

Short Communication

Polymorphisms at codons 141 and 154 in the ovine prion protein gene are associated with scrapie Nor98 cases

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Until June 2004, thirty-eight scrapie cases with unusual features, designated Nor98, have been diagnosed in Norway. This study investigated the distribution of PrP genotypes among Nor98 cases, their flock-mates and a random sample of Norwegian slaughtered sheep. The PrP genotype distribution of Nor98 cases differed markedly from that of previous cases of classical scrapie. A leucine/phenylalanine polymorphism at codon 141 with hitherto unknown significance to scrapie was strongly associated with Nor98 cases. Twenty of 38 (52.6 %) cases were either homozygous or heterozygous for phenylalanine at codon 141. In contrast, this allele was present in only 10.5 % of the flock-mates and 4.5 % of the random sample of slaughtered sheep. Moreover, the H₁₅₄ allele was represented in 24 of 38 (63.2 %) of Nor98 cases, as opposed to 27.0 % of Nor98 flock-mates and 17.0 % of the slaughtered sheep.

Scrapie, a fatal nervous disease of sheep and goat, is the prototype of transmissible spongiform encephalopathies (TSE) or prion diseases. Prion diseases manifest as infectious, sporadic and/or inherited disorders, characterized by the accumulation of an abnormal, protease-resistant isoform (PrP^{Sc}) of the prion protein (PrP) in some tissues of infected animals. Amino acid polymorphisms in the endogenous PrP of sheep affect the relative susceptibility and clinical course of scrapie. At least 20 aa polymorphisms have been reported (Heaton *et al.*, 2003). However, only three codons have been linked to host susceptibility to scrapie, namely codons 136 (A/V), 154 (R/H) and 171 (Q/R/H), where alternative amino acids are indicated by their single-letter symbols (reviewed by Hunter, 1997). The V₁₃₆R₁₅₄Q₁₇₁ (hereafter VRQ) allele confers high susceptibility, while the ARQ allele is associated with high to moderate susceptibility to scrapie and experimental bovine spongiform encephalopathy (BSE). In contrast, the ARR allele is associated with resistance and prolonged incubation periods (Goldmann *et al.*, 1994; Hunter *et al.*, 1994, 1996; Baylis *et al.*, 2002a, b). The AHQ allele may be associated with increased resistance and incubation periods in some breeds (Hunter *et al.*, 1996; Elsen *et al.*, 1999; Thorgeirsdottir *et al.*, 1999; O'Doherty *et al.*, 2002), while the ARH allele seems to be neutral (Dawson *et al.*, 1998; Baylis *et al.*, 2002a).

Experimental challenge has shown that susceptibility to scrapie is also determined by sheep breed and the infective isolate used (Goldmann *et al.*, 1994; O'Rourke *et al.*, 1997; Dawson *et al.*, 1998).

Since their first discovery in 1998 an increasing number of scrapie cases with atypical characteristics, designated Nor98, have been diagnosed in Norway. Nor98 cases differ from classical scrapie and BSE in several features, including the neuroanatomical distribution of the histopathological lesions and of PrP^{Sc} in the brain, and the pattern of PrP^{Sc} deposits. The distinction between classical scrapie and scrapie Nor98 is based on these features and confirmed by the observation of the Nor98 Western blot electrophoretic signature, characterized by a fast migrating band at about 12 kDa (Benestad *et al.*, 2003).

The aim of this study was to compare the PrP genotype distribution of scrapie Nor98 cases to that of their flock-mates and a random sample of the Norwegian sheep population. All

Norwegian scrapie cases classified as scrapie Nor98 until June 2004 were included ($n=38$). All of the adult flock-mates older than one year of age have been blood-sampled and genotyped on a routine basis, and were available for all but two flocks. Ages for individual flock-mates were available for 29 flocks. Until 2004, all sheep of affected flocks were culled, with the exception of several animals from a single flock that is kept for monitoring. In 2004, a new management scheme was introduced, allowing farmers to keep animals with certain genotypes. As a result, examination for PrP^{Sc} has not been completed for all animals in a total of nine flocks.

