

## PrP gene polymorphism and natural scrapie in Icelandic sheep

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The association between scrapie and polymorphism of the prion protein (PrP) gene was studied in the Icelandic sheep breed. Polymorphism of the three codons, 136, 154 and 171, that are important for scrapie susceptibility was determined. A *Bsp*HI restriction analysis was used to study the alleles of codons 136 and 154, while density gradient gel electrophoresis (DGGE) was used to analyse codon 171 and detect new polymorphisms. The PrP allelic variant, VRQ (amino acids at codons 136, 154 and 171), was found to be highly statistically associated with scrapie, whereas the allelic variant, AHQ, was never found in scrapie-affected animals, a finding that is statistically significant. Iceland has a few scrapie-free regions, which are a part of a quarantine network. Homozygotes for the VRQ variant were found there at a low frequency, indicating that genetic susceptibility is not enough for scrapie to develop and further evidence for the infectious nature of the disease. A comparison of PrP genotypes between sheep outside and within the scrapie-free zones revealed an increase in the AHQ allelic variant in the latter. No polymorphism was found at codon 171 in a total of 932 sheep studied, all individuals having the glutamine allele. Two novel, rare PrP alleles were found using DGGE at codons 138 and 151, i.e. S138N and R151C. Their relevance to scrapie is still unclear, but the former was found in scrapie-affected sheep as well as healthy sheep, whereas the latter was only found in healthy sheep.

### Introduction

Transmissible spongiform encephalopathies (TSE) are fatal degenerative disorders of the central nervous system that occur naturally in man and sheep. Common to all TSE diseases is the accumulation of an abnormal form of the normal prion protein (PrP), primarily in the brain and spinal cord. In its abnormal configuration the PrP (PrP<sup>Sc</sup>) is infectious and extremely resistant to proteolytic enzymes (Prusiner, 1991, 1998). The normal PrP protein (PrP<sup>C</sup>) is expressed in most tissues of the body, with the highest expression in the nervous tissues (Bendheim *et al.*, 1992; Horiuchi *et al.*, 1995).

Scrapie in sheep appears to be entirely an infectious disease with genetic susceptibility playing an important role (Dickinson *et al.*, 1965; Hunter *et al.*, 1997*a*). This susceptibility seems to be determined largely by genotypes of the PrP gene (Hunter *et al.*, 1989; Goldmann *et al.*, 1990). In sheep, polymorphisms of codons 136, 154 and 171 are the most important parameters (Belt *et al.*, 1995; Clouscard *et al.*, 1995; Hunter *et al.*, 1996). This polymorphism might influence the conversion of PrP<sup>C</sup> into PrP<sup>Sc</sup> (Bossers *et al.*, 1997). The study

of scrapie susceptibility is complicated by different PrP genotypes found in the different breeds and due to the possibility that several infectious scrapie strains may exist, each with a different affinity to host genotypes (Smits *et al.*, 1997).

There is only one breed of sheep in Iceland. It is an old breed which has been genetically isolated from other sheep breeds since its introduction by settlers from Scandinavia about 11 centuries ago. Scrapie of sheep was apparently brought to Iceland by an imported ram 120 years ago. The disease was confined to the northern part of the country for 70 years (Sigurdsson, 1954; Pálsson, 1979). In the late 1930s the country was divided by fences into 36 quarantine zones when a programme for eradication of the lentiviral disease of sheep, visna and maedi, was initiated. During the eradication all sheep in the endemic scrapie area were culled. Scrapie recurred following restocking with healthy sheep from outside the endemic area. In the early 1950s scrapie began to spread to other regions, but six quarantine zones have always remained scrapie-free. These six scrapie-free zones, located in three remote parts of the country, have according to records never been affected by this disease (Pálsson, 1979; Sigurdarson, 1991). In the last 20 years a scrapie eradication programme has been in force. The disease is notifiable and any scrapie case

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results in the culling of the entire flock, disinfection of the pens and a sheep-free period of 3 years, followed by restocking with sheep from the scrapie-free regions. Nevertheless, scrapie has recurred once, twice or three times on some farms, especially in the old endemic area (Sigurdarson, 1991).

