

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

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A total of 216 local crossbred sheep from 16 scrapie-affected Greek flocks and 210 purebred sheep of the milk breeds Chios and Karagouniko from healthy flocks were analysed for scrapie-linked polymorphisms in the prion protein (PrP) gene. Of the 216 sheep in this case-control study, 96 sheep were clinical cases, 25 subclinical cases (asymptomatic at the moment of euthanasia but positive by histopathology and/or ELISA detecting proteinase-resistant PrP) and 95 healthy controls (negative by all evaluations). Polymorphisms at codons 136, 154 and 171 were determined by denaturing gradient gel electrophoresis, followed by RFLP and sequencing. Scrapie, both clinical and subclinical, was associated with the genotypes ARQ/ARQ (88 of 110 sheep of that genotype), ARQ/TRQ (9 of 13), ARQ/AHQ (15 of 38) and VRQ/VRQ (9 of 17). Histopathological lesions were more severe in the clinical cases. Genotypes ARQ/ARR (26 sheep), ARQ/ARK (seven sheep), AHQ/ARR (one sheep), ARH/ARH (one sheep) and ARR/ARH (three sheep) were detected exclusively in healthy control sheep. In the purebred survey, four genotypes were present in the Chios sheep (ARQ/ARQ, ARQ/TRQ, ARQ/AHQ and ARQ/ARR) and four in the Karagouniko sheep (ARQ/ARQ, ARQ/AHQ, ARQ/ARR and ARQ/ARH).

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INTRODUCTION

Scrapie is an infectious neurodegenerative fatal disease of sheep and goats belonging to the group of transmissible subacute spongiform encephalopathies (TSEs), along with bovine spongiform encephalopathy (BSE), chronic wasting disease and Creutzfeldt–Jakob disease. All TSEs are characterized by long incubation periods, disturbances in behaviour and movement, degeneration in tissues of the central nervous system (CNS) and accumulation of an abnormal isoform of the host-encoded cellular prion protein in tissues of the CNS. It has been hypothesized that these diseases are attributable to a conformational change in the prion protein (PrP), which results in a change from a predominantly α -helical protein to a β -sheet form (Prusiner, 1996). Normal PrP protein (PrP^c) is a cell-surface

glycoprotein, the expression of which is necessary for the production of prions (Büeler *et al.*, 1993; Prusiner *et al.*, 1993). PrP^c is expressed in most tissues of the body, with the nervous tissues showing the highest PrP^c expression levels (Bendheim *et al.*, 1992; Horiuchi *et al.*, 1995).

In several animal species and in humans, polymorphisms within the open reading frame of the PrP gene are associated with the occurrence and the pathological lesions of TSEs (Pocchiari, 1994). The incidence of natural scrapie is strongly influenced by alterations in the host gene that encodes the PrP (Hunter, 1997). Such polymorphisms may influence the conversion of PrP^c into the pathogenic isoform (Bossers *et al.*, 1997). The mechanism by which the individual allelic variants lead to altered susceptibility or incubation periods has not been elucidated. It has been proposed that in humans PrP polymorphisms may be present at critical sites involved in the conformational transition from PrP^c to PrP^{Sc} (Glockshuber *et al.*, 1999). The study of predisposition to scrapie is complicated by the fact that there are many different PrP genotypes within different individual animals and breeds (Bossers *et al.*, 2000).

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It is also difficult to predict the relationship between conformational changes and the existence of several infectious scrapie strains, each of which has an affinity for host genotypes (Smits *et al.*, 1997).

