172) interfered with this RFLP analysis. Therefore, additional sequence analysis for genotype determination was used. When DNA from paraffin-embedded tissues was used as a template, an increase to 35 amplification cycles was necessary for PCR-RFLP analysis.

Sequencing. The complete PrP open reading frame has been sequenced in 697 sheep including 34 scrapie-affected sheep, the complete scrapie-affected flocks RNS, RAS2 and RAS3, 46 % of RAS1, 50 % of Ojinegra sheep, 30 % of Rasa Aragonesa, 25 % of Cartera, 20 % of Maellana, 25 % of Roya Bilbilitana, 30 % of Ansotana and 20 % of Churra Tensina. The whole coding region was amplified using the following primers: 20fwd, 5′-ATGGTGAAAAGCCACATA GGCAGT-3′ (codons 1–8) and 767rev, 5′-CTATCCTACTATGAG AAAAATGAG-3′ (250–stop codon). PCR fragments were purified using the MALDIspot kit and the vacuum manifold from Millipore and sequenced with the Big Dye kit from Applied Biosystems. The same PCR primers were used for bi-directional sequencing and chromatograms were analysed using BioEdit v.4.8.6 (Hall, 1999).

Statistical analysis. The genotype distributions obtained for the different populations were compared statistically using the χ^2 test for independence and the Yates correction for continuity for $N \times K$ contingency tables. $P<0{\cdot}05$ was considered statistically significant. Haplotypic and genotypic frequencies for each population were calculated using the GENEPOP program (Raymond & Rousset, 1995). This program was also used to carry out a statistical test to determine possible deviations from the Hardy–Weinberg proportion. A Markov-chain method was applied to calculate exact *P*-values; the length of the chain was set to 100 000 iterations. Some flocks showed Hardy–Weinberg disequilibrium, probably because of non-random mating common in domestic populations. The χ^2 statistic treating alleles (or haplotypes) rather than genotypes as individual entities, is only appropriate when the Hardy–Weinberg equilibrium holds (Sasieni, 1997). As some of the analysed populations were not

in equilibrium, we decided to analyse genotypic data. In order to avoid a possible flock effect, before pooling together the data for breed comparison, the distribution of genotypic frequencies was compared between each flock belonging to the same breed using the χ^2 test for independence and the Yates correction for continuity for $N \times K$ contingency tables.

RESULTS

Polymorphism in codons 136, 154 and 171

Retrospective study of scrapie samples. Fifteen sheep from confirmed cases, sampled between 1997 and 2002, were PrP genotyped. These animals belonged to the Rasa Aragonesa (10), Rasa Navarra (4) and Ojinegra breeds (1) and represented five flocks from five different scrapie areas in Spain. Fourteen animals had the genotype ARQ/ARQ and only one Rasa Aragonesa sheep was heterozygous for ARR/ARQ.

Four-flock case–control study. Scrapie cases: 23 sheep with clinical scrapie signs were sacrificed and the diagnosis was confirmed for all of them. Four animals were from the RNS flock and all showed the homozygous ARQ/ARQ genotype. The other 19 sheep were from the Rasa Aragonesa flocks (15 from RAS1, two from RAS2 and two from RAS3), with 18 sheep of ARQ/ARQ genotype and only one of ARR/ARQ genotype. The frequency of 95·5 % for ARQ/ARQ genotype sheep in this study was not significantly different from the 93 % of the same genotype in the retrospective study and therefore all scrapie-affected animals ($n = 38$) have been combined into one group for haplotype frequency comparison.

Healthy animals: six PrP haplotypes, ARQ, ARR, AHQ, ARH, VRQ and ARK, were found in these four flocks. The ARQ haplotype is the most common in all four flocks, with frequencies between 66·2 and 78·7 % (Table 1). The ARR haplotype is the second most common (14·5–25·7 %), and the AHQ, ARH and VRQ haplotypes were detected at low frequencies (AHQ 0–5·4 %, ARH 0·6–6·1 % and VRQ 0·6–4·7 %). The rare ARK variant was observed in one heterozygous sheep from the RAS3 flock.

Haplotype and genotype survey of native healthy flocks. Only the ARQ and ARR haplotypes were found in all seven Spanish sheep breeds from this survey of healthy, scrapie-unaffected flocks. The ARQ variant was the most frequent in all flocks with haplotype frequencies ranging between 45·8 and 73·9 % in Ansotana and Ojinegra flocks, respectively. The ARR variant was found at frequencies ranging from 11·4 to 46 %, which was seen in the Cartera breed.

