

## Prion protein gene polymorphisms in sheep with natural scrapie and healthy controls in Norway

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Two-hundred and forty healthy sheep and 32 cases of natural scrapie in Norway were analysed for disease-linked polymorphisms in the prion protein (PrP) gene. Scrapie was strongly associated with the presence of a valine polymorphism at codon 136 (V<sub>136</sub>), as 68·8% of the cases were homozygous (VV<sub>136</sub>) and 15·6% were valine/alanine heterozygous (VA<sub>136</sub>). All cases were homozygous arginine/arginine at codon 154 (RR<sub>154</sub>), except two which were homozygous histidine/histidine (HH<sub>154</sub>). All cases except two were homozygous glutamine/glutamine at codon 171 (QQ<sub>171</sub>), the two exceptions being heterozygous glutamine/arginine (QR<sub>171</sub>). More than 80% of all scrapie cases in Norway have occurred in a Cheviot-related crossbred type of sheep called Rygja. This type of sheep, which is largely restricted to the south-western coast, carries the V<sub>136</sub> allele at a higher frequency than do other breeds of Norwegian sheep. Polymorphisms at codons 138 and 151 are also described.

### Introduction

The occurrence of natural scrapie in sheep is strongly influenced by polymorphisms in the host gene that encodes the prion protein (PrP), as reviewed by Hunter (1997). The PrP gene is identical to the scrapie incubation period (*Sip*) gene (Dickinson & Outram, 1988; Moore *et al.*, 1998). In all breeds studied, an amino acid change from glutamine (Q) to arginine (R) at codon 171 in the PrP gene renders the animal more resistant towards natural and experimental scrapie as well as experimental bovine spongiform encephalopathy (Goldmann *et al.*, 1994), and only a single case of scrapie has been reported in sheep with a homozygous RR<sub>171</sub> PrP genotype (Ikeda *et al.*, 1995). In several breeds, scrapie susceptibility is greatly enhanced by a valine (V) substitution for alanine (A) at codon 136. This polymorphism is very rare in Suffolk and Lacaune sheep (Westaway *et al.*, 1994; Clouscard *et al.*, 1995), but of the greatest importance in breeds such as Swaledale, Shetland, Cheviot, Romanov, Texel, Ile de France and Blue du Maine (Laplanche *et al.*, 1993; Hunter *et al.*, 1994; Westaway *et al.*, 1994; Belt *et al.*, 1995).

From about 1860 until the beginning of this century,

important changes took place in the Norwegian sheep industry that were of relevance to the genetic background of today's Norwegian sheep as well as to scrapie. There were extensive imports of sheep from England and Scotland. South and North Country Cheviots, Border Leicester, Leicester Longwool and Oxford Down were the most popular breeds. Phenotypically distinct and stable crosses between the old, short-tailed Norse Spel sheep and the imported sheep were gradually established as new 'breeds' in different parts of the country.

Although a disease very similar to scrapie was mentioned in 1890 by a veterinary surgeon named Jenssen (Kjos-Hansen & Holmboe, 1926) and was also described in the 1930s in a Cheviot ram (Anonymous, 1958–1979), the diagnosis was never verified, and it was not until 1958 that the disease was discovered in two Suffolk rams imported from England (Naerland, 1970; Ulvund & Bratberg, 1994). Scrapie was not diagnosed in indigenous sheep, however, until the first outbreak was verified in 1981. Since then, several outbreaks have been diagnosed (Ulvund *et al.*, 1996). A series of single-flock outbreaks occurred in 1985, 1987, 1991, 1992 and 1993. Two flocks came down with scrapie in 1994, eight in 1995, 31 in 1996 and seven in 1997, and up to August 1998 only two flocks had been diagnosed with the disease.

In this report, we compare polymorphisms in part of the PrP gene of 240 Norwegian sheep, representing the three

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major types of crossbred sheep and the Spel breed, with the PrP genotypes of 32 cases of natural scrapie, covering outbreaks of scrapie in Norway from 1981 to 1998.

## Methods

■ **Animals.** We included 189 crossbred sheep among the healthy controls. These included 38 Dala sheep, originating from four flocks from widely separated geographical regions, mean age 3·0 years (range 1–6). The Dala sheep, which today constitutes nearly 50% of the total population of about 1 million winter-fed sheep, is distributed nationwide. It is a large type of sheep, influenced by the Leicester Longwool breed (Trodahl, 1989). We included 53 sheep of the Steigar type, sampled from two well-separated flocks in Nordland, mean age 3·0 years (1–8). The Steigar was developed by crossing local breeds with mostly North Country Cheviot (Sutherland). It amounts to about 10–12% of the total population and is generally restricted to northern Norway.