In sheep, several polymorphisms in the open reading frame of PrP are associated with differences in the phenotypic expression of prion diseases, such as in the incubation period, pathology and clinical signs. Amino acid polymorphisms at positions 112 (M→T), 136 (A→V), 137 (M→T), 138 (S→N), 141 (L→F), 151 (R→C), 154 (R→H), 171 (Q→H or R), 176 (N→K) and 211 (R→Q) have been described (Thorgeirsdottir *et al.*, 1999; Bossers *et al.*, 1996; Tranulis *et al.*, 1999; Hunter *et al.*, 1989, 1994; Laplanche *et al.*, 1993; Westaway *et al.*, 1994; Belt *et al.*, 1995; Vaccari *et al.*, 2001). The polymorphisms at codons 136, 154 and 171 are the most important. The polymorphisms at other codons are rare and have not yet been associated with scrapie susceptibility. The genotype AA₁₃₆RR₁₅₄RR₁₇₁ is associated with the maximum resistance to natural and experimental infections with scrapie and BSE, while the genotype VV₁₃₆RR₁₅₄QQ₁₇₁ is associated with the highest susceptibility to scrapie (Hunter *et al.*, 1994; Goldmann *et al.*, 1994; Bossers *et al.*, 1996, 2000; Clouscard *et al.*, 1995).

The first case of scrapie in Greece was diagnosed in a sheep in 1986 (Leontides *et al.*, 2000). As of the middle of 2001, the disease has been diagnosed in 18 flocks. In goats the first case was diagnosed in late 1997 (Leontides *et al.*, 1999). The scrapie eradication scheme applied in Greece mandates the eradication of the affected flocks.

The aim of this study was to determine the PrP polymorphisms with regard to scrapie incidence in Greek milking sheep. Thus, sheep affected with natural scrapie were compared with healthy sheep from the same affected flocks. Finally, we also examined the PrP gene polymorphisms in healthy purebred sheep of two of the most common Greek milking breeds, Chios and Karagouniko.

METHODS

Animals and samples used for the study. The total population of sheep in Greece is approximately 9.5 million, the great majority of which are crosses of Greek milking breeds (such as Chios, Karagouniko and others) or are crosses of these with imported milking breeds, such as East Friesian and recently Lacaune. They are mainly reared under semi-intensive management (Zygyiannis, 1999).

Our case-control study was carried out on 216 sheep originating from 16 flocks from nine different geographical regions. Of these animals, 96 'clinically suspect' sheep in the advanced stages of the disease were submitted to be killed within a 3-year period of observation. The remaining 120 sheep were selected randomly for the study from 'clinically healthy' animals during the eradication procedure. The number of sheep studied per flock ranged from one (three flocks) to 64 (median six). Their ages ranged from 1 to 10 years (mean=3.8, SD=1.6). Scrapie-affected sheep (age range 1-8 years, mean=3.3, SD=1.4) were younger ($P<0.0001$) than non-affected ones (age range 1-10 years, mean=4.3, SD=1.7).

For the survey of the PrP gene polymorphisms, we studied 110 Chios sheep and 100 Karagouniko sheep, each from five non-affected flocks.

From each animal, EDTA-treated blood was collected for genotyping. The brains from the sheep of the affected flocks were removed and, except for portions that were stored at -70°C for ELISA, were fixed immediately in saline/10% formalin for histopathology.

Scrapie diagnosis. The affected sheep were initially suspected as having scrapie on the basis of the most common clinical signs, i.e. hyperexcitability, restlessness and almost always pruritus starting from the base of the tail extending to the whole body, extensive wool loss followed by muscle tremor and ataxia, and often progressive emaciation (Leontides *et al.*, 2000). The clinical diagnosis was confirmed by histopathological examination and/or ELISA (Platelia BSE detection Kit; Bio-Rad).

The histopathological examination protocol included the selection of eight, 3-4 mm thick coronal slices of the brain (cerebrum, brainstem and cerebellum) from the medulla at the obex and at the caudal cerebellar peduncles including the trapezoidal body, the middle of the pons, the mesencephalon through the rostral colliculi just posterior to the pineal body, the middle transverse section of the cerebellum, the diencephalon at the mamillary body and at the hypophyseal infundibulum-optic tract levels and the frontal cortex rostral to corpus callosum. The slices were processed and embedded in paraffin and sections 4-6 μm thick were stained with haematoxylin and eosin. The density of vacuolation of neuronal perikarya and status spongiosus was graded on a scale of 0-5 as described previously (Fraser & Dickinson, 1967, 1968).