The Norwegian sheep population was represented by PrP genotype analysis of every 100 normal slaughtered sheep sampled for TSE-examination at two of three laboratories in accordance with the EU commission directive 999/2001 ($n=200$).

A 393 bp fragment of the coding region of the PrP gene, encompassing codons 92–222, was amplified via PCR for all samples, including cases and flock-mates, with primers F294 (5'-AGGCTGGGGTCAAGGTGGTAGC-3') and R642 (5'-TGGTACTGGGTGATGCACATTTGC-3'). In addition, the complete coding region was amplified in Nor98 cases with flanking primers 33F (5'-TTTTACGTGGGCATTTGATG-3') and 56R (5'-TACAGGGCTGCAGGTAGACA-3').

PCR products from scrapie Nor98 cases were sequenced on both strands using the ABI automated sequencing system and Big Dye Terminator chemistry (Applied Biosystems). Flock-mates were either sequenced ($n=1380$) or analysed for single nucleotide polymorphisms (SNP; $n=1016$) at codons 136, 154, 171, and, eventually 141, using the SNaPshot Multiplex system (Applied Biosystems; specific SNaPshot primers have been submitted to JGV Online). PCR-RFLP was designed for convenient detection of the polymorphism at codon 141. In short, the 393 bp PCR product was digested to completion with *Mnl*I and the resulting fragments separated on a 2100 Bioanalyser (Agilent Technologies). The PCR-RFLP analysis was applied to SNP analysed samples not previously analysed at codon 141 ($n=276$) and a number of samples with inconclusive readings of codon 141 from sequence analysis ($n=17$). All of the abattoir samples were amplified with primers F294 and R642 modified by 5' attachment of M13-21 and M13 rev tails, respectively, allowing the use of commercially available fluorescence labelled primers, and sequenced using Big Dye Primer chemistry (Applied Biosystems).

A cloning strategy was applied to determine the specific combinations of polymorphisms on each chromosome, haplotypes, of all scrapie Nor98 cases heterozygous at more than one of the codons 136, 141, 154 and 171. PCR products were obtained by primers F294 and R642 with M13 tails and cloned into the pCR4-TOPO vector using the TOPO TA Cloning kit (Invitrogen). PCR products from five to eight single colonies were sequenced using DYEnamic sequence chemistry (Amersham Biosciences). Haplotypes for the flock-mates and the sample of normal slaughtered sheep were inferred according to the common combinations of codon variants reported in this study and other studies, notwithstanding the observation of rare haplotypes VRR and AHR in sheep from Germany (Kutzer *et al.*, 2002).

Haplotyping of the Nor98 cases revealed that the F₁₄₁ variant was only found with ARQ, constituting an allele designated AF₁₄₁RQ. Non-haplotyped sheep homozygous for the F₁₄₁ substitution were always homozygous ARQ ($n=18$) and all heterozygous animals (F/L₁₄₁) had at

least one ARQ allele ($n=238$), suggesting that the F₁₄₁ variant always occurs in the combination AF₁₄₁RQ, in accordance with previous reports (Hunter *et al.*, 1996; Bossers *et al.*, 1996).

Genotyping of Nor98 cases revealed five alleles: the four common AHQ, ARQ, ARR and ARH alleles, in addition to the AF₁₄₁RQ allele (Table 1). The C₁₅₁ allele was found at an allele frequency of 2.0 % in Nor98 flocks and a C→G nucleotide substitution resulting in a glutamine to glutamate amino acid substitution was detected at codon 175 in eight Black face sheep from one flock. This polymorphism has also been recently reported in Spanish sheep of the Ojinegra breed (Acín *et al.*, 2004).