In south-western Norway, in the county of Rogaland, the Rygja type of crossbred sheep was developed. This is a breed influenced by the South Country Cheviot. We included 98 Rygja sheep representing five ram-circles (80 rams), in addition to 18 sheep belonging to the Department of Sheep and Goat Research, Norwegian College of Veterinary Medicine, Sandnes, mean age 2·1 years (1–11). In the Norwegian ram-circle scheme, farmers co-operate in progeny-testing of ram lambs, because the individual flocks are too small to allow such testing within flocks. Normally, about 20 rams are included in each circle, which serves some 1100 ewes in about 20 flocks per year. Based upon a calculated index, a few proven lambs are selected from the circles each year (Eikje, 1995). In 1995, the Rygja breed constituted about 12% of the sheep population, but in 1996 this breed suffered a serious setback, as a large proportion of the population was destroyed in a dramatic cull of about 70000 healthy sheep from flocks that had experienced various degrees of contact with scrapie-positive flocks. To get the best possible view of the present genetic situation in this type of sheep, we have PrP-genotyped 64 young breeding rams born after the cull, while the remaining 16 rams were born in the period 1989–1993.

We also included 51 animals of the Spel breed, which is the major pure-bred sheep in Norway, making up some 25% of the total population, sampled from the same regions as the Dala sheep, but with the addition of 13 rams from two ram-circles in Rogaland, mean age 3·4 years (1–7).

The 32 cases of natural scrapie cover 21 separate outbreaks during the period 1981–1998, mean age 3·3 years (2–6). Twenty-five of the cases (78%) were of the Rygja breed, four (12·5%) were of the Steigar breed and there was one (3·1%) from each of the breeds Dala, Spel and Pels. The Pels breed (Norwegian fur sheep) accounts for less than 2% of the total population and no sheep of this breed was included in the control group.

■ **Scrapie diagnosis.** All cases showed scrapie suspect clinical signs (Ulvund *et al.*, 1996). The scrapie diagnosis was confirmed by histopathological examination. Furthermore, all cases had to be positive according to at least one additional supportive diagnostic test. Fourteen of the cases were confirmed at Weybridge, UK, by using the detection of scrapie-associated fibrils (SAF) or Western-blot analysis as outlined in Cooley *et al.* (1998). The remaining cases were verified at the National Veterinary Institute, Oslo, and at the Department of Sheep and Goat Research, Sandnes, by Western blots or by immunohistochemistry as described by van Keulen *et al.* (1995), with polyclonal antibodies generously provided by L. J. M. van Keulen (DLO-Institute for Animal Science and Health, Lelystad, The Netherlands).

■ **Genetic analysis.** Genomic DNA was isolated from blood leukocytes or frozen brain tissue by a standard phenol–chloroform protocol, basically as described by Sambrook *et al.* (1989), or by use of a

DNA isolation kit for mammalian blood (Boehringer). For seven of the scrapie cases, paraffin-embedded, formalin-fixed brain tissue was the only available source of DNA. The tissue (200–500 mg) was deparaffinized with xylene and alcohol and cut into small pieces before transfer to distilled water for 24 h. After centrifugation and removal of the supernatant, the tissue was resuspended in 2 ml lysis buffer containing 10 mM Tris–HCl, pH 8·0, 100 mM EDTA and 1% (w/v) SDS. The samples were incubated at 37 °C in the presence of 500 µg/ml proteinase K for 24 h, with the addition of the same amount of proteinase K after 12 h. Final DNA isolation was accomplished by the routine phenol–chloroform protocol.

Part of the PrP open reading frame (GenBank accession no. M31313) was amplified by PCR, by using 0·5–1 µg genomic DNA in standard PCR buffer containing 2 mM MgCl<sub>2</sub>, 300 µM dNTPs, 2·5 units Amplitaq Gold (Perkin Elmer) and 40 pmol PrP-specific, tailed primer oligonucleotides, 5' TGTAAAACGACGGCCAGTAGGCTGGGGTCAAG-GTGGTAGC and 5' CAGGAAACAGCTATGACCTGGTACTGGG-TGATGCACATTTGC (sequencing tails, for –21M13 and M13Rev, are underlined), in a total volume of 100 µl. A nested PCR was designed to allow amplification from low-quality DNA derived from formalin-fixed brain tissue. The outer primers (with tails underlined) were 5' TGTAAAACGACGGCCAGTCAACCGCTATCCACCTCAGGG and 5' CAGGAAACAGCTATGACCGGAAAGAGATGAGGAGGATC-ACAGG. One µl of the first reaction mixture was used as template for the second PCR. Products were visualized by staining with ethidium bromide after electrophoresis of 10 µl reaction mixture on 2% agarose gels and were then purified with Microspin S-400 HR columns (Pharmacia).

