Short Communication

Identification of prion protein gene polymorphisms in goats from Italian scrapie outbreaks

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Susceptibility to scrapie in sheep is influenced by polymorphisms of the prion protein (*PrP*) gene, whereas no strong association between genetics and scrapie has yet been determined in goats due to the limited number of studies on these animals. In this case–control study on 177 goats from six Italian scrapie outbreaks, the association between *PrP* alleles and the occurrence of scrapie was studied. Three silent mutations and 11 *PrP* polymorphisms were identified, of which two polymorphisms (L133Q and M137I) and one silent mutation (T202T) have not been reported previously. Twelve alleles were determined by cloning. Statistical analysis suggested a possible protective role against scrapie for the glutamine to lysine mutation at codon 222.

Scrapie is a neurodegenerative disease affecting sheep and goats and belongs to the group of transmissible spongiform encephalopathies (TSEs) or prion diseases. Although scrapie is an infectious disease, the susceptibility of sheep is influenced by genotypes of the prion protein (*PrP*) gene (Hunter *et al.*, 1997). *PrP* allelic variants valine/arginine/glutamine (VRQ) and alanine/arginine/glutamine (ARQ) at codons 136, 154 and 171, respectively, are generally associated with high susceptibility to scrapie, whereas the ARR allele has been linked to decreased susceptibility or even resistance (Belt *et al.*, 1995; Bossers *et al.*, 1996; Hunter *et al.*, 1996, 1997). In compliance with European Union Decision 2003/100/EC, each member state has introduced a breeding programme to select for resistance to TSEs in sheep populations to increase the frequency of the ARR allele. A similar breeding programme cannot yet be applied to control TSEs in goats, due to the limited knowledge of the genetics of scrapie in this species.

The polymorphisms (amino acid substitutions) described so far in caprine *PrP* are V21A, L23P, G37V, G49S, W102G, T110N, T110P, G127S, I142M, H143R, N146S, R154H, P168Q, R211Q, I218L, Q220H, Q222K and S240P (Goldmann *et al.*, 1996, 1998, 2004; Wopfner *et al.*, 1999; Billinis *et al.*, 2002; Agrimi *et al.*, 2003; Zhang *et al.*, 2004; Kurosaki *et al.*, 2005). The W102G polymorphism has been found only in combination with a variation in *PrP* containing only three instead of the usual five octapeptide repeats (Goldmann *et al.*, 1998). Silent mutations have been described at codons 42 (a→g), 107 (g→a), 138 (c→t), 207 (g→a) and 231 (a→c) (Goldmann *et al.*, 1996; Billinis *et al.*, 2002; Zhang *et al.*, 2004). The most common polymorphism is S240P, which has not yet been found in other species. P240 has been found in mink, ferret, the domestic dog and dingo PrP (Bartz *et al.*, 1994; Wopfner *et al.*, 1999). This leads to the presence of two main *PrP* variants in goats (S240 and P240) that can be linked to other mutations on other codons; consequently, 17 *PrP* alleles have been inferred so far. Based on phylogeny within goats and on sequence conservation over different species, S240 is regarded as the phylogenetic wild type for goats.

No strong association has been established between *PrP* polymorphisms and susceptibility to TSEs in goats. The presence of methionine at codon 142 and the three-repeat/G102 variant were associated with increased incubation periods after experimental challenge with bovine spongiform encephalopathy (BSE) and scrapie strains (Goldmann *et al.*, 1996, 1998). Some protection offered by the R143 and H154 variants against natural scrapie infection has been suggested in Greek goats (Billinis *et al.*, 2002) and an association between scrapie susceptibility and the distribution of genotype at codons 37, 143 and 240 was observed in the Ionica goat breed in Italy (Agrimi *et al.*, 2003). Several factors have been suggested (Baylis & Goldmann, 2004) to explain the difficulties in associating goat *PrP* gene polymorphisms with prion diseases, including the low frequency of the detected mutations, the few experimental studies and the limited availability of case–control studies due to the rarity of goat scrapie in many countries.

In Italy, the first case of natural scrapie in goats was diagnosed in 1997 (Capucchio *et al.*, 1998). Since then, 27 goat

scrapie outbreaks have been reported, 11 of which were mixed flocks of sheep and goats and 16 of which were goat-only herds. A sudden rise in the incidence of scrapie, involving an exceptionally large number of goats, was reported in 1997. Implicated as a cause of the outbreak was an accidental infection from a vaccine against *Mycoplasma agalactiae* (Agrimi *et al.*, 1999; Caramelli *et al.*, 2001).