DNA extraction and amplification. Genomic DNA was isolated from the EDTA-treated blood using a DNA isolation kit for mammalian blood (Promega). PCR amplifications of the PrP gene were performed in a 100 μl reaction volume containing 0.5-1 μg genomic DNA, 200 μM dNTPs, 2 mM MgCl_2 , 2.5 units *Taq* DNA polymerase and 30 pmol each of the primers P3(+) (5'-GCAACCGCTATC-CACCTCAG-3') and P2(-) (5'-CTGTGTGTTGCTTGACTGTG-3'), which refer respectively to nt 217-236 and nt 645-626 of the PrP gene (Goldmann *et al.*, 1990). The amplification reactions were performed in an MJR Cyclor for 32 cycles of 1 min at 94°C , 1 min at 59°C and 2 min at 72°C . The products were visualized by staining with ethidium bromide after electrophoresis of 10 μl reaction mixture on a 2% agarose gel.

Genetic analysis

Denaturing gradient gel electrophoresis (DGGE) analysis. Initially, to detect polymorphisms at codon 171 and also to screen for any other polymorphisms, DGGE was performed according to Belt *et al.* (1995). Using this method, a mismatch of only one nucleotide can be detected as the formation of new heteroduplex bands located above the homoduplex bands. Fifteen μl of each PCR product was loaded on a 6.5% polyacrylamide gel containing a linearly increasing gradient from 20 to 80% (v/v) denaturant and electrophoresed overnight at 42 V in a temperature-controlled bath at 60°C . Samples of nine allotypes of codons 136, 154 and 171 were added as controls in each gel. Gels were stained with ethidium bromide and examined by UV transillumination.

RFLP analysis. Polymorphisms at codons 136 and 154 were also confirmed by RFLP analysis using the restriction enzyme *Bsp*H1 (New England Biolabs) as described by Hunter *et al.* (1993).

Sequencing. DNA sequencing was performed on both strands of the PCR products (MWG Biotech). The PrP gene was amplified by PCR using the primers G1(+) (5'-ATGGTGAAGCCACATAGG-CAGT-3') and G2(-) (5'-CTATCCTACTATGAGAAAAATGAG-3'),

which refer respectively to nt 72–90 and 842–819 of the PrP gene (Goldmann *et al.*, 1990). Only high-quality sequences were analysed and about 10% of the samples were reanalysed. In the case of new polymorphisms, the amplification and sequencing of the PrP coding region was repeated, starting with a new DNA isolation.

Descriptions of PrP genotypes. Genotypes are described by the single letter amino acid code, whereas nucleotides are indicated with lower-case letters.

Statistical analysis. The data were initially described in SAS version 8 (Statistical Analysis Systems, 2000). The agreement between the results of the histopathological examination and the ELISA and that between the results of the former examination and the occurrence of typical scrapie clinical signs was evaluated for significance by McNemar's chi-squared test for symmetry (SAS PROC FREQ). The age profiles of scrapie-affected sheep were compared among the genotypes with one-way ANOVA; multiple comparisons were done with the Tukey's test (SAS PROC GLM). The same tests were used for the comparison of the age profiles of clinically affected sheep among the genotypes. The age profiles of scrapie-affected or of clinically affected sheep were compared with those of healthy sheep with the *t*-test for unequal variances (SAS PROC TTEST). The same test was applied for the comparison between the age profiles of the clinically and subclinically affected sheep.

To identify the pairs of alleles of scrapie-susceptible sheep at each of codons 136 and 154, we evaluated the significance of the associations between the pairs of alleles and the results of the ELISA tests in generalized linear models fitted in SAS PROC MIXED (GLIMMIX macro) with a probit link function. Age was included in the models as a control variable. A random-effects term for flock of origin, controlling the likely flock effect, was also included. The same analysis could not be done for the pairs of alleles found at codon 171 because all scrapie-affected sheep had the pair Q/Q.

To identify differences in the risk of scrapie infection among the genotypes that occurred both in scrapie-affected and healthy sheep, we fitted a generalized linear model with a probit link function (SAS PROC MIXED, GLIMMIX macro). The model included the age of the sheep as a control variable and a random-effects term for flock controlling the likely flock effect. The same statistical methodology was used to investigate whether there was a difference in the risk of clinical expression of scrapie among the genotypes of affected sheep. Therefore, in the latter model, the dependent variable comprised clinically and subclinically affected sheep.