We found scrapie Nor98 to be strongly associated with PrP alleles AF₁₄₁RQ and AHQ. Either of these alleles, or both, was present in 36 of 38 cases (Table 1). The two cases with neither AF₁₄₁RQ nor AHQ, were both homozygous ARQ and no substitutions were detected in the analysed coding region of these cases. In the statistical comparisons of PrP genotype distribution of cases to their flock-mates we omitted two cases (AF₁₄₁RQ/AF₁₄₁RQ and AF₁₄₁RQ/AHQ) for which flock-mates were not available for analyses. We compared the genotype distributions by using six genotype categories, i.e. without any AF₁₄₁RQ or AHQ alleles, heterozygous AF₁₄₁RQ/XXX, homozygous AF₁₄₁RQ, heterozygous AHQ/XXX, homozygous AHQ and heterozygous AF₁₄₁RQ/AHQ, where XXX represents any allele except AF₁₄₁RQ and AHQ. In scrapie Nor98 affected flocks the prevalence of scrapie Nor98 among sheep without any AF₁₄₁RQ or AHQ alleles was only 0.1 %, whereas it was 3.2 % among heterozygous AF₁₄₁RQ/XXX, 23.5 % among homozygous AF₁₄₁RQ, 1.4 % among heterozygous AHQ/XXX, 10.7 % among homozygous AHQ and 20.6 % among heterozygous AF₁₄₁RQ/AHQ animals (Table 2). This is a highly significant association ($\chi^2=208.7$, $P<0.001$). Similarly, the genotype distribution among cases using the six genotype categories was significantly different from that of the random sample of 200 slaughtered sheep ($\chi^2=145.7$, $P<0.001$).

The odds ratios (OR, corresponding to the relative risk) for scrapie Nor98 in affected flocks were calculated, comparing animals with the AF₁₄₁RQ allele and/or the AHQ allele to those lacking either (Table 2). Using those animals that lacked both alleles as a reference, the ORs for being affected were: 25.3 for heterozygous AF₁₄₁RQ/XXX ($P<0.001$), 234.6 for homozygous AF₁₄₁RQ ($P<0.001$), 11.0 for heterozygous AHQ/XXX ($P=0.002$), 91.0 for homozygous AHQ ($P<0.001$), and 197.7 for heterozygous AF₁₄₁RQ/AHQ ($P<0.001$). This suggests that the AF₁₄₁RQ allele is associated with a higher risk than AHQ of acquiring scrapie Nor98 and that the effects of the two alleles are additive. The earliest onset of scrapie Nor98 seems to be at about 30 months of age. Consequently, we also calculated the ORs for scrapie Nor98 in 29 flocks for which ages of flock-mates were available, using only sheep at least 30-months old ($n=1307$). Due to the recent change in management practice, some animals from Nor98 affected flocks are kept and have not yet been examined for Nor98 pathology. Although the chances of finding scrapie Nor98 in these animals seem to be very small (based on the previous experience from 1998 to 2003), we also calculated the ORs for a sample confined to only 20 completely examined flocks with sheep of known ages ($n=873$). Furthermore, the same comparison was made controlling for any association between age and genotype. These conservative analyses showed that the associations between scrapie Nor98 and genotypes with

AF₁₄₁RQ and/or AHQ alleles remained significant, with the exception of heterozygotes AHQ/XXX using the more restricted sample (Table 2). Statistics were carried out using the SPSS software, version 12.0.1.

The VRQ allele has so far not been detected among Nor98 cases, although it is present in 22.0 % of the sheep in Nor98 flocks and 18.5 % of normal sheep (allele frequencies 12.3 and 9.8 %, respectively; Table 1). This is in sharp contrast to previous cases of classical scrapie in Norway, which have been strongly associated with the VRQ allele. Conversely, the AF₁₄₁RQ allele was not reported among 32 cases of classical scrapie in Norway, although it was found at a moderate level (~7 %) in control animals of cross-bred sheep (Tranulis *et al.*, 1999). The present data suggest that the VRQ allele confers partial or complete resistance to scrapie Nor98, whereas it is highly susceptible to classical scrapie. Based on the contrasting PrP genotype distributions among Nor98 and classical scrapie cases described here, it is conceivable that the persistence of several polymorphisms in the ovine PrP gene may in part be due to the opposing selective regimes imposed by two (or more) types of scrapie.