By comparison, the haplotype frequencies for the other four PrP variants were considerably lower (VRQ 0·3–13·5 %, AHQ 0–7·6 %, ARH 0–9·4 % and ARK $\leqslant$1 %). Table 1 shows the frequencies obtained for each breed. By means of the PCR-RFLP technique, it is not possible to discriminate between the ARQ and ARK variants (see Methods), which may lead to incorrect estimates of the ARQ frequency if the ARK haplotype is present. Sequencing of 100 ARQ/ARQ sheep from the 182 Ojinegra samples confirmed only four heterozygous ARK/ARQ animals. The ARK haplotype was not found in 197 sheep sequences from the other six breeds in this survey (26 % of total). Assuming that the selection of sequencing samples from the total was random, we estimate that the ARK frequency is 0–4 %. The impact of the genotyping method on the ARQ frequency is therefore very small.

Statistical differences were not observed in the genotype distribution between the flocks that belong to the same breed, except for the Maellana population. The two Maellana flocks have been considered independently, whereas data from the remaining flocks that belong to the same breed have been pooled together. The differences observed between populations allow the classification of these breeds into different groups (Table 1). First, a group that contains the Rasa Aragonesa, Ojinegra, Roya Bilbilitana and Maellana (only flock 2) breeds, where the most common genotype is ARQ/ARQ and the susceptible VRQ variant is only present in low frequencies ($<$3 %). The second group includes the Ansotana, Churra Tensina and Maellana (only flock 1) breeds, with intermediate frequencies for the ARQ/ARQ genotype and relatively high frequencies for VRQ. A third group, which in this study only contains the Cartera population, was different from any other breed because of the very high frequency of the resistance-associated ARR/ARQ and ARR/ARR genotypes (66 %).

Association of PrP genotypes with natural scrapie. The frequency of the ARQ/ARQ genotype in scrapie-affected animals was considerably higher than in the healthy sheep from RAS2 and RNS scrapie-affected flocks ($P < 0{\cdot}05$) and the frequency of ARQ/ARQ was not significantly different between the scrapie-free and scrapie-affected animals in the RAS1 and RAS3 flocks. Genotype frequencies in healthy animals from scrapie-affected flocks and the native breed group containing Rasa Aragonesa, Ojinegra and Roya Bilbilitana were similar, whereas the frequency for the genotype ARQ/ARQ was significantly higher in the scrapie-affected animals than the frequency values obtained for the second and third groups. We can therefore conclude that the ARQ/ARQ genotype confers risk of scrapie infection to the Spanish sheep breeds. The scrapie-affected sheep showed low variability, with only four observed genotypes (ARQ/ARQ, ARR/ARQ, AR^{143}RQ/ARQ and R^{101}ARQ/ARQ; see Table 3).

Other PrP polymorphisms

PrP variants. Complete sequencing results were achieved for 697 sheep (alleles = 1394). A very complex genetic PrP profile emerged in these native Spanish sheep breeds, with additional amino acid polymorphisms found at codons 101, 112, 141, 143, 151, 172, 175 and 176. At codon 101,

Table 1. Haplotype and genotype frequencies (%) for PrP genotypes in scrapie-affected sheep compared with healthy animals in scrapie-affected sheep flocks and native healthy flocks

Genotypes were obtained by RFLP or sequence analysis as described in Methods. Statistical comparison of genotype distribution between scrapie animals and the remaining population using N × K (χ^2) contingency tables. Breeds: RA, Rasa Aragonesa; OJ, Ojinegra; RO, Roya Bilbilitana; MA, Maellana; Ans., Ansotana; Ch., Churra Tensina; CA, Cartera; RAS, Rasa Aragonesa Scrapie; RNS, Rasa Navarra Scrapie; Pos., Positive scrapie. NS, Not significant.