A generalized linear model was fitted in SAS PROC MIXED to investigate whether there was an association between the grading of the histological lesions and the genotype of the affected sheep. In this model, the histological grading was the dependent variable and the genotype of the sheep the independent, with age included as a fixed-effect control variable and the flock of origin as a random-effect term. Finally, a similar model was fitted to investigate the likely association between the intensity of histological lesions and the clinical or subclinical status of the affected sheep. In all tests described, significance was evaluated at the 5% level.

RESULTS

Neuropathological analysis and PrP^{Sc} detection

Histopathological examination revealed lesions in the brains of 118 (54.7%) sheep, including all 96 of the 'clinically suspect' sheep, plus 22 'clinically healthy' sheep. The lesions were scored as grade 1 in 39 (33%), grade 2

in 33 (27.9%), grade 3 in 29 (24.6%) and grade 4 in 17 (14.4%) sheep. Large numbers of vacuoles with some confluence (grade 4) were detected in the brains of 13 sheep carrying the PrP genotype ARQ/ARQ, two ARQ/AHQ sheep and two VRQ/VRQ sheep (Fig. 1A). Moderate numbers of evenly scattered vacuoles (grade 3) were detected in the brains of 18 sheep carrying the PrP genotype ARQ/ARQ and in six, three and two sheep carrying genotypes ARQ/AHQ, ARQ/TRQ and VRQ/VRQ, respectively (Fig. 1B). The remaining sheep had few vacuoles, which were either evenly scattered (grade 2) or widely and unevenly scattered (grade 1) (Fig. 1C and D).

In the ELISA test, all 118 sheep with histopathological lesions were positive for PrP^{Sc}. In addition, three sheep (two ARQ/ARQ and one ARQ/AHQ) with neither histopathological lesions nor clinical signs gave a positive result. In all, out of the 216 sheep tested, 121 were assessed as scrapie-affected because they received a positive score in at least one of the two diagnostic evaluations. Of the scrapie-affected animals, 25 (20.6%) could be classified as sub-clinical or pre-clinical cases, since in the absence of any clinical signs of scrapie they were positive for PrP^{Sc} with or without accompanying histopathological changes in the brain. The remaining 95 sheep were assessed as healthy, as they received negative scores in the histopathology, ELISA and clinical evaluations.

PrP genotypes detected in natural scrapie cases and healthy controls

The polymorphisms detected in the prion gene make up seven allelic variants, VRQ, ARQ, AHQ, TRQ, ARK, ARR and ARH for the three codons 136, 154 and 171, resulting in nine different genotypes, two of which (ARQ/ARK, cag→aag, and ARQ/TRQ, gcc→acc) are novel (Fig. 2). The genotype ARQ/AHQ occurred in 38 (17.6%) sheep, AHQ/ARR in one sheep (0.5%), ARH/ARR in one sheep (0.5%), ARQ/ARK in seven sheep (3.2%), ARQ/ARQ in 110 sheep (50.9%), ARQ/ARR in 26 sheep (12%), ARR/ARR in three sheep (1.4%), ARQ/TRQ in 13 sheep (6%) and VRQ/VRQ in 17 sheep (7.9%) (Table 1).

Comparison of the distribution of PrP genotypes in natural scrapie cases and healthy controls

At codon 136, the frequency of the scrapie-affected sheep was not associated with the pairs of alleles ($P=0.4$). At codon 154, the chance of being scrapie-affected was 2.2 times higher in sheep with the RR compared with the RH pair of alleles (95% CI 1.06–4.4; $P=0.003$). At codon 171, all scrapie cases were homozygous QQ.