PrP polymorphisms were detected by automated DNA sequencing, by using either dye-primer or dye-terminator cycle sequencing, and subsequent analysis by capillary electrophoresis on an ABI Prism 310 Genetic analyser (Perkin Elmer). With the inner pair of primers, we could normally detect polymorphisms covering codons 93 to 216. Only high-quality sequences were analysed and about 10% of the samples were analysed more than once. In the cases of new or rare polymorphisms, the complete PrP genotyping protocol was repeated, starting with new DNA isolations.

■ **Statistical analysis.** PrP codon 136, 154 and 171 polymorphisms were compared by use of  $N \times K$  ( $\chi^2$ ) contingency tables.

## Results

### PrP genotypes detected in this study

We found the well-known dimorphisms at codons 136, 141 and 154 and the trimorphism at codon 171. We also found polymorphisms at codons 138 and 151. In three Spel sheep that were derived from the same flock as the single scrapie case found in this breed, we detected a G → A nucleotide substitution at codon 138 in the second position leading to an amino acid codon change from serine (S) to asparagine (N). Two of the animals had AA<sub>136</sub>SN<sub>138</sub>RH<sub>154</sub>QQ<sub>171</sub> PrP genotypes, while the third carried the AA<sub>136</sub>SN<sub>138</sub>RR<sub>154</sub>QQ<sub>171</sub> PrP genotype. The codon 151 polymorphism, detected in six Dala, one Steigar and three Rygja sheep, consists of a C → T nucleotide substitution in the first codon position leading to an amino acid change from R to cysteine (C). All of these animals were heterozygous RC<sub>151</sub>. Polymorphisms previously described at codons 112, 137 and 211 (Laplanche *et al.*, 1993; Bossers *et al.*, 1996) were not found. The phenylalanine → leucine polymorphism at codon 141 was found at a moderate

**Table 1.** PrP codon 136, 154 and 171 genotypes of 32 cases of natural scrapie compared with healthy controls of the four major breeds of sheep in Norway

Control animals were clinically healthy as judged by a veterinary surgeon at the time of blood sampling. Scrapie has never been diagnosed in any of the control flocks. The criteria for scrapie diagnosis are given in Methods.

PrP genotype			Clinically healthy controls									
			Scrapie		Rygja		Steigar		Dala		Spel	
136	154	171	n = 32	%	n = 98	%	n = 53	%	n = 38	%	n = 51	%
VV	RR	QQ	22	68.8	3	3.1	1	1.9	0	0.0	0	0.0
VA	RR	QQ	5	15.6	13	13.3	5	9.4	1	2.6	1	2.0
VA	HR	QQ	0	0.0	3	3.1	0	0.0	0	0.0	0	0.0
VA	RR	QR	0	0.0	9	9.2	0	0.0	2	5.3	0	0.0
AA	RR	QQ	1	3.1	11	11.2	9	17.0	5	13.2	15	29.4
AA	HR	QQ	0	0.0	7	7.1	5	9.4	3	7.9	10	19.6
AA	HH	QQ	2	6.3	1	1.0	0	0.0	0	0.0	1	2.0
AA	RR	QH	0	0.0	2	2.0	6	11.3	1	2.6	0	0.0
AA	RR	QR	2	6.3	31	31.7	15	28.3	14	36.8	16	31.3
AA	RR	RH	0	0.0	2	2.0	1	1.9	2	5.3	0	0.0
AA	HR	RQ	0	0.0	4	4.1	2	3.8	4	10.5	5	9.8
AA	RR	RR	0	0.0	12	12.2	9	17.0	6	15.8	3	5.9

**Table 2.** PrP codon 136, 154 and 171 genotypes and breed distribution of 32 cases of natural scrapie in Norway

PrP genotype			Natural scrapie					
			Rygja		Steigar	Dala	Spel	Pels
136	154	171	n = 25	%	n = 4	n = 1	n = 1	n = 1
VV	RR	QQ	20	80	1	0	0	1
VA	RR	QQ	3	12	2	0	0	0
AA	RR	QQ	1	4	0	0	0	0
AA	HH	QQ	0	0	1	0	1	0
AA	RR	QR	1	4	0	1	0	0

level (~ 7%) in the three types of crossbred sheep, but not in the Spel breed (data not shown).