	RA	OJ	RO	MA1	MA2	Ans.	Ch.	CA	RAS1	RAS2	RAS3*	RNS	Pos.
Total†	296	182	96	58	57	48	32	136	246	64	155	37	38
Haplotypes													
ARR	15	14·6	21·4	19	11·4	28·1	27·3	46	15·3	18	14·5	25·7	2·6
AHQ	4·8	3	1	0	5·3	3·2	7·6	0·3	1·4	0	1·6	5·4	0
ARH	6·4	8·2	2·6	1·7	0	9·4	3	1·5	6·1	4·7	0·6	1·4	0
ARQ	70·9	73·9	72·4	60·3	82·5	45·8	54·5	50·7	76·5	72·7	78·7	66·2	97·4
VRQ	2·9	0·3	2·6	19	0·9	13·5	7·6	1·5	0·6	4·7	4·2	1·4	0
Genotypes													
ARR/ARR	2	2	2	2	0	6	9	21	2	5	2	8	0
ARR/AHQ	2	0	1	0	2	4	3	1	1	0	0	3	0
AHQ/AHQ	0	0	0	0	0	0	0	0	0	0	0	0	0
ARR/ARH	3	3	2	0	0	13	3	2	3	1	0	2	0
AHQ/ARH	0	0	0	0	0	0	0	0	1	0	0	0	0
ARR/ARQ	21	21	34	26	21	19	28	45	23	25	24	30	10
AHQ/ARQ	6	1	0	0	9	0	12	0	1	0	2	8	0
ARH/ARH	0	1	0	2	0	0	0	0	1	1	0	0	0
ARH/ARQ	9	10	3	0	0	4	3	1	7	5	2	0	0
ARQ/ARQ	51	56	53	36	66	32	30	26	60	53	61	46	90
ARR/VRQ	0	1	1	9	0	8	0	1	0	2	1	0	0
AHQ/VRQ	0	0	1	0	0	2	0	0	0	0	0	0	0
ARH/VRQ	1	2	0	0	0	2	0	0	0	0	1	0	0
ARQ/VRQ	5	3	3	22	2	6	12	1	1	8	5	3	0
VRQ/VRQ	0	0	0	3	0	4	0	0	0	0	1	0	0
χ^2	27·70	17·20	17·66	23·36	8·12	27·47	23·57	55·19	15·04	14·65	13·88	15·56	–
d.f.‡	8	9	8	6	4	10	7	7	9	7	8	6	–
P	<0·01	<0·05	<0·05	<0·001	NS	<0·01	<0·01	<0·001	NS	<0·05	NS	<0·05	–

*ARK haplotype at 0·3 % frequency.

†Number of analysed sheep of each breed.

‡d.f., Degrees of freedom (number of comparisons−1).

the second codon position is dimorphic (A→G transition) leading to a novel change from glutamine (CAG) to arginine (CGG). The dimorphisms M112T (Laplanche *et al.*, 1993), L141F (Hunter *et al.*, 1996) and H143R (Stephens *et al.*, 1998) have been described previously in other sheep breeds. Codon 151 exhibited two new polymorphisms: a transversion C→G in the first codon position, resulting in an amino acid substitution of arginine (CGT) with glycine (GGT) and a G→A transition in the second codon position, resulting in an amino acid substitution of arginine with histidine (CAT). The replacement of tyrosine (TAT) with aspartic acid (GAT) at codon 172 is the result of a T→G transversion in the first codon position; the first position was also polymorphic in codon 175 (C→G), leading to a change from glutamine (CAG) to glutamic acid (GAG). Both dimorphisms are new, but the Q175E change has also been detected in UK breeds (W. Goldmann, unpublished). Finally, the N176K dimorphism has also been described by Vaccari *et al.* (2001) in Italian sheep. The sequence analysis revealed that all additional polymorphisms segregated with the ancestral ARQ haplotype, and all new polymorphisms are mutually exclusive. Two silent mutations were detected, at codons 83 and 231. In the first case, we found a nucleic acid transversion in the third nucleotide (GGC→GGG); in the second case, a cytosine replaces the adenine at the first codon position (AGG→CGG). The frequency of the polymorphism at codon 231 was very high and also segregated with the ARQ haplotype (data not shown). In summary, a total of 16 single nucleotide polymorphisms (SNPs) have been observed in the coding region of the sheep PrP gene, including 7 transitions and 9 transversions. These 16 SNPs within the alleles encode 14 protein variants of which five polymorphisms (Q101R, R151G, R151H, Y172D and

Table 2. Frequencies (%) of PrP polymorphisms in native flocks and healthy sheep in scrapie-affected flocks compared with scrapie-affected sheep

Breeds: RA, Rasa Aragonesa; OJ, Ojinegra; CA, Cartera; MA, Maellana; RO, Roya Bilbilitana; Ans., Ansotana; Ch., Churra Tensina; RAS, Rasa Aragonesa Scrapie; RNS, Rasa Navarra Scrapie; Pos., Positive scrapie.