The distribution of genotypes of the scrapie-affected and non-affected sheep is shown in Table 1. Five genotypes, AHQ/ARR, ARH/ARR, ARQ/ARK, ARQ/ARR and ARR/ARR, did not occur among scrapie-affected sheep. For the genotypes that occurred in both scrapie-affected and

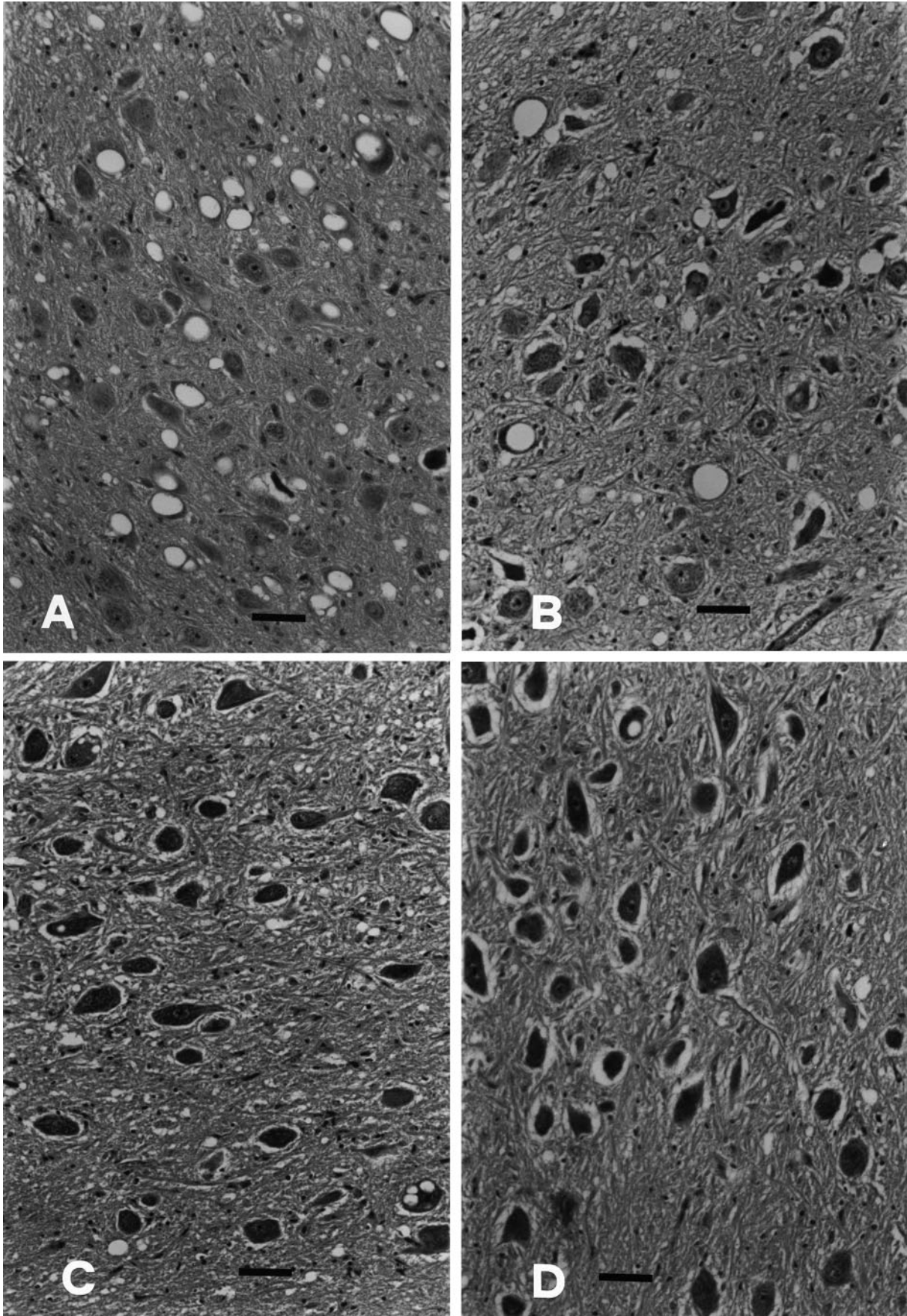


Fig. 1. Medulla oblongata at the obex. (A) Large numbers of vacuoles with some confluence. (B) Moderate numbers of evenly scattered vacuoles. (C) A few evenly scattered vacuoles. (D) A few vacuoles widely and unevenly scattered. Sections were stained with haematoxylin and eosin. Bar, 50 μ m.