The aim of this study was to determine the PrP polymorphism in Icelandic sheep, especially in regard to scrapie incidence. For analysis of genotypes relating to risk, sheep affected with natural scrapie were compared to clinically healthy sheep from scrapie farms. Information on PrP genotype frequencies in the Icelandic sheep breed could possibly be used in breeding programmes as an additional strategy in the fight against this disease (Schreuder *et al.*, 1997; Dawson *et al.*, 1998). Of special interest was to compare PrP gene polymorphism in sheep from scrapie-free areas and healthy sheep from scrapie areas in regard to the question of whether scrapie can arise spontaneously in sheep with susceptible genotypes or if the disease is solely of infectious nature.

## Methods

■ **Sheep.** The sheep used in this study ( $n = 932$ ) were all of the Icelandic short-tailed breed, *Ovis brachyura borealis pall.* For the breed survey two categories of control sheep were used: sheep from three regions in Iceland where scrapie has never been detected ( $n = 171$ ) and healthy sheep from scrapie-free farms within regions that have in the past or are currently experiencing scrapie outbreaks ( $n = 286$ ). The scrapie-affected sheep ( $n = 101$ ) came from 77 flocks affected between 1987 and 1998. In 17 cases, scrapie had reappeared on the farm for the second or third time. Symptomless sheep from scrapie-affected flocks served as a control to test for association between PrP polymorphism and scrapie incidence ( $n = 374$ ). Those sheep, collected from 15 affected flocks in the years 1995–1998, matched 30 of the scrapie sheep in age and origin.

The diagnosis of scrapie was based on clinical signs and confirmed by histological examination of three planes of section from medulla oblongata. In some cases immunohistochemical staining for PrP and/or Western blotting for PrP<sup>Sc</sup> was also done.

■ **DNA extraction and amplification.** High molecular mass DNA was isolated from blood or frozen ( $-20\text{ }^{\circ}\text{C}$ ) brain tissue (Sambrook *et al.*, 1989). DNA was later isolated from EDTA blood using Puregene kits (Gentra Systems) or from citrate blood using Chelex 100 (Walsh *et al.*, 1991). PCR amplifications of the PrP gene were performed in a 100  $\mu\text{l}$  reaction volume containing 0.5–1  $\mu\text{g}$  genomic DNA, 200  $\mu\text{M}$  dNTPs, 1  $\mu\text{M}$  of each primer, p8(+) (5' CAGGTTAACGATGGTGAAAAG-CCACATAGG 3') and p143(–) (5' CTGGGATTCTCTCTGGTACTG 3') (Bossers *et al.*, 1996) and 2 U Dynazyme I DNA polymerase (Finnzymes). For Chelex DNA, 40  $\mu\text{l}$  of solution was used in a 100  $\mu\text{l}$  reaction. The amplification reactions were performed in a Techne thermal cycler II, one cycle of 1.5 min at 95  $^{\circ}\text{C}$ , 1.5 min at 58  $^{\circ}\text{C}$  and 1.5 min at 72  $^{\circ}\text{C}$  followed by 39 cycles of 1 min at 94  $^{\circ}\text{C}$ , 1.5 min at 58  $^{\circ}\text{C}$  and 1.5 min at 72  $^{\circ}\text{C}$ , ending with an extension incubation for 10 min at 72  $^{\circ}\text{C}$ .

### ■ Genotype analysis

**RFLP analysis.** Polymorphisms at codons 136 and 154 were detected by RFLP analysis as described by Hunter *et al.* (1993) using the restriction enzyme *Bsp*HI (New England Biolabs). Amplified DNA was digested without further purification. In each reaction pUC19 plasmid DNA was included as an internal control for complete diges-

tion. Polymorphism at codon 151 in the PrP gene was detected by digestion of the PCR product p8-p143 (10  $\mu\text{l}$ ) with 4 U of the restriction enzyme *Ava*II (Pharmacia Biotech). In the wild-type (151-R/R), two fragments (447 and 231 bp) are produced by digestion with *Ava*II. When arginine (CGT) at codon 151 is replaced with cysteine (TGT), the *Ava*II restriction site is lost. Digests of 151-R/C heterozygotes therefore show three fragments (678, 447 and 231 bp), while 151-C/C homozygotes are uncut (678 bp only).

**DGGE analysis.** Density gradient gel electrophoresis (DGGE) was performed, firstly, to detect polymorphism at codon 171, and also to screen for other polymorphisms. By this method a mismatch of only one nucleotide can be detected as a formation of new heteroduplex bands located above the homoduplex bands. Ten  $\mu\text{l}$  of denatured PCR products covering codons 1–226 of the PrP gene, produced by using primers p8 and p143, was used directly in denaturant gradient gels which were prepared and run according to Belt *et al.* (1995). Samples of known allotypes in regard to codons 136, 154 and 171 (ARR, ARQ, ARH and AHQ) were added as controls in each gel.