Codon	Sequence	Amino acid	RA	OJ	CA	MA	RO	Ans.	Ch.	RAS	RNS	Pos.
101	CAG	Q	97·3	100	93·8	100	100	89·1	100	99·3	100	97·1
	CGG	R	2·7	0	6·3	0	0	10·9	0	0·7	0	2·9
112	ATG	M	100	99·5	100	100	100	93·5	100	99·4	100	100
	ACG	T	0	0·5	0	0	0	6·5	0	0·6	0	0
136	GCC	A	98·4	97·5	100	90	97·9	84·8	91·7	99·7	100	100
	GTC	V	1·6	2·5	0	10	2·1	15·2	8·3	0·3	0	0
141	CTT	L	98·4	93·6	100	85	100	100	100	98·8	87·5	100
	TTT	F	1·6	6·4	0	15	0	0	0	1·2	12·5	0
143	CAT	H	90·8	83·7	96·9	95	95·8	93·5	100	91·3	98·4	94·1
	CGT	R	9·2	16·3	3·1	5	4·2	6·5	0	8·7	1·6	5·9
151	CGT	R	100	98	100	100	100	100	100	100	98·4	100
	GGT	G	0	0	0	0	0	0	0	0	1·6	0
	CAT	H	0	2	0	0	0	0	0	0	0	0
154	CGT	R	97·3	100	100	100	100	97·8	66·7	98·5	92·2	100
	CAT	H	2·7	0	0	0	0	2·2	33·3	1·5	7·8	0
171	CAG	Q	78·8	97·5	42·2	77·5	75	71·7	66·7	83·8	70·3	98·5
	CGG	R	15·8	0·5	57·8	22·5	22·9	28·3	25	12·5	29·7	1·5
	CAT	H	5·4	0	0	0	2·1	0	8·3	3·4	0	0
	AAG	K	0	2	0	0	0	0	0	0·3	0	0
172	TAT	Y	98·9	100	100	100	100	100	100	99·8	98·4	100
	GAT	D	1·1	0	0	0	0	0	0	0·2	1·6	0
175	CAG	Q	100	98·5	100	100	100	100	100	100	100	100
	GAG	E	0	1·5	0	0	0	0	0	0	0	0
176	AAC	N	98·4	94·6	100	95	100	100	100	99·2	100	100
	AAA	K	1·6	5·4	0	5	0	0	0	0·8	0	0
Total*			92	100	32	20	20	23	6	334	32	34

*Number of sequenced sheep of each breed.

Q175E) have to our knowledge not been published before. Table 2 shows the nucleic acid changes at the different codons and the allelic frequencies for the different flocks and breeds.

Haplotype and genotype frequencies. In general, haplotypes carrying the additional amino acid changes showed low frequencies (<5 %), although some breeds had a significant frequency of some haplotypes, for example 16·3 % $AR^{143}RQ$ in Ojinegra sheep. All haplotypes were detected more than once in the selection of 697 samples; the rarest haplotypes were $AG^{151}RQ$ ($n=5$), $AH^{151}RQ$ ($n=4$), $ARQD^{172}$ ($n=3$), $ARQE^{175}$ ($n=3$) and $ARQK^{176}$ ($n=6$). On the other hand, the haplotypes $R^{101}ARQ$ ($n=21$), $AF^{141}RQ$ ($n=39$) and $AR^{143}RQ$ ($n=114$) were more common. $AR^{143}RQ$ especially had relatively high frequencies (>10 %) in several populations and homozygous genotypes were found; it was also present in two of the scrapie-affected sheep (Table 3). In addition to the 15 known genotypes related to the codons 136(A/V), 154(R/H) and 171(Q/R/H), we have detected 27 additional genotypes in these sheep, although some were only detected in individual animals. About half of the sequenced animals were heterozygous in one codon ($n=348$). Only 64 of 697 animals showed heterozygosity in two codons simultaneously, and the remaining animals were homozygous for all polymorphic sites ($n=285$).