Since 2002, a number of scrapie cases with characteristics similar to scrapie Nor98 have been diagnosed in several European countries. While the distinctive Western blot signal at 12 kDa is present in several of these cases, it is apparently missing in others, suggesting the existence of yet another form of atypical scrapie. A significant association between AHQ and atypical scrapie cases was recently reported in eight Merinoland sheep from Germany (Lühken *et al.*, 2004). No significant association between PrP genotype and atypical scrapie cases was reported in a comprehensive study of sheep from Germany and France (Buschmann *et al.*, 2004). Information on codon 141 for these cases is presently not available. However, the genotype distribution would seem to resemble the pattern found among Nor98 cases, in that ARQ (which may or may not turn out as AF₁₄₁RQ) and/or AHQ is always present while VRQ is missing.

The mean age of the scrapie Nor98 cases is approximately 6 years, varying from 36 to 100 months (Fig. 1). The youngest animals are found among animals with the genotypes AF₁₄₁RQ/AF₁₄₁RQ, AF₁₄₁RQ/AHQ or AHQ/AHQ. However, no significant difference between the age distributions of the genotypes was observed.

Scrapie Nor98 cases in Norway seem to occur sporadically throughout the country and with only a single case within each sheep flock. There have been no obvious contacts between the positive flocks, suggesting that the infectious agent might not have been transmitted by direct contact between infectious animals as for classical scrapie (Hopp *et al.*, 2001). Though scrapie is generally considered to be an infectious disease, the observed pattern has led to the speculation of scrapie Nor98 being a spontaneous prion disease analogous to sporadic Creutzfeldt–Jakob disease in humans (Benestad *et al.*, 2003). The strong association with certain alleles and the generally high age of the diseased animals would be in agreement with such a hypothesis, though other study designs will be needed to establish a spontaneous aetiology.

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Fig. 1. Diagram depicting the age and genotype distribution of the Norwegian scrapie Nor98 cases. The circle sizes are proportional to the number of cases (one to three cases).

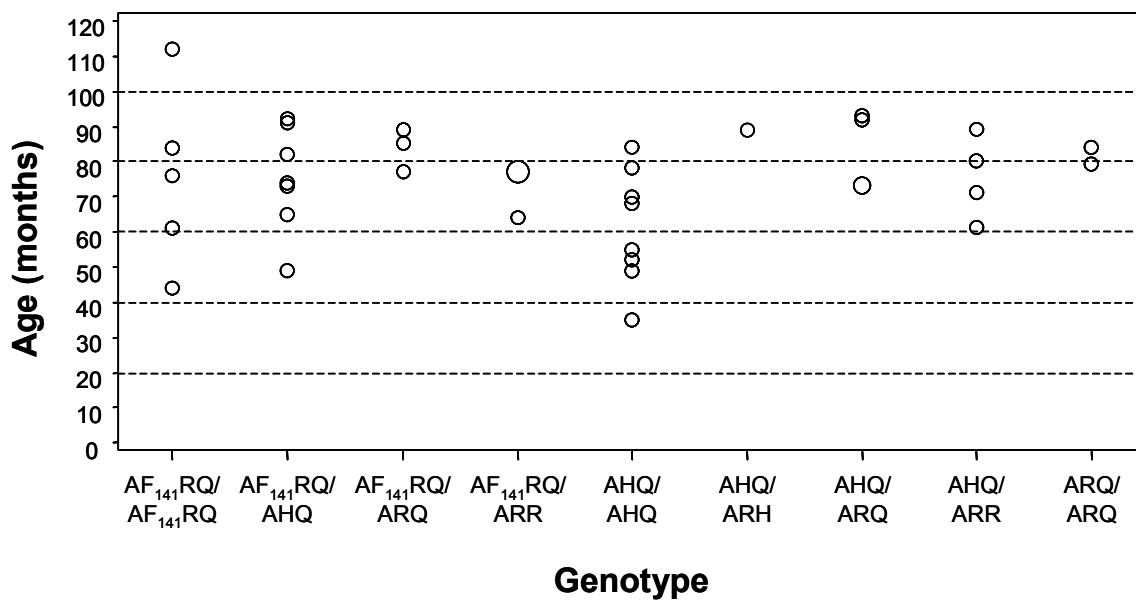


Table 1. Haplotype and genotype frequencies (%) for the PrP gene in Nor98 cases (observed), their adult flock-mates (inferred) and a random sample of Norwegian slaughtered sheep (inferred)

The PrP haplotype is given by the amino acid code at codon 136, 154 and 171, and at codon 141 when the phenylalanine substitution is detected.