In our study, we analysed the *PrP* genes of goats from several Italian scrapie outbreaks to detect *PrP* polymorphisms and to determine *PrP* haplotypes by cloning. A case–control study was carried out to look for associations between *PrP* alleles and the occurrence of scrapie.

Material for the study was available from 177 goats taken from six herds (herd 1, 64 animals; herd 2, 11; herd 3, 150; herd 4, 172; herd 5, 246; herd 6, 75) that had scrapie outbreaks between 1998 and 2003. In four of these herds (nos 1, 3, 4 and 5), the vaccine against *M. agalactiae* had been administered. The animals were primarily of the Maltese, Camosciata and crossed breeds. Their age ranged from 1 to 10 years. Twenty-five scrapie-positive cases (age range, 4·5–9 years) were present and distributed among the six different outbreaks as follows: herd 1, 2/61; herd 2, 1/11; herd 3, 0/35; herd 4, 13/14; herd 5, 7/39; herd 6, 2/17 (cases/sample size). Material from the one positive case of the third outbreak was unavailable. For scrapie diagnosis, the obex region was examined by histopathology, immunohistochemistry and/or Western blotting.

Genomic DNA was isolated from 108 frozen brain tissue and 69 EDTA-treated blood samples by using manual Qiagen kits or Thermo Labsystems KingFisher kits, respectively. PCR amplification of the entire open reading frame of the *PrP* gene was performed according to a protocol described previously (Acutis *et al.*, 2004), using the primers p8(+) (5′-CAGGTTAACGATGGTGAAAAGCCACATAGG-3′) and p9(−) (5′-GGAATTCTATCCTACTATGAGAAAAATGAGG-3′) (Bossers *et al.*, 1996). *PrP* polymorphisms were detected by direct DNA sequencing on both strands of the PCR products by using dye terminator cycle sequencing and an ABI Prism 310 Genetic Analyser (Applied Biosystems). Sequencing primers were p8(+), p61(+) (5′-AACCAACATGAAGCATGTGG-3′), p60(−) (5′-GATAGTAACGGTCCTCATAG-3′) and p9(−) (Belt *et al.*, 1995). The primers hybridized to the target *PrP* DNA at codons 1–7, 109–116, 147–154 and 249–257, respectively. To link the detected polymorphisms into an allele sequence (haplotype), PCR-amplified products of selected samples were cloned in a TA cloning vector (Invitrogen). At least five clones each were analysed by sequencing to identify the polymorphisms per allele. Several polymorphism combinations (haplotypes) were checked from different animals to exclude potential heterogeneous coupling.

A χ^2 test was performed to look for associations between each allele and scrapie status. This was done by comparing the frequencies of genotypes with and without an allele between cases and controls: heterozygotes and homozygotes for the same allele were combined in a single group. When data were sufficient for multivariate analysis, a mixed logistic regression model with a binomially distributed error term was fitted to the outcome variable (i.e. the scrapie status). Age and vaccination were included in the model as covariates, whereas 'herd' was included as a random effect to control for the effect of clustering of goats within each outbreak. All descriptive statistics and data manipulation were performed by using Stata Statistical Software version 9 (Stata Corporation); the Stata macro *gllamm* was used to fit the mixed logistic model.

Eleven polymorphisms were identified (Table 1). Two of these polymorphisms had not been reported previously: at codon 133, a ctg→cag substitution caused an amino acid

Table 1. *PrP* polymorphisms and alleles detected in the analysed goats and their frequencies

Allele	Codon											Allele frequency (%)
	37	110	127	133	137	142	143	154	168	222	240	
1	G	T	G	L	M	I	H	R	P	Q	S	21·75
2	–	–	–	–	–	–	–	–	–	–	P	39·55
3	V	–	–	–	–	–	–	–	–	–	–	5·6
4	–	P	–	–	–	–	–	–	–	–	–	1·4
5	–	–	S	–	–	–	–	–	–	–	P	1·1
6	–	–	–	Q	–	–	–	–	–	–	P	0·6
7	–	–	–	–	I	–	–	–	–	–	P	2·8
8	–	–	–	–	–	M	–	–	–	–	P	2·0
9	–	–	–	–	–	–	R	–	–	–	P	7·6
10	–	–	–	–	–	–	–	H	–	–	–	8·5
11	–	–	–	–	–	–	–	–	Q	–	P	2·0
12	–	–	–	–	–	–	–	–	–	K	–	7·1