**Sequencing.** DNA sequencing was performed on both strands of the PCR products using an ALFexpress automated sequencer (Pharmacia Biotech). Before sequencing, the PCR products were treated with Exonuclease I and shrimp alkaline phosphatase (Amersham). Cycle sequencing (Amersham kit US 78500) was performed using either one of the fluorescent-labelled primers, p95(+) (5' F CAAGGTGGTAGC-CACAGTCAG 3') or p182(–) (5' F ACAGTCATGCACAAAGTTG 3'). The primers p95 and p182 refer to bp 354–374 and 597–617 of the PrP sheep sequence, respectively, (Goldmann *et al.*, 1990).

■ **Statistical analysis.** The results were analysed statistically using the  $\chi^2$  test for independence or the Fisher's exact test to compare frequencies of alleles and allelic variants between groups.

■ **Descriptions of PrP genotypes.** In tables and text genotypes are described by the single letter amino acid code: A, alanine; C, cysteine; H, histidine; N, asparagine; Q, glutamine; R, arginine; T, threonine; V, valine. If not otherwise indicated the letters refer to amino acids at codons 136, 154 and 171, in that order.

## Results

### Breed survey

A total of 457 sheep from various parts of the country was studied with regard to the PrP gene polymorphism. The sheep were divided into two groups on the basis of their geographical origin with regard to scrapie incidence, i.e. sheep from scrapie-free regions ( $n = 171$ ) and healthy sheep from scrapie-free farms within regions with scrapie outbreaks either currently or in the past ( $n = 286$ ).

Table 1 shows genotype frequencies at codons 136, 154 and 171 of the sheep PrP gene. These results were derived from RFLP analysis using the restriction enzyme *Bsp*HI and DGGE.

**Codon 136.** At codon 136 the alanine (A) allele predominated, with 80.8–86% of sheep being A/A homozygotes. Valine (V) homozygotes on the other hand were rare, occurring in only three of the 457 sheep tested. One V/V ewe (60 months old) came from a scrapie-free region (0.6%), while two V/V lambs were detected among the 286 sheep (0.7%) tested from scrapie regions. In the scrapie-free regions 13.5% were A/V hetero-

**Table 1.** Breed survey: frequency of different genotypes at codons 136, 154 and 171 in the PrP gene in Icelandic sheep

Codon	Genotype*	Scrapie-free regions	Scrapie regions	$\chi^2$ †	P‡
136	V/V	1 (0.6%)	2 (0.7%)	1.54	0.2151 (0.1896)
136	A/V	23 (13.5%)	53 (18.5%)		
136	A/A	147 (86.0%)	231 (80.8%)		
154	H/H	1 (0.6%)	0 (0%)	6.95	0.0084 (0.0069)
154	H/R	19 (11.1%)	14 (4.9%)		
154	R/R	151 (88.3%)	272 (95.1%)		
171	Q/Q	171 (100%)	286 (100%)		

\* Polymorphism at codons 136 and 154 was determined by *Bsp*HI digestion, and that at codon 171 by DGGE.

† The result of a  $\chi^2$  test (Yates corrected) on allele frequency is shown (df = 1).

‡ The P values shown were derived from a  $\chi^2$  test and the Fisher's exact test (in parentheses).