#### PrP genotypes in natural scrapie cases and healthy controls

We found natural scrapie in Norway to be strongly associated with a valine polymorphism at codon 136 in the PrP gene (Table 1), similar to that described in several breeds, most notably in Scottish Cheviots (Hunter *et al.*, 1996). Twenty-two of the scrapie cases (68.8%) carried the otherwise very rare VV<sub>136</sub>RR<sub>154</sub>QQ<sub>171</sub> PrP genotype, while five (15.6%) cases were heterozygous VA<sub>136</sub>RR<sub>154</sub>QQ<sub>171</sub>. We found no difference in the mean age between these two groups. The

mean ages in months ( $\pm$  SEM) were  $39.4 \pm 3.3$  and  $35.2 \pm 0.8$  in the VV<sub>136</sub> and VA<sub>136</sub> cases. In the VV<sub>136</sub> scrapie group, the two oldest animals were 6 years old, while three were 5 years old at the time of disease onset. The largest group consisted, however, of animals about 2 years old. As shown in Table 2, two of the scrapie cases, one Rygja and one Dala, the latter being the only scrapie case in this type of sheep, presented AA<sub>136</sub>RR<sub>154</sub>QR<sub>171</sub> PrP genotypes, known to be more scrapie resistant. Both cases were negative when tested for SAF and by immunohistochemistry, but they were weakly positive on Western blots (data not shown). One scrapie case, a Rygja–Spel cross, had the AA<sub>136</sub>RR<sub>154</sub>QQ<sub>171</sub> PrP genotype.

One of the scrapie cases with the AA<sub>136</sub>HH<sub>154</sub>QQ<sub>171</sub> PrP genotype is to date the only case seen in pure-bred Spel sheep,

**Table 3.** Comparison of PrP codon 136 and 171 polymorphisms in healthy animals of the four major breeds of Norwegian sheep

PrP codon 136 and 171 alleles (a) and the results of a statistical comparison (b) using  $N \times K$  ( $\chi^2$ ) contingency tables.

(a)	Breed	n	PrP codon 136			PrP codon 171				
			AA	VA	VV	QQ	QR	RR	QH	RH
	Rygja	98	70	25	3	38	44	12	2	2
	Dala	38	35	3	0	9	20	6	1	2
	Steigar	53	47	5	1	20	17	9	6	1
	Spel	51	50	1	0	32	16	3	0	0

  

(b)	PrP codon 136		PrP codon 171	
	$\chi^2$	P	$\chi^2$	P
Rygja vs Dala	6.8	0.03	3.4	0.49
Rygja vs Steigar	6.0	0.05	7.6	0.11
Rygja vs Spel	15.2	< 0.001	9.1	0.06
Spel vs Dala	1.8	0.18	15.8	0.003
Spel vs Steigar	3.7	0.16	12.8	0.01
Dala vs Steigar	0.8	0.67	6.6	0.16

while the other occurred in a Steigar sheep. Both cases were diagnosed in 1998. All cases of natural scrapie were homozygous at codon 154 (Table 2).

As shown in Table 3, the Rygja sheep presented a significantly higher level of polymorphism at codon 136 ( $\chi^2 \geq 6.0$ ,  $P \leq 0.05$ ) compared with the other types of crossbred sheep and Spel, while at codon 171, the Spel breed was different from the rest in not carrying the H<sub>171</sub> allele. We could not detect any significant difference in PrP genotypes in Rygja sheep sampled before and after the cull in 1996 (data not shown).

## Discussion

Twenty-nine (90.6%) of the cases of natural scrapie were from the Rygja and Steigar types of crossbred sheep, which are both descendants of Cheviot sheep imported from England and Scotland. The strong 'Cheviot-type' association between natural scrapie and the V<sub>136</sub> allele is therefore not surprising. It is, however, interesting to note that the most common type of crossbred sheep in Norway today, the Dala sheep, seems to be less influenced by the Cheviot imports. This is in agreement with old records describing the Dala as a descendant of the Leicester Longwool breed, a breed for which relevant PrP genotype data seems to be lacking.