non-affected sheep, the risk of being scrapie-affected appeared to be 3.75 times higher for sheep with the ARQ/ARQ genotype than for those with the ARQ/AHQ genotype (95% CI 1.2–10.5; $P=0.001$). It was also 3.5 times higher in sheep with ARQ/TRQ compared with those with the ARQ/AHQ genotype (95% CI 1.26–9.93; $P=0.02$) and 2.7 times higher for sheep with the VRQ/VRQ genotype compared with those with the ARQ/AHQ genotype (95% CI 1.05–6.75; $P=0.04$). The risk did not differ among sheep with the other genotypes (ARQ/ARQ vs VRQ/VRQ, VRQ/VRQ vs ARQ/TRQ and ARQ/ARQ vs ARQ/TRQ).

The histopathological grading was not associated ($P=0.16$) with the genotype of the sheep. It was higher in the clinical compared with the subclinical scrapie cases ($P<0.0001$).

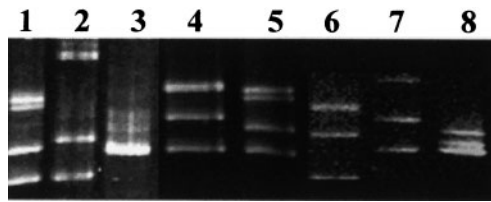


Fig. 2. Denaturing gradient gel electrophoresis analysis of PCR-amplified DNA showing different banding patterns of various PrP genotypes, confirmed by DNA sequencing. Lane 1, ARR/ARQ; lane 2, AF¹⁴¹RQ/ARR; lane 3, ARQ/ARQ; lane 4, ARQ/ARK; lane 5, ARQ/ARH; lane 6, ARR/ARH; lane 7, ARQ/AHQ; lane 8, ARQ/TRQ.

Comparison of the distribution of PrP genotypes in clinical and subclinical cases

The highest risk of clinical scrapie was found in sheep with the VRQ/VRQ genotype (Table 1). Affected-sheep with ARQ/ARQ, ARQ/TRQ and ARQ/AHQ genotypes had a three (1.02–8.7), 4.4 (1.2–16.7) and 4.2 (1.02–17.4) times lower risk, respectively, of developing clinical scrapie than those with the VRQ/VRQ genotype.

Crude effect of age in scrapie-affected sheep

The age of the affected sheep differed ($P=0.03$) among the genotypes. Scrapie-affected sheep belonging to the genotype ARQ/TRQ were older ($P<0.05$) than those belonging to the genotype ARQ/AHQ. All other pairwise genotype comparisons with respect to age were non-significant. In contrast, there was no difference ($P=0.2$) in the age of the clinically affected sheep among the genotypes. No difference ($P=0.6$) was found in the age profiles of clinically and subclinically affected sheep. Both the affected and the clinically affected sheep were younger ($P=0.001$) than the healthy ones.

PrP polymorphisms detected in purebred sheep (Chios and Karagouniko)

Within the Chios breed, the polymorphisms detected in the prion gene presented as four allelic variants, ARR, ARQ, TRQ, AHQ, for codons 136, 154 and 171, resulting in four different genotypes. The genotype ARQ/ARR occurred in 16 (14.5%) sheep, ARQ/TRQ in 11 sheep (10%), ARQ/AHQ

Table 1. Distribution of PrP codons 136, 154 and 171 genotypes and histopathological grading of scrapie-affected (clinical and subclinical cases) and non-affected sheep

Genotype	N	ELISA positive	Histopathological grading								
			Clinical cases				Subclinical cases				Non-affected
			+	++	+++	++++	+	++	+++	++++	
ARQ/ARQ	110	88	12	27	18	12	15	1	0	1	22
ARQ/TRQ	13	9	3	0	3	0	3	0	0	0	4
ARQ/AHQ	38	15	3	2	6	2	1	0	0	0	23
VRQ/VRQ	17	9	1	3	2	2	1	0	0	0	8
ARQ/ARR	26										26
ARQ/ARK	7										7
AHQ/ARR	1										1
ARH/ARH	1										1
ARR/ARH	3										3
Subtotal...			19	32	29	16	20	1	0	1	
Total...	216	121			96				22		95