**Table 2.** Breed survey: frequencies of PrP genotypes and allelic variants in Icelandic sheep

	Scrapie-free regions	Scrapie regions	$\chi^2$ †	P
<b>Genotype*</b>				
ARQ/ARQ	107 (62.6%)	192 (67.1%)		
ARQ/VRQ	18 (10.5%)	50 (17.5%)		
VRQ/VRQ	1 (0.6%)	2 (0.7%)		
ARQ/AHQ	16 (9.4%)	12 (4.2%)		
AHQ/AHQ	1 (0.6%)	0 (0%)		
AHQ/VRQ	2 (1.2%)	1 (0.4%)		
ARQ/AT <sup>137</sup> RQ	0 (0%)	6 (2.1%)		
VRQ/AT <sup>137</sup> RQ	0 (0%)	2 (0.7%)		
ARQ/AN <sup>138</sup> RQ	17 (9.9%)	19 (6.6%)		
VRQ/AN <sup>138</sup> RQ	2 (1.2%)	0 (0%)		
AHQ/AN <sup>138</sup> RQ	1 (0.6%)	1 (0.4%)		
ARQ/AC <sup>151</sup> RQ	5 (2.9%)	1 (0.4%)		
VRQ/AC <sup>151</sup> RQ	1 (0.6%)	0 (0%)		
<b>Allelic variant</b>				
ARQ	270 (78.9%)	472 (82.5%)	1.56	0.2117
VRQ	25 (7.3%)	57 (10%)	1.54	0.2151
AHQ	21 (6.1%)	14 (2.5%)	6.95	0.0084
AT <sup>137</sup> RQ	0 (0%)	8 (1.4%)	3.35	0.0673‡
AN <sup>138</sup> RQ§	20 (5.8%)	20 (3.5%)	2.29	0.1299
AC <sup>151</sup> RQ§	6 (1.8%)	1 (0.2%)	5.10	0.0239

\* If not specifically noted, the genotype refers to the amino acids coded by the triplet sequences present at codons 136, 154 and 171. Polymorphism was determined by *Bsp*HI digestion (codons 136 and 154), *Ava*II digestion (codon 151) and DGGE (codons 137, 138 and 171).

† The result of a  $\chi^2$  test (Yates corrected) on the frequency of a given allelic variant compared to all other variants is given (df = 1).

‡ Statistically significant by the Fisher's exact test,  $P = 0.0284$ .

§ Novel allelic variants described in this study.

zygotes, compared to 18.5% in scrapie regions. This difference was, however, not statistically significant ( $P > 0.05$ ).

**Codon 154.** The large majority of the sheep, between 88% and 95%, were carrying arginine (R) on both alleles at this location of the PrP gene. 154-Histidine (H)/R heterozygotes made up the rest, i.e. 11.1% and 4.9% of sheep from scrapie-free and scrapie regions, respectively. Statistical analysis showed a significant difference between the two groups in regard to allelic frequencies at this codon ( $\chi^2 = 6.95$ ,  $P < 0.01$ ) because of the higher incidence of 154-H in scrapie-free areas. Only one 154-H/H homozygote was found among the 932 sheep (from all groups) included in this study. An additional 154-H/H homozygote has been detected in the same flock from a scrapie-free region (unpublished results).

**Codon 171.** No polymorphism was found at this codon. Only the glutamine (Q) allele was found at codon 171 in a total of 932 sheep included in this study. Because of this apparent lack of polymorphism, a special effort was made to collect samples from as many different farms as possible.

This limited polymorphism in the prion gene of the Icelandic sheep breed described above makes up only three allelic variants, ARQ, VRQ and AHQ, in regard to the three codons 136, 154 and 171, resulting in six different genotypes (Table 2).

### Other polymorphisms

The DGGE analysis showed band patterns that indicated new polymorphisms (Fig. 1). DNA sequencing confirmed the presence of two novel allelic variants, AN<sup>138</sup>RQ and AC<sup>151</sup>RQ, and one rare and previously described variant, AT<sup>137</sup>RQ (Table 2). For each of the two new variants, PCR fragments from eight different sheep were sequenced in both directions

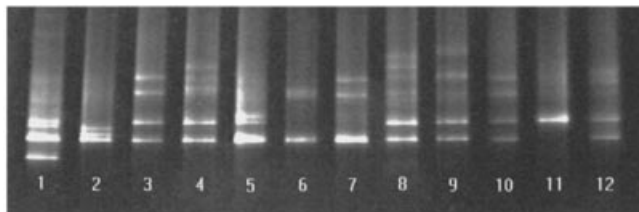


Fig. 1. DGGE analysis of PCR-amplified DNA showing different banding patterns of various PrP genotypes. Lane 1, a combined control of four allelic variants that were loaded without denaturing. From bottom: ARR, ARQ, ARH (courtesy of A. Bossers and M. A. Smits) and AHQ. Lanes 2–4, known banding pattern of the PrP genotypes ARQ/VRQ, ARQ/AHQ and AHQ/VRQ, respectively. Lanes 5–12, eight novel genotypes resulting from various combinations of the two novel allelic variants AN<sup>138</sup>RQ and AC<sup>151</sup>RQ, confirmed by DNA sequencing, showing distinct patterns in the DGGE. Genotypes by lanes: 5, ARQ/AN<sup>138</sup>RQ; 6, AN<sup>138</sup>RQ/AT<sup>137</sup>RQ; 7, VRQ/AN<sup>138</sup>RQ; 8, AHQ/AN<sup>138</sup>RQ; 9, AN<sup>138</sup>RQ/AC<sup>151</sup>RQ; 10, ARQ/AC<sup>151</sup>RQ; 11, AC<sup>151</sup>RQ/AC<sup>151</sup>RQ; 12, VRQ/AC<sup>151</sup>RQ.