	Nor98 cases		Nor98 flock-mates		Slaughtered sheep	
	<i>n</i>	Frequency	<i>n</i>	Frequency	<i>n</i>	Frequency
Haplotypes						
AF ₁₄₁ RQ	25	32.9	263	5.5	9	2.3
AHQ	32	42.1	714	14.9	34	8.5
ARQ	9	11.8	1854	38.7	159	39.8
ARR	9	11.8	1226	25.6	137	34.3
VRQ	–	–	598	12.5	39	9.8
ARH	1	1.3	137	2.9	22	5.5
Total	76	100	4792	100	400	100
Genotypes						
AF ₁₄₁ RQ/AF ₁₄₁ RQ	5	13.2	13	0.5	–	–
AF ₁₄₁ RQ/AHQ	8	21.1	27	1.1	–	–
AF ₁₄₁ RQ/ARQ	3	7.9	67	2.8	3	1.5
AF ₁₄₁ RQ/ARR	4	10.5	90	3.8	4	2.0
AF ₁₄₁ RQ/VRQ	–	–	45	1.9	2	1.0
AF ₁₄₁ RQ/ARH	–	–	9	0.4	–	–
AHQ/AHQ	8	21.1	67	2.8	–	–
AHQ/ARQ	2	5.3	298	12.4	13	6.5
AHQ/ARR	5	13.2	144	6.0	13	6.5
AHQ/VRQ	–	–	91	3.8	6	3.0
AHQ/ARH	1	2.6	21	0.9	2	1.0
ARQ/ARQ	2	5.3	446	18.6	30	15.0
ARQ/ARR	–	–	380	15.9	59	15.0
ARQ/VRQ	–	–	167	7.0	16	8.0
ARQ/ARH	–	–	48	2.0	8	4.0
ARR/ARR	–	–	210	8.8	22	11.0
VRQ/ARH	–	–	16	0.7	1	0.5
VRQ/ARR	–	–	155	6.5	10	8.0
VRQ/VRQ	–	–	62	2.6	2	1.0
ARR/ARH	–	–	37	1.5	7	3.5
ARH/ARH	–	–	3	0.1	2	1.0
Total	38	100	2396	100	200	100

Table 2. ORs for scrapie Nor98 in affected flocks and the percentages of affected animals within each of six genotype categories, comparing animals with the AF₁₄₁RQ allele and/or the AHQ allele to those lacking either

Analyses were based on 36 cases and all of their flock-mates, as well as restricted samples, as indicated (see text for further details). XXX, Any allele other than AF₁₄₁RQ and AHQ.

Genotype	Cases and flock-mates in 36 flocks (n=2432)		Cases and flock-mates in 29 flocks with sheep of known age* (n=1307)		Cases and flock-mates in 20 completely examined flocks with sheep of known age* (n=873)		Cases and flock-mates in 20 completely examined flocks with sheep of known age† (n=873)	
	Cases (%)	OR	Cases (%)	OR	Cases (%)	OR	Cases (%)‡	OR
XXX/XXX (reference)	0.1	1.0	0.2	1.0	0.4	1.0	0.6	1.0
Heterozygous AF ₁₄₁ RQ/XXX	3.2	25.3§	5.1	21.9§	6.8	19.8§	7.0	22.2§
Homozygous AF ₁₄₁ RQ	23.5	234.6§	16.7	81.7§	20.0	67.6§	17.6	22.7
Heterozygous AHQ/XXX	1.4	11.0¶	2.0	8.3¶	1.0	2.8	0.7	1.7
Homozygous AHQ	10.7	91.0§	18.6	93.4§	17.2	56.4§	16.2	37.7§
AF ₁₄₁ RQ/AHQ	20.6	197.7§	33.3	204.3§	33.3	135.3§	31.4	68.6§

*Only sheep 30 months and older.

†Only sheep 30 months and older, controlled linearly for age.

‡Estimated by Ordinary Least Squares (procedure General Linear model).

§ $P \leq 0.001$.

|| $P \leq 0.05$.

¶ $P \leq 0.01$