in 17 sheep (15.5%) and ARQ/ARQ in 66 (60%) sheep. In the Karagouniko breed, the polymorphisms in the prion gene also made up four allelic variants, ARR, ARQ, AHQ and ARH, resulting in four different genotypes. The genotype ARQ/ARR occurred in 57 (57%) sheep, ARQ/AHQ in six sheep (6%), ARQ/ARQ in 32 sheep (32%) and ARQ/ARH in five sheep (5%). Sequence analysis of the PrP gene in the Karagouniko breed sheep revealed the presence of a non-silent polymorphism at codon 141 (L to F, ctt→ttt) in 12 sheep with the ARQ/ARR genotype (Fig. 2).

DISCUSSION

Genotyping of the two most important Greek sheep breeds in the present survey and the crossbred sheep of this case-control study demonstrated the extremely low prevalence of the susceptibility V_{136} allele (0–7%) and a variable prevalence of the resistant R_{171} allele, which was present mostly in heterozygous form (14–57%). Similar results have been reported for the East Friesian milk sheep breed in Germany (Junghans *et al.*, 1998; Drogemuller *et al.*, 2001), which was used in several areas for crossing with the local sheep in Greece. Therefore, the susceptibility of the Greek sheep should depend on the polymorphism at codon 171, as has been established for other breeds (Hunter, 1997).

In this study, differences in the frequency distribution of the codon 136 polymorphisms (A/A, A/T and V/V) between scrapie-affected and healthy controls were not found. The genotype AT_{136} , which was detected in nine scrapie-affected and four healthy sheep, has, to our knowledge, never been previously reported. This polymorphism does not seem to affect susceptibility to scrapie. On the other hand, at codon 154 the risk of being scrapie-affected was two times higher in sheep with the R/R compared with the R/H pair of alleles. The influence of AHQ on scrapie resistance varies between breeds. In Greek sheep and in Romanov, Suffolk and the Finn Dorset breeds, the H_{154} allele is present in both scrapie-affected and healthy sheep (Hunter *et al.*, 1997a; Dawson *et al.*, 1998; Elsen *et al.*, 1999), while in Cheviot and Texel breeds it seems to be associated with resistance (Belt *et al.*, 1995; Hunter *et al.*, 1996; Thorgeirsdottir *et al.*, 1999). The correlation between QQ_{171} homozygosity and scrapie has already been established for a number of ovine breeds (Westaway *et al.*, 1994; Cloucard *et al.*, 1995; O'Rourke *et al.*, 1997). All our scrapie cases were homozygous QQ_{171} . In addition, the non-affected sheep had four polymorphisms at this codon, which were absent from their scrapie-affected counterparts. The genotype QK_{171} , which was detected in seven healthy sheep, was novel. However, during the submission process of our paper, the lysine at codon 171 has also been reported in one healthy Mongolian Khalkh sheep (Gombojav *et al.*, 2003). The association of the QK_{171} genotype with scrapie susceptibility is still unknown, due to the low frequency of this variant.