between codons 95 and 182 of the PrP gene. Various combinations of these allelic variants resulted in ten additional genotypes, eight of which are novel (Fig. 1, lanes 5–12). They were found at different frequencies in the four research groups, but most of them were very rare (Tables 2 and 4). The frequencies of AT<sup>137</sup>RQ and AN<sup>138</sup>RQ were not found to be significantly different ( $P > 0.05$ ) when control sheep from scrapie-free and scrapie regions were compared (Table 2). The allelic variant, AC<sup>151</sup>RQ, was significantly more frequent in scrapie-free areas ( $\chi^2 = 5.10$ ,  $P < 0.05$ ), but its incidence there was restricted to only one farm.

The polymorphism found at codon 137 (ATG → ACG), resulting in a replacement of methionine with threonine, has been reported before in the ovine PrP gene of Texel and Flemish breeds in Holland (Bossers *et al.*, 1996). Sheep with the allelic variant AT<sup>137</sup>RQ were detected at a low frequency (2.8%) on three farms within the scrapie regions (Table 2).

A new polymorphism at codon 138 was detected by DNA sequencing of samples showing two new heterozygote bands in the DGGE analysis (Fig. 1, lane 5). A nucleic acid transition, G → A, results in a substitution of the amino acid serine (AGC) with asparagine (AAC). This allelic variant, AN<sup>138</sup>RQ, was found in approximately 10% of the sheep (Table 2).

The second novel allelic variant we discovered at codon 151 was a nucleic acid transition (C → T), resulting in an amino acid substitution, with cysteine (TGT) replacing arginine (CGT). The polymorphism at this location was initially discovered because of conflicting results from the RFLP and DGGE analysis. Samples undigested by the restriction enzyme *Bsp*HI gave a DGGE band pattern identical to that of 154-H/R heterozygotes of the genotype ARQ/AHQ. The ARQ/AC<sup>151</sup>RQ genotype was therefore indistinguishable from ARQ/AHQ by DGGE analysis (Fig. 1, compare lanes 3 and 10). After using DNA sequencing to determine the polymorphism at this codon, it became clear that the C<sup>151</sup> allele could also be detected by RFLP analysis using the restriction enzyme *Av*aII, which recognizes the sequence G ↓ GACC. The restriction site, on the PrP wild-type allele (ARQ/ARQ), spanning codons 149–151, is lost when codon 151 changes from CGT to TGT. A few individuals carrying this allele were found in sheep from scrapie-free (3.5%) and scrapie regions (0.4%) (Table 2).

#### Association of PrP genotypes with natural scrapie

**Codon 136.** The frequency of the 136-V allele in 101 scrapie sheep was considerably higher than in the healthy sheep from scrapie flocks. The difference was highly significant ( $\chi^2 = 71.14$ ,  $P < 0.0001$ ) (Table 3). While 12.9% of the scrapie sheep were homozygotes for valine at codon 136, only 1.9% of the control group carries this genotype. In a similar manner the

**Table 3.** Scrapie association: frequency of different genotypes at codons 136, 154 and 171 in the PrP gene in scrapie-affected sheep compared to healthy control sheep from scrapie flocks

Codon	Genotype*	Scrapie-affected sheep	Healthy sheep in scrapie flocks	$\chi^2$ †	<i>P</i> ‡
136	V/V	13 (12.9%)	7 (1.9%)	71.14	< 0.0001
136	A/V	41 (40.6%)	56 (15.0%)		
136	A/A	47 (46.5%)	311 (83.2%)		
154	H/H	0 (0%)	0 (0%)	4.57	0.0325 (0.0121)
154	H/R	0 (0%)	21 (5.6%)		
154	R/R	101 (100%)	353 (94.4%)		
171	Q/Q	101 (100%)	374 (100%)		

\* Polymorphism at codons 136 and 154 was determined by *Bsp*HI digestion, and at codon 171 by DGGE.

† The result of a  $\chi^2$  test (Yates corrected) on allele frequency is shown (df = 1).