DISCUSSION

The first Spanish scrapie focus was diagnosed in a flock of the Rasa Aragonesa breed (García de Jalón *et al.*, 1987). Although there are no confirmed records, it is thought that scrapie was introduced in this population by Suffolk sheep imported from the UK in the 1980s. Recently, several foci of scrapie infection have been detected in the Rasa Aragonesa breed and in other related populations. The aims of this study were to determine the PrP genotypes associated with Spanish scrapie cases in a case–control study of four flocks,

Table 3. Rare genotypes in native breeds compared with Rasa Aragonesa healthy and scrapie-affected sheep

Rare genotype	Native breed (survey)*	RA† sheep (survey)	Healthy sheep in RAS‡	Healthy sheep in RNS§	Scrapie-affected sheep
ARK/ARQ	4	0	1	0	0
R^{101}ARQ/ARQ	6	3	3	0	1
R^{101}ARQ/ARR	4	1	2	0	0
R^{101}ARQ/VRQ	0	1	0	0	0
T^{112}ARQ/ARQ	3	0	3	0	0
T^{112}ARQ/ARR	1	0	1	0	0
AF^{141}RQ/ARQ	14	0	5	5	0
AF^{141}RQ/ARR	1	1	1	2	0
AF^{141}RQ/VRQ	1	0	0	0	0
AF^{141}RQ/AR^{143}RQ	3	0	2	1	0
AF^{141}RQ/ARQK^{176}	1	1	0	0	0
AF^{141}RQ/AG^{151}RQ	0	0	0	1	0
AR^{143}RQ/ARQ	25	7	30	0	2
AR^{143}RQ/ARR	4	4	5	0	0
AR^{143}RQ/VRQ	2	0	3	0	0
AR^{143}RQ/ARK	1	0	0	0	0
AR^{143}RQ/AR^{143}RQ	5	2	5	0	0
AR^{143}RQ/AG^{151}RQ	1	0	0	0	0
AG^{151}RQ/ARQ	3	0	0	0	0
AH^{151}RQ/ARQ	3	0	0	0	0
AH^{151}RQ/AH^{143}RQ	1	0	0	0	0
ARQD^{172}/ARQ	0	0	1	0	0
ARQD^{172}/ARR	0	1	0	1	0
ARQE^{175}/ARQ	3	0	0	0	0
ARQK^{176}/ARQ	9	1	5	0	0
ARQK^{176}/ARR	0	1	0	0	0
ARQK^{176}/ARK	3	0	0	0	0
Total‖	205	92	334	32	34

*Excluding Rasa Aragonesa.
†Rasa Aragonesa healthy flocks.
‡Rasa Aragonesa scrapie-affected flocks.
§Rasa Navarra scrapie-affected flock.
‖Number of sheep.

as well as to perform a PrP genotype survey in healthy native sheep from several related breeds from the scrapie regions.

Nearly 95 % of the scrapie cases in Rasa breeds were homozygous ARQ/ARQ and the frequency of this genotype was significantly higher in scrapie animals than in healthy sheep. This is unexpected, as our analysis revealed about 20 animals with the VRQ haplotype in healthy flockmates. The VRQ haplotype is generally classified as a high-risk factor for scrapie infection (Hunter *et al.*, 1993, 1994, 1996; Thorgeirsdottir *et al.*, 1999). However, our results are similar to studies of so-called 'alanine breeds' (sheep breeds with no or only occasional VRQ carriers), where scrapie cases occur in the ARQ/ARQ, ARQ/ARH genotypes and rarely in the ARR/ARQ genotype (Hunter *et al.*, 1997; Elsen *et al.*, 1999). Therefore, it remains to be established whether the PrP haplotype risk classification for Spanish sheep breeds needs to be different from the classification used for UK sheep breeds.

The survey of the native breeds showed a similar genotype distribution for the Rasa Aragonesa (in both, scrapie and healthy flocks), Ojinegra and Roya Bilbilitana flocks. The haplotype frequencies in these breeds resembled the frequencies published for Suffolk and Lacaune sheep, in which the VRQ haplotype is absent or its frequency is very low (Westaway *et al.*, 1994; Clouscard *et al.*, 1995; Ikeda *et al.*, 1995; Hunter *et al.*, 1997). The detected haplotype frequencies are also similar to those reported for the Spanish Latxa sheep (Hurtado *et al.*, 2002).