change of L→Q and at codon 137, an atg→ata substitution led to the amino acid change M→I. As reported previously by Goldmann *et al.* (1996), silent mutations were also found at codon 42 (cca→ccg) (134 goats, 55 of which were homozygotes) and at codon 138 (agc→agt in 142 goats, 57 of which were homozygotes), and a new silent nucleotide change was detected at codon 202 (acc→act) (four heterozygous goats). The three-octarepeat variant was not found in any of the examined animals. Twelve alleles were determined (Table 1) by single-allele sequencing (by cloning), which were found combined in 37 different genotypes (Table 2). The most frequent alleles were 1 (corresponding to the phylogenetic wild type of sheep) and 2, which were distinguished only by the codon 240 S/P dimorphism. Predominant genotypes were 1/2 and 2/2. According to Goldmann *et al.* (1996), silent mutations at codons 42 and 138 were found in linkage with the dimorphism at codon 240: 42a and 138c were linked to codon S240, whereas 42g and 138t were linked to codon P240. Similarly, the novel c→t substitution at codon 202 was found solely in linkage with P240.

Table 3 shows the χ^2 comparisons. Only two alleles (2 and 12) showed a significant association with scrapie status. Allele 12 was not present in any scrapie case, thus suggesting a potential protective effect. The complete absence of cases with this allele precluded any further statistical analysis. Univariate analysis showed that the presence of allele 2 was associated with an increased risk of scrapie [odds ratio (OR), 6·4; 95 % confidence interval (CI), 1·8–22·4]. This positive effect was still evident after adjusting for age and vaccine (OR, 8·6; 95 % CI, 1·8–40·2), but lost its statistical significance when the mixed logistic model included the herd as a random effect (OR, 2·4; 95 % CI, 0·4–15·2).

In agreement with previous studies, we found that the caprine *PrP* gene is highly variable at positions different from those in sheep. We also discovered several novel coding and silent mutations in a relatively small group of animals. The alleles were not inferred; instead, they were assessed precisely by single-allele cloning and sequencing, thus confirming that each detected polymorphism other than the one at codon 240 was always in the same linkage with the 240 polymorphism and gave rise to one allele only. No double mutation was detected together with the 240 dimorphism in any of the examined samples.

Our case–control study included a relatively high number of scrapie outbreaks and animals; however, a possible selection bias cannot be ruled out, as case and control recruitment was restricted to the limited material available. Even so, taking into account data from other studies, several suggestions can be made. Both alleles 1 (S240) and 2 (P240) were found in scrapie-positive animals; the significant positive association between allele 2 and scrapie positivity revealed by univariate analysis was unconfirmed by multivariate analysis. An association between the 240 polymorphism and scrapie has been excluded by some authors (Goldmann *et al.*, 1996; Billinis *et al.*, 2002), who hypothesized that this codon is probably eliminated during post-translational processing of the caprine PrP. It could well be, however, that the polymorphisms at codon 240 modulate disease susceptibility by interfering with mRNA stability or they may be linked to another quantitative trait of the animal.

Allele 3 (37V) did not appear to be associated with scrapie status. Alleles 4 (P110), 5 (S127), 6 (Q133) and 7 (I137) were found only in healthy animals, but at a frequency too low to establish an association. Allele 5 was found only in the Camosciata breed; the same amino acid change was also found in Mongolian sheep (Gombojav *et al.*, 2003) with an

Table 2. *PrP* genotypes detected in the analysed goats and their frequencies

Genotype		Frequency/ frequency in scrapie cases (%)	No. goats/no. scrapie-positive goats
Allele 1	Allele 2		
1	1	3·95/4	7/1
	2	17·51/24	31/6
	3	2·82	5
	4	1·13	2
	5	1·13	2
	7	1·13	2
	8	0·56	1
	9	2·82	5
	10	3·39	6
	11	1·13	2
	12	3·95	7
2	2	20·9/40	37/10
	3	3·39/12	6/3
	4	0·56	1
	8	1·13	2
	9	5·65/4	10/1
	10	2·82/4	5/1
	11	1·69/4	3/1
	12	4·52	8
3	3	0·56	1
	9	2·26	4
	10	0·56	1
	11	0·56	1
	12	0·56	1
4	10	1·13	2
5	10	1·13	2
6	7	0·56	1
	8	0·56	1
7	7	0·56	1
	8	1·13	2
	12	1·69	3
8	12	0·56	1
9	10	3·95/8	7/2
	12	0·56	1
10	10	1·13	2
	12	1·69	3
11	12	0·56	1
Total		100/100	177/25