‡ The *P* values shown were derived from a  $\chi^2$  test and the Fisher's exact test (in parentheses).

**Table 4.** Scrapie association: frequencies of PrP genotypes and allelic variants in scrapie-affected sheep and healthy control sheep from scrapie flocks

	Scrapie-affected sheep	Healthy sheep in scrapie flocks	$\chi^2$ †	P
<b>Genotype*</b>				
ARQ/ARQ	42 (41.6%)	246 (65.8%)		
ARQ/VRQ	40 (39.6%)	44 (11.8%)		
VRQ/VRQ	13 (12.9%)	7 (1.9%)		
ARQ/AHQ	0 (0%)	17 (4.5%)		
AHQ/VRQ	0 (0%)	3 (0.8%)		
VRQ/AT <sup>137</sup> RQ	0 (0%)	2 (0.5%)		
ARQ/AN <sup>138</sup> RQ	5 (5%)	29 (7.8%)		
VRQ/AN <sup>138</sup> RQ	1 (1%)	3 (0.8%)		
AHQ/AN <sup>138</sup> RQ	0 (0%)	1 (0.3%)		
ARQ/AC <sup>151</sup> RQ	0 (0%)	14 (3.7%)		
VRQ/AC <sup>151</sup> RQ	0 (0%)	4 (1.1%)		
AN <sup>138</sup> RQ/AT <sup>137</sup> RQ	0 (0%)	1 (0.3%)		
AC <sup>151</sup> RQ/AC <sup>151</sup> RQ	0 (0%)	1 (0.3%)		
AN <sup>138</sup> RQ/AC <sup>151</sup> RQ	0 (0%)	2 (0.5%)		
<b>Allelic variant</b>				
ARQ	129 (63.9%)	596 (79.7%)	21.15	< 0.0001
VRQ	67 (33.2%)	70 (9.4%)	71.14	< 0.0001
AHQ	0 (0%)	21 (2.8%)	4.57	0.0325
AT <sup>137</sup> RQ	0 (0%)	3 (0.4%)	0.04	0.8455
AN <sup>138</sup> RQ‡	6 (3%)	36 (4.8%)	0.88	0.3485
AC <sup>151</sup> RQ‡	0 (0%)	22 (2.9%)	4.85	0.0276

\* If not specifically notified, the genotype refers to the amino acids coded by the triplet sequences present at codons 136, 154 and 171. Polymorphism was determined by *Bsp*HI digestion (codons 136 and 154), *Avu*II digestion (codon 151) and DGGE (codons 137, 138 and 171).

† The result of a  $\chi^2$  test (Yates corrected) on the frequency of a given allelic variant compared to all other variants is given (df = 1).

‡ Novel allelic variants described in this study.

frequency of 136-V/A heterozygotes increased from 15.0% in the control group, to 40.6% of the scrapie sheep. We can therefore conclude that the 136-V allele confers a definite risk of scrapie infection to the Icelandic sheep breed.

**Codon 154.** All scrapie sheep studied were found to carry the 154-R allelic variant, whereas the 154-H allelic variant was detected in 5.6% of the healthy control sheep from scrapie farms. This difference in allelic variant frequency between the two groups was statistically significant ( $\chi^2 = 4.57$ ,  $P < 0.05$ ). Therefore, the possibility exists that the 154-H allele offers some protection against scrapie infection in Icelandic sheep.

**Codon 171.** No polymorphism was found at this location in Icelandic sheep, as shown in Tables 1 and 3.

### Genotypes and allelic variants

We detected altogether 14 genotypes in scrapie sheep and healthy sheep from scrapie flocks, five of which were shared by both groups (Table 4). Comparison between the groups of the

frequency as well as statistical analysis of the six allelic variants was done with the following results (Table 4).

**ARQ.** The percentage of scrapie sheep that are homozygous for the 136-A allele was very high. Still this allele seems to offer some protection against scrapie as the A/A homozygotes constituted 83.2% of the control sheep but only 46.5% of the scrapie sheep (Table 3). When comparing the frequency of ARQ between scrapie-affected and healthy sheep, a statistically significant difference was found ( $\chi^2 = 21.15$ ,  $P < 0.0001$ ), suggesting an effect of lower risk by this allelic variant (Table 4). In six cases where the scrapie-affected sheep was an ARQ homozygote, ARQ/VRQ individuals were found among the healthy sheep in the affected flock.