Two native populations (Churra Tensina and Ansotana) could be considered as belonging to the so-called 'valine breeds' (sheep breeds with a significant number of VRQ

carriers; Hunter *et al.*, 1996). The haplotype and genotype frequencies are similar to the values reported for other European, Australian and New Zealand 'valine breeds' (Hunter *et al.*, 1997; Hunter & Cairns, 1998; Thorgeirsdottir *et al.*, 1999; Drögemüller *et al.*, 2001). In these breeds, VRQ is associated with a very high risk of scrapie disease and healthy homozygote VRQ/VRQ sheep of old age in scrapie-affected sheep flocks are rare. Sheep of these breeds are less likely to be affected if they are heterozygous at codon 154 (Q/H) or codon 171 (Q/R) (Hunter *et al.*, 1996). In this survey, the AHQ haplotype was only found at low frequencies (Table 1). However, the ARR haplotype was found at relative high frequency in these two flocks; they also showed the highest frequency in all flocks of the ARR/VRQ genotype. Besides presenting susceptible genotypes, no scrapie case has been reported in the Churra Tensina and Ansotana breeds, which may reflect the possibility that they have not come into contact with the scrapie agent. Genetically susceptible sheep are present in scrapie-free areas, such as New Zealand and Australia (Hunter *et al.*, 1997), and can stay healthy after import into countries with high incidence of scrapie, such as the UK, if they are not brought into contact with the scrapie agent (Hunter & Cairns, 1998). It will therefore be of major importance to investigate the epidemiology of Spanish scrapie cases in conjunction with the genetic profiling presented here. The Churra Tensina and Ansotana breeds are close to extinction, and the application of a scrapie eradication programme based on the susceptible VRQ and ARQ haplotypes could reduce the effective breeding population to a dangerously low level, with serious consequences for the survival of the breed. However, it should be possible as a first step to eliminate VRQ-carrying rams from breeding and to increase the number of ARR carriers.

The sequencing analysis has revealed the complexity of PrP genetics of these native sheep populations. In addition to the known polymorphisms, we have detected five more amino acid changes, but most of these new polymorphisms have very low frequencies ($<5\%$) and all of them seem to have originated from a mutation in an ancestral (ARQ) haplotype. It is tempting to speculate that the codon 151 polymorphism, resulting in an amino acid substitution of arginine with glycine or histidine, could have a similar association with disease as has been described for the adjacent codon 154 dimorphism (Goldmann *et al.*, 1991). An equivalent polymorphism (R148H) has recently been found in chimpanzees (Soldevila *et al.*, 2004). The $ARQD^{172}$ and $ARQE^{175}$ haplotypes are of special interest as the amino acid changes are very close to the disease-associated codon position 171. Whether they have a similar disease effect remains to be established. Only the $R^{101}ARQ$ and $AR^{143}QR$ haplotypes were present in scrapie-affected animals, but their frequency was not significantly different from expectation. Four of the new dimorphisms have not been found in other species and the codon 151–175 region of PrP is N-terminal to the region linked to human prion diseases such as familial Creutzfeldt–Jacob disease and Gerstmann–Sträussler–Scheinker syndrome (GSS) (Windl *et al.*, 1999). However, codon 101 (equivalent to codon 98 in human PrP) is adjacent to two genetic mutations (P102L and P105L) in human GSS and is therefore of special interest.

In conclusion, we report here the high variability of the PrP gene found in Spanish sheep. The genotype distribution indicates that the breeds that form large populations in Spain, such as the Ojinegra and Rasa Aragonesa, could be highly susceptible to scrapie. Although a larger population study needs to be conducted based on these PrP genotype frequencies, the application of a breeding programme to control scrapie appears to be a challenging task. The aim of most breeding programmes is to control scrapie susceptibility by the gradual elimination of haplotypes associated with high scrapie susceptibility and to encourage the use of breeding rams with the ARR/ARR genotype. The Rasa and Ojinegra populations present ARR/ARR genotype frequencies lower than 5 % and heterozygous frequencies are around 25 %, presenting ARQ in rather high frequencies. Therefore, it may be important to establish the properties of some of the new PrP variants, as they may provide selectable resistance-associated haplotypes. This paper has shown the importance of a full, sequence-based genotype study before making recommendations for the most suitable sheep-breeding programme.

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