Journal of General Virology (2009), **90**, 769–776 DOI 10.1099/vir.0.006114-0

Identification of seven haplotypes of the caprine *PrP* gene at codons 127, 142, 154, 211, 222 and 240 in French Alpine and Saanen breeds and their association with classical scrapie

F. Barillet,[1] D. Mariat,[2] Y. Amigues,[3] R. Faugeras,[3] H. Caillat,[1] K. Moazami-Goudarzi,[2] R. Rupp,[1] J. M. Babilliot,[3] C. Lacroux,[4] S. Lugan,[4] F. Schelcher,[4] C. Chartier,[5] F. Corbière,[4] O. Andréoletti[4] and C. Perrin-Chauvineau[5]

Correspondence
F. Barillet
francis.barillet@toulouse.inra.fr

[1]INRA, UR 631, Station d'amélioration génétique des animaux, BP 52627, 31326 Castanet-Tolosan Cedex, France

[2]INRA, UR 339, Laboratoire de génétique biochimique et cytogénétique, 78352 Jouy-en-Josas Cedex, France

[3]GIE Labogena, Domaine de Vilvert, 78352 Jouy-en-Josas Cedex, France

[4]INRA, UMR 1225, Interactions Hôtes Agents Pathogènes, Ecole Nationale Vétérinaire de Toulouse, 23 chemin des Capelles, 31076 Toulouse Cedex, France

[5]AFSSA-Niort, Laboratoire d'études et de recherches caprines, BP 3081, 79012 Niort Cedex, France

Received 31 July 2008
Accepted 17 November 2008

In sheep, susceptibility to scrapie is mainly influenced by polymorphisms of the *PrP* gene. In goats, there are to date few data related to scrapie susceptibility association with *PrP* gene polymorphisms. In this study, we first investigated *PrP* gene polymorphisms of the French Alpine and Saanen breeds. Based on *PrP* gene open reading frame sequencing of artificial insemination bucks (n=404), six encoding mutations were identified at codons 127, 142, 154, 211, 222 and 240. However, only seven haplotypes could be detected: four ($GIH_{154}RQS$, $GIRQ_{211}QS$, $GIRRK_{222}S$ and $GIRRQP_{240}$) derived from the wild-type allele ($G_{127}I_{142}R_{154}R_{211}Q_{222}S_{240}$) by a single-codon mutation, and two ($S_{127}IRRQP_{240}$ and $GM_{142}RRQP_{240}$) by a double-codon mutation. A case–control study was then implemented in a highly affected Alpine and Saanen breed herd (90 cases/164 controls). Mutations at codon 142 (I/M), 154 (R/H), 211 (R/Q) and 222 (Q/K) were found to induce a significant degree of protection towards natural scrapie infection. Compared with the baseline homozygote wild-type genotype $I_{142}R_{154}R_{211}Q_{222}$/IRRQ goats, the odds of scrapie cases in $IRQ_{211}Q$/IRRQ and $IRRK_{222}$/IRRQ heterozygous animals were significantly lower [odds ratio (OR)=0.133, P<0.0001; and OR=0.048, P<0.0001, respectively]. The heterozygote M_{142}RRQ/IRRQ genotype was only protective (OR=0.243, P=0.0186) in goats also PP_{240} homozygous at codon 240. However, mutated allele frequencies in French Alpine and Saanen breeds were low (0.5–18.5 %), which prevent us from assessing the influence of all the possible genotypes in natural exposure conditions.

INTRODUCTION

Scrapie in small ruminants is the archetype of transmissible spongiform encephalopathies (TSE) or prion disease. These neurodegenerative diseases affect a large spectrum of mammalian species including cattle (bovine spongiform encephalopathy, BSE), cervids (chronic wasting disease) or humans (Creutzfeldt–Jakob disease, fatal familial insomnia and Gerstman–Scheincker–Straussler syndrome).