**VRQ.** As the 136-V allele always occurred on the VRQ allelic variant it is not possible to separate the effect of having 136-V from the risk of having the 154-R allele. A highly significant difference ( $P < 0.0001$ ) was found when the frequency of

VRQ in scrapie-affected sheep was compared to the control sheep (Table 4). The VRQ allelic variant can therefore be classified as a high-risk PrP genotype in Icelandic sheep.

**AHQ.** The AHQ allelic variant was not found in the scrapie sheep. Statistical analysis showed a significant difference between the scrapie-affected and control animals ( $\chi^2 = 4.57$ ,  $P < 0.05$ ). The AHQ allelic variant can therefore be classified as a low-risk PrP genotype, at least in Icelandic sheep.

**AT<sup>137</sup>RQ.** Individuals carrying AT<sup>137</sup>RQ were seen at low frequency (0.8%) in healthy sheep from scrapie flocks but were not found among the scrapie sheep, but the frequency of this variant in the control group is so low (0.4%) that nothing can be concluded about association with scrapie susceptibility.

**AN<sup>138</sup>RQ.** The incidence of the N<sup>138</sup> allele was 3% in the scrapie-affected sheep (6% of individuals) and 4.8% in the control (9.7% of individuals). This difference was not statistically significant ( $P > 0.05$ ) (Table 4).

**AC<sup>151</sup>RQ.** C<sup>151</sup> was not found in scrapie sheep, whereas 5.6% of healthy sheep from scrapie-affected flocks were carrying this allele (Table 4). Comparable numbers from the breed survey were 0.4% and 3.5% (Table 2). The difference in frequency between the control groups is derived from an unusually high incidence of C<sup>151</sup> in one scrapie-affected flock (26%, data not shown), probably caused by inbreeding. For example, two new genotypes, in which C<sup>151</sup> is included (AC<sup>151</sup>RQ/AC<sup>151</sup>RQ and AN<sup>138</sup>RQ/AC<sup>151</sup>RQ), were only found in this particular flock. The frequency of this allelic variant was found to be significantly different in the two groups compared in Table 4 ( $P < 0.05$ ), but because of the high incidence of C<sup>151</sup> in this one particular scrapie flock, these results can not be used to draw any conclusions about Icelandic sheep in general.

## Discussion

The situation in Iceland offers a unique opportunity to compare the PrP genotypes within a single breed of sheep either living in scrapie-free zones within quarantine areas or exposed to scrapie infection to a variable degree as in the rest of the country. No statistically significant difference was found in the frequency of the codon 136-V/A polymorphism of the two groups. On the other hand, with regard to the codon 154-H/R polymorphism, a statistically significant difference was found, with histidine being more common in the scrapie-free regions compared to the scrapie regions.

Only the glutamine allele was found at codon 171. The absence of a codon 171 polymorphism in the PrP gene in the Icelandic sheep is striking and could be due to a founder effect dating back to the time of settlement, about 870 AD, or the result of genetic bottlenecks since. It is also possible that 171-R (or H) is so rare in Iceland that present sampling missed it

even though we have now sampled almost 0.2% of the total sheep population of Iceland, making a special effort to get samples from all regions. This is the only sheep breed known to lack polymorphism at codon 171. All other breeds of sheep studied so far have shown a polymorphism at this codon, with arginine being a common allele and histidine being a rare variant, except in the Texel and the Lacaune breeds (Belt *et al.*, 1995; Cloucard *et al.*, 1995).

The use of DGGE enabled us to detect two new alleles within the prion gene, i.e. asparagine and cysteine at codons 138 and 151, respectively, which were confirmed by DNA sequencing. It is interesting to note that the only codon 151-C homozygote found looked like the AHQ homozygote on the DGGE gel but could be detected using the *Ava*II restriction analysis. The amino acid substitution arginine to cysteine is expected to be in the middle of the first  $\alpha$ -helix of the PrP<sup>C</sup> protein by comparison with the mouse PrP protein (Riek *et al.*, 1996), and the unpaired cysteine could lead to an unstable protein. However, preliminary studies of the codon 151-C homozygote by Western blotting indicate that the PrP<sup>C</sup> levels are normal (unpublished results). Both amino acid substitutions were found on a PrP ARQ background, which seems to be the ancestral genotype in sheep. A cluster of polymorphic amino acids in the ovine PrP gene, spanning the amino acids 136–171, is beginning to emerge.