Table 3. Univariate analysis by allele for scrapie case/control status

Allele		No. cases	No. controls	χ^2	*P* value
1	Present	7	63		
	Absent	18	89	1·62	0·203
2	Present	22	81		
	Absent	3	71	10·63	0·001
3	Present	3	16		
	Absent	22	136	0·05	0·825
4	Present	0	5		
	Absent	25	147	0·85	0·358
5	Present	0	4		
	Absent	25	148	0·67	0·412
6	Present	0	2		
	Absent	25	150	0·33	0·564
7	Present	0	9		
	Absent	25	143	1·56	0·212
8	Present	0	7		
	Absent	25	145	1·2	0·274
9	Present	3	24		
	Absent	22	128	0·24	0·625
10	Present	3	25		
	Absent	22	127	0·32	0·572
11	Present	1	6		
	Absent	24	146	0·002	0·99
12	Present	0	25		
	Absent	25	127	4·79	0·029

unknown association with scrapie. In Dutch Swifter and Icelandic sheep, a mutation at the same amino acid position of allele 7 (137) was found, but with a change from M to T instead of M to I (Bossers *et al.*, 1996; Thorgeirsdottir *et al.*, 1999). No association between this codon and scrapie in sheep has been established, although the M137T polymorphism also seems to modulate sheep PrP conversion (Bossers *et al.*, 2000). Allele 8 (M142) has been shown to prolong incubation periods in goats challenged experimentally with scrapie or BSE (Goldmann *et al.*, 1996), thus suggesting that this allele may confer partial resistance to the disease. In our study, we were unable to assess this association because of the low frequency of this allele in general and because no scrapie-positive goats with allele 8 were present in our sample: the frequency in our Italian goats (2 %) was much lower than that reported by Goldmann *et al.* (2004) for the UK (28 %). Should more extensive studies confirm this frequency, allele 8 might not be a practical target for genetic selection in goats in Italy. Alleles 9 (R143) and 10 (H154) are thought to offer some protection against scrapie infection in Greek goats (Billinis *et al.*, 2002). Our results differed in that the two alleles were found to occur at similar frequencies in both scrapie-affected and healthy animals. Furthermore, the age of the positive animals carrying these alleles was no higher than that of the other cases (data not shown), which suggests no alteration in the incubation period. The reasons for these differences may depend on the small sample size, the different susceptibility profiles in the goat populations by other breed- or country-specific factors or the presence of a different isolate/strain of scrapie agent. In sheep, the H154 polymorphism seems to have different effects. It is associated with resistance in some breeds (Dawson *et al.*, 1998; Thorgeirsdottir *et al.*, 1999), but with susceptibility in other breeds and countries (Dawson *et al.*, 1998; Acutis *et al.*, 2004; Vascellari *et al.*, 2005).

Allele 11 is characterized by a polymorphism at codon 168 (P→Q), which is also polymorphic in sheep, with a change from P to L. In sheep, this mutation appears to prolong the incubation period of the disease (Baylis & Goldmann, 2004). What is remarkable is that, despite its very low frequency in the study sample, allele 11 was found in one scrapie-positive goat. The animal was 8 years old, but was not the oldest case. This result suggests that amino acid changes at codon 168 have a different effect on susceptibility to scrapie in sheep and in goats, as has been demonstrated, for instance, in sheep having codon A136 or V136, that results in different disease susceptibility.

A noteworthy result of our study is the significant association between allele 12 (K222) and healthy animals. No goats carrying this allele were found to be scrapie-positive, even though the frequency was not low. In contrast, all other alleles with similar frequencies were also actually present in the group of cases. Moreover, allele 12 had a relatively high frequency in all but one herd, whether vaccinated or not (4·9 % in herd 1; 9 % in herd 2; 7·14 % in herd 3; 0 % in herd 4; 11·5 % in herd 5; 8·8 % in herd 6). A multivariate analysis to assess further the possible protection given by allele 12 in goats could not be done precisely because of the absence of scrapie-affected animals, so potential confounders on χ^2 analysis could not be explored. It is noteworthy that a similar change is found in human PrP (Q219K) (Shibuya *et al.*, 1998), where it serves as a unique protective factor against sporadic Creutzfeldt–Jakob disease. This could support our hypothesis for a protective role of lysine at codon 222 in goats, assuming that the same single amino acid change seems to have the same effect in different species. *In vitro* experiments to study the efficacy of this polymorphism in converting normal PrP into pathological PrP are now under way. More genetic data on goats in scrapie outbreaks will need to be collected to confirm whether K222 can really be a practical target for breeding towards scrapie resistance. The possibility that a genetic influence on TSE susceptibility may also be present in the goat, but conferred by alleles different from those of sheep, may also be useful generally for TSE research in other species (with similar or even identical alleles) in the same way that sheep genetics originally revealed disease associations and potential mechanisms of disease.

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