In sheep, polymorphisms of the gene encoding the PrP protein (PRP) strongly modulate susceptibility to scrapie (Hunter *et al.*, 1996; Elsen *et al.*, 1999). In particular the $A_{136}R_{154}R_{171}$ allele is associated with high resistance to natural and experimental infections with classical scrapie and BSE agents, while other haplotypes like $V_{136}R_{154}Q_{171}$ or $A_{136}R_{154}Q_{171}$ are associated with a high susceptibility to

A supplementary table is available with the online version of this paper.

scrapie (Baylis & Goldmann, 2004). Selection of the ARR allele in the sheep population is now widely used for TSE control and eradication in several EU countries, like the Netherlands, France and the UK. In scrapie-affected sheep flocks, breeding for resistance represents an alternative approach to stamping-out and similar prevention and eradication of TSE in goats would be a valuable solution.

Studies carried out in several European and Asian goat breeds have led to the discovery of 30 polymorphisms of the *PrP* encoding gene of which nine are silent mutations (at codons 42, 107, 138, 179, 181, 202, 207, 219 and 231) and 21 are amino acid substitutions in the open reading frame (ORF) (V21A, L23P, G37V, G49S, W102G, T110P or T110N, G127S, L133Q, M137I, I142M or I142T, H143R, N146S or N146D, R151H, R154H, P168Q, T194P, R211Q, I218L, Q220H, Q222K and S240P), indicating high variability in this species (Goldmann *et al.*, 1996, 2004; Billinis *et al.*, 2002; Agrimi *et al.*, 2003; Zhang *et al.*, 2004; Kurosaki *et al.*, 2005; Acutis *et al.*, 2006, 2008; Vaccari *et al.*, 2006; Papasavva-Stylianou *et al.*, 2007). The W102G substitution has been found only in combination with a *PrP* variant containing only three instead of the usual five octapeptide repeats (Goldmann *et al.*, 1998). So far 21 of 256 codons (8 %) are polymorphic, generally only about six to 12 are described as polymorphic for a given goat breed. While some PRP polymorphisms are similar in sheep and goats, others like $A_{136}R_{154}R_{171}$ in sheep are specific to each species (Baylis & Goldmann, 2004).

To date, only a few significant association analyses between goat *PrP* gene polymorphisms and susceptibility to TSE agents have been published. When goats were challenged experimentally with different TSE isolates or BSE strains, the heterozygote IM_{142} genotype and the three-repeat/G102 variant were associated with increased incubation periods (Goldmann *et al.*, 1996, 1998). In naturally affected goats, partial protective effects of heterozygote HR_{143} and RH_{154} genotypes have been suggested (Billinis *et al.*, 2002) and a lower susceptibility was demonstrated in QK_{222} heterozygous individuals (Acutis *et al.*, 2006; Vaccari *et al.*, 2006). Additionally, compared with homozygote NN_{146} genotypes, NS_{146}, SS_{146}, ND_{146} and DD_{146} genotypes have been associated with resistance to classical scrapie in Cypriot goats (Papasavva-Stylianou *et al.*, 2007). In all these studies, however, the power of the analyses aiming at identifying associations between resistance/susceptibility to scrapie and *PrP* gene polymorphisms was often impaired by the relatively low number of scrapie cases or control involved.

Currently, in the absence of clearly identified polymorphisms associated with major TSE resistance in goats, genetic selection as an approach to control scrapie in affected herds and the general goat population is not foreseen.

In this study our objectives were the following: (i) to describe the polymorphisms at the *PrP* locus in French Alpine and Saanen breeds, which represent more than 90 % of the goat population in France and (ii) to investigate associations between *PrP* gene polymorphisms and scrapie susceptibility through a case–control study in a high prevalence scrapie herd.

METHODS

***PrP* gene sampling in French Alpine and Saanen goats.** In France, the selection schemes for the Alpine and Saanen breeds rely on two open nuclei totalling 274 000 goats in 2500 herds genetically connected via 80 000 artificial inseminations (AI) per year, which allows on-farm AI progeny-testing for production and functional traits of 70 young bucks per year (Barillet, 2007). The ORF of the *PrP* gene was sequenced in 404 AI Alpine (n=220) and Saanen (n=184) bucks born between 1998 and 2005, representing 82 % of all the AI buck progeny tested in France between 1999 and 2006.

Scrapie-affected herd. A scrapie endemic goat herd (n=409), composed of Alpine, Saanen and Alpine × Saanen individuals born between 1992 and 2001, was followed-up from 2000. Stamping out of this herd was decided by Animal Health Authorities in 2003. During this period, the eliminated animals (either at the end of their economic life or at stamping out) were sampled for (i) blood and (ii) various tissues (spleen, ileum, mesenteric lymph node, tonsil and obex).