A total of nine genotypes were found, four of which were

associated with scrapie-affected sheep. The high percentage of Greek sheep homozygous for the ARQ allelic variant (110 of 216, or 50.9%) is reflected in the high number of scrapie sheep of this genotype (88 of 110, or 80%). Sheep with this genotype appeared more susceptible than those with ARQ/AHQ but not than those with VRQ/VRQ or ARQ/TRQ. Additionally, sheep with ARQ/TRQ or VRQ/VRQ genotypes appeared more susceptible than those with ARQ/AHQ. Among the scrapie-affected sheep (clinical and subclinical cases), those with VRQ/VRQ genotype were the most likely to develop clinical disease. In other breeds, scrapie incidence in the ARQ/ARQ homozygotes is very variable. As in our case, A_{136} homozygotes are most susceptible in the Suffolk and in some French breeds, where the V_{136} allele is extremely rare and the codon 171 polymorphism is more important (Hunter *et al.*, 1997a; Laplanche *et al.*, 1993; Westaway *et al.*, 1994). In V_{136} encoding breeds, such as the Poll Dorset and the Romanov, A_{136} homozygotes are susceptible if they are also homozygous for Q_{171} (Hunter *et al.*, 1997b). Our results demonstrated that neither the 96 sheep with clinical scrapie nor the 25 asymptomatic sheep with either vacuolation and/or positivity for PrP^{Sc} were of the protective genotype ARR/ARR. This genotype is significantly associated with resistance to natural infection and experimental oral challenge with scrapie and BSE, probably in all sheep breeds (Belt *et al.*, 1995; Bossers *et al.*, 1996; Cloucard *et al.*, 1995; Goldmann *et al.*, 1994, 1996; Hunter *et al.*, 1996). In our study, the absence of the heterozygous genotype QR_{171} , which was detected in approximately 30% of the healthy sheep, lends further support to previous findings that the ARR genotype is associated with resistance to scrapie infection. Thus, previous recommendations (Junghans *et al.*, 1998; Bossers *et al.*, 2000) for controlling scrapie by increasing the national herd resistance by breeding to ARR homozygosity are most likely useful and applicable in the Greek population. However, it is at present uncertain whether homozygosity of animals in the ARR allele leads to silent agent carriers or to true scrapie resistance. Recently, Houston *et al.* (2003) reported that sheep with the ARR genotype in the homozygous form that were inoculated intracerebrally with a high dose of BSE developed clinical symptoms. These important findings should be further elucidated to clarify whether sheep with this genotype are susceptible when exposed to the natural route of infection. Nevertheless, it is anticipated that minimizing the number of clinical scrapie cases in infected flocks would reduce new infections by potentially lowering the incidence rate. Suitable breeding strategies based on PrP genotyping of sheep may therefore be the only currently available strategy to control scrapie in endemically infected sheep populations.

The proportion of scrapie-affected sheep detected by histopathology was not statistically different from that detected by ELISA. However, the difference between the proportion of histopathology and/or ELISA and clinical sign positive sheep was significant. The small numerical, but not statistical, discrepancy between the results of pathology

and ELISA might be attributed to sampling of different parts of the brain (Hope, 2000) or to the early detection of PrP^{Sc} in pre-clinical cases with no progression of lesions in the CNS. This later hypothesis is supported by the observation that the mean score histopathological grading of subclinical cases was significantly lower compared with the clinical cases.

Our findings of subclinical or pre-clinical cases in approximately one quarter of the 'clinically healthy' asymptomatic sheep in our scrapie-affected flocks is similar to or higher than other related studies (Begara-McGorum *et al.*, 2000; Ryder *et al.*, 2001; Thorgeirsdottir *et al.*, 2002). The infection of these asymptomatic sheep is of concern as they may be spreading the agent for long periods, before clinical scrapie is detected in the flock. The histopathological examination of the brain and the ELISA were equally efficient in identifying these subclinical cases, and were far better than the clinical evaluation alone.

The histopathological grading was not statistically associated with the genotype of the sheep. The severity of lesions was significantly greater in the younger sheep. Also, the age of the scrapie-affected sheep appeared significantly lower than that of the non-affected ones. This finding is in agreement with the relatively low age of the affected sheep. Thorgeirsdottir *et al.* (2002) detected PrP^{Sc} in a 1-year-old lamb but reported clinical scrapie only in older ones. We detected an affected 1-year-old lamb with clinical signs. We can hypothesize that the genotype of the animal influences the risk of becoming infected but not the progression of the disease in the CNS when it becomes infected.

In the purebred Chios and Karagouniko sheep, there were only five PrP genotypes present, with no animal carrying the highly scrapie-susceptible VRQ allele. In addition, a high ARQ allele frequency was found in a homozygous or heterozygous form. These breeds should, therefore, be considered sufficiently susceptible to sustain scrapie endemically. Interestingly, despite the high prevalence of the ARQ allele, a considerable number of animals, especially in the Karagouniko breed, also carried the ARR allele in heterozygous form. Purebred individuals with this genotype could be part of a nucleus flock to be developed to supply scrapie-resistant genetic material.

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