A total of 16 genotypes were found in Icelandic sheep, of which nine occur at frequencies less than 1%. All sheep studied were found to have the same length of PCR products, indicating that all sheep had the same number of octarepeats in the PrP gene. Neither the 112-T (threonine) allele found in the Ile de France breed (Laplanche *et al.*, 1993a) nor the 141-F (phenylalanine) allele found in the Cheviot breed (Hunter *et al.*, 1996) were found.

As can be seen from Table 3 the 136-V allele can be classified as a high-risk for scrapie infection in Icelandic sheep. In this respect the Icelandic sheep resemble breeds with valine at codon 136, i.e. Cheviots, Swaledales and the Shetlands, where the VRQ homozygotes are extremely susceptible to scrapie (Hunter *et al.*, 1993, 1994, 1996). Sheep of these breeds are less likely to get scrapie if they are heterozygous for codon 154-H or codon 171-R (Hunter *et al.*, 1996).

One VRQ homozygote (60 months old) was found in a total of 171 genotyped sheep in the scrapie-free regions (at a frequency of 0.6%). An experimental breeding programme for increasing scrapie resistance by selecting for low-risk PrP genotyped animals has recently been started in Iceland. Genotyping of 442 sheep from scrapie-free regions using the *Bsp*HI analysis only identified six (1.4%) individuals, three ewes and three rams, homozygous for 136-V and 154-R. They were between 12 and 84 months old, with a mean of 48 months (unpublished results). This means that in the scrapie-free zones, with approximately 47 000 sheep and an average frequency of 1%, there are estimated to be 470 VRQ homozygotes. In the last two decades the scrapie-free zones

have traditionally been used to restock scrapie-infected farms after total culling of sheep. The scrapie-free areas in Iceland are equivalent to New Zealand and Australia, where scrapie has been averted due to strict quarantine measures (Hunter *et al.*, 1997a). Scrapie-susceptible sheep do indeed exist in these scrapie-free countries (Hunter *et al.*, 1997a; Hunter & Cairns, 1998). Our results lend further support to the view that scrapie is solely an infectious disease, where incidence is controlled by the availability of the infectious agent and genetically susceptible sheep.

The AHQ allelic variant was not found in scrapie sheep. All scrapie sheep studied so far were homozygous for the codon 154-R allele compared to 94.4% of the control group, i.e. healthy sheep in scrapie flocks. This difference in allele frequency is statistically significant. The same applies when sheep from scrapie-free farms located in scrapie regions are compared to sheep from the scrapie-free regions in the breed survey, where a slightly lower incidence of the 154-R allele is found in the latter (97.5% and 93.9%, respectively). In Cheviot sheep and the Texel breed the AHQ allelic variant seems to be associated with resistance to scrapie (Hunter *et al.*, 1996; Belt *et al.*, 1995). In other studies it is absent in scrapie sheep (Laplanche *et al.*, 1993a; Ikeda *et al.*, 1995), but in each case the numbers were too low to be significant. On the other hand, in Romanov, Suffolk and the Finn Dorset sheep the codon 154-H allele is present in both scrapie sheep and healthy animals (Elsen *et al.*, 1999; Hunter *et al.*, 1997b; Dawson *et al.*, 1998).

The high percentage of Icelandic sheep homozygous for the ARQ allelic variant (65%) is reflected in the high number of scrapie sheep (42%). In other breeds, scrapie incidence in the ARQ/ARQ homozygotes is very variable. In 136-V-encoding breeds, such as the Poll Dorset and the Romanov breeds, 136-A homozygotes are susceptible if they are also homozygous for 171-Q (Hunter *et al.*, 1997c). Similarly, 136-A homozygotes are most susceptible in the Suffolk and in some French breeds, where the 136-V allele is extremely rare and the codon 171 polymorphism is more important (Hunter *et al.*, 1997b; Laplanche *et al.*, 1993b; Westaway *et al.*, 1994).

The 138-N allele seems to be neutral with regard to scrapie susceptibility, while the association of the 151-C allele to scrapie susceptibility is still unknown due to the low frequency of this variant. An analysis of one particular scrapie flock showed an unusually high incidence of this allele (26%). In this flock the 151-C allele was neither found in sheep with definite scrapie nor in the 23% of sheep with questionable scrapie lesions according to histological and immunohistochemical examination (unpublished results). Thus, the 151-C allele may have a protective effect with regard to scrapie susceptibility.

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