For each animal, PrP^{Sc} detection was carried out on all samples using immunohistochemistry. Immunohistochemistry was carried out as already described previously (Andréoletti *et al.*, 2002). An animal was considered scrapie positive when at least one tissue was found positive. A total of 90 scrapie-positive cases, including 20 clinical cases, were identified.

A case–control study was designed within this herd, based on about two PrP^{Sc}-negative goats (n=174) per one scrapie case (n=90). The 264 goats included in the case–control groups were Alpine (16 %), Saanen (5 %) and Alpine × Saanen (79 %) individuals. To ensure a similar exposure length and account for the possible effect of age, scrapie cases and controls were chosen within the same birth cohorts, with the constraint moreover that they were born in the herd. Breed, birth date and herd of birth information was extracted from the milk recording national database.

***PrP* gene sequencing.** Genomic DNA was isolated from blood by using the alkaline-lysis method. Goat PRP ORF was PCR amplified with primers designed from the published goat PrP sequence (GenBank accession no. X91999): PCR fragments were amplified with the forward primer PRP1: 5′-ATTTTGCAGAGAAGTCATCATGGTGA-3′ and the reverse primer PRP2: 5′-AACAGGAAGGTTGCCCCTATCCTA-3′. PCR amplifications were performed in a Qiagen Master Mix Multiplex PCR. Reactions were set up in a 70 µl reaction volume, containing 200 ng genomic DNA, 1 × Qiagen Master Mix, 0.2 µM each primer. Amplification was performed in a DNA thermal cycler PTC200 (BioRad) with heat steps 15 min at 95 °C, two cycles of 45 s at 94 °C, 30 s at 66 °C, 60 s at 72 °C, two cycles of 45 s at 94 °C, 30 s at 63 °C, 60 s at 72 °C, 26 cycles of 45 s at 94 °C, 30 s at 57 °C, 60 s at 72 °C and an elongation step of 15 min at 72 °C.

Both strands of the PCR fragments were sequenced with forward and reverse primers by using a 3730XL sequencer (Applied Biosystems). Sequences were analysed using the Variant Reporter software (Applied Biosystems).

Haplotype determination. The presence of double-heterozygous loci in part of the population precluded the identification of the allelic phase by direct sequence analysis. Given the frequencies of allelic variants of the codons included in the analysis and under the assumption of the Hardy–Weinberg equilibrium, maximum-likelihood estimates of haplotypes were therefore obtained using the expectation-maximization (EM) method (Excoffier & Slatkin, 1995). These haplotype frequencies

were estimated by using the Arlequin software version 3 when the gametic phase was not known (Excoffier *et al.*, 2006).

Computed haplotypes were then checked by direct cloning and sequencing of the PRP ORF amplified from goat harbouring the mutations. DNA extract from 35 animals carrying identified mutations was amplified using the forward 5′-TCAGCCCCA-TGGTGGTGGCT-3′ and reverse 5′-CTGCAGGTAGACACTCCC-TCC-3′ primers (annealing, 61 °C, 40 cycles). PCR fragments were cloned in the TOPO TA Cloning vector kit (reference K4560-01; Invitrogen), before transformation of competent *Escherichia coli* (DH5α). After bacterial growth and isolation, plasmids were isolated from 10 individual colonies and PCR amplified before sequencing (M13 forward and M13 reverse primers).

Statistical analysis. The frequencies of the allelic variants or haplotypes observed for different populations (breeds or case–control groups) were compared using χ^2 tests or Fisher's exact tests when needed.

Association analyses based on relevant *PrP* genotypes according to haplotypes were carried out using logistic regression models fitted to the scrapie status of the goats in the affected herd, with *PrP* genotypes as the main covariate (SAS version 9.1; SAS Institute, 1999). To further account for the potential effect of age, the models were also adjusted for three classes of birth cohorts (1993–1995, 1996–1998 and 1999–2001). When there were no positive scrapie cases in some genotypes the Fisher's exact test was used instead.

RESULTS

Goat *PrP* gene ORF polymorphisms in