Our data show that the Rygja sheep carry the V<sub>136</sub> allele at a significantly higher frequency than the other breeds. This may explain, at least partly, the high incidence of scrapie in this

type of sheep. However, similar to NPU Cheviots (Hunter *et al.*, 1996), only a sub-population of the VA<sub>136</sub> heterozygous animals seems to be at high risk, as no scrapie cases with PrP genotypes VA<sub>136</sub>HR<sub>154</sub>QQ<sub>171</sub> or VA<sub>136</sub>RR<sub>154</sub>QR<sub>171</sub> were found. Furthermore, all scrapie cases were homozygous at codon 154, supporting the notion that heterozygosity at codon 154 is moderately protective against scrapie (Hunter *et al.*, 1996).

The two scrapie cases with the AA<sub>136</sub>RR<sub>154</sub>QR<sub>171</sub> PrP genotype highlight the problem of diagnosing scrapie in sheep with more-resistant PrP genotypes, as the supportive diagnostic tests based on detection of partially proteinase-resistant prion protein (PrP<sup>Sc</sup>) may lack the desired sensitivity, thus making negative results difficult to interpret, especially in combination with vague clinical signs and inconclusive or atypical brain histopathology. Inoculations in mice with various materials from cases such as this, in order to search for scrapie infectivity, may be important to clarify whether resistant sheep can harbour and possibly spread the scrapie agent.

The single scrapie case diagnosed in the Spel breed carried the AA<sub>136</sub>HH<sub>154</sub>QQ<sub>171</sub> PrP genotype and was found in eastern Norway, where scrapie has never been seen before. Interestingly, in this flock we found three animals with the susceptible VA<sub>136</sub>RR<sub>154</sub>QQ<sub>171</sub> PrP genotype, which appears to be a very rare PrP genotype in this breed (Table 1). Two of these animals were 5 years old, while the third was 6 years old at the time scrapie appeared in the animal with the AA<sub>136</sub>HH<sub>154</sub>QQ<sub>171</sub> PrP genotype. Apparently, the finding of healthy, old and supposedly scrapie-susceptible animals in a flock must be evaluated with great care when it comes to classifying a flock as scrapie free. Although only a single case, this might indicate that in the Spel breed the scrapie agent can target PrP genotypes different from those at a high risk in crossbred types of sheep. The scrapie case with the AA<sub>136</sub>RR<sub>154</sub>QQ<sub>171</sub> PrP genotype, which was a Rygja–Spel cross, can also be interpreted as supporting this suggestion.

The H<sub>171</sub> allele detected in the Norwegian crossbred sheep is probably a Texel influence, since the Texel, which carries the H<sub>171</sub> allele at a high frequency (~ 20%) (Belt *et al.*, 1995), has been used for crossbreeding in Norway during the last few decades, along with the Finnish Landrace. As the Texel is also a breed in which scrapie susceptibility is governed by the V<sub>136</sub> allele, crossing with Texel might be of relevance for the scrapie-susceptible PrP genotypes described here, particularly in the Steigar type of sheep, which seems to carry the V<sub>136</sub> allele at about the same frequency as the Texel (Belt *et al.*, 1995).

Polymorphisms at codons 138 and 151 have also been reported recently in Icelandic sheep (Thorisson *et al.*, 1998). Interestingly, we found the R → C codon 151 polymorphism only in crossbred Norwegian sheep and not in the short-tailed Spel, which in contrast to the crossbred sheep is thought to be a rather close relative of Icelandic sheep. Another striking

difference between the Norwegian Spel and the Icelandic sheep is the apparent total lack of polymorphism at codon 171 in the Icelandic sheep (Thorisson *et al.*, 1998), while some 30% of the Norwegian Spel are heterozygous QR<sub>171</sub>.

On the basis of information on imports of British sheep, especially around the turn of the century and up to around 1960, both scrapie-susceptible animals and infectivity may well have been introduced into Norway on several occasions. The delayed appearance of clinical scrapie in Norwegian sheep remains an enigma, but it might be related to the need for a long build-up of infectivity in the environment. Lack of diagnostic awareness, because of the notion that scrapie was an inheritable disease that did not occur in Norwegian sheep, may have prevailed. However, Norwegian sheep flocks are small and are kept indoors for 6 months of the year, making scrapie, at least in its classical manifestation, easier to discover. Additionally, the interaction between sheep owners and veterinary services in Norway is very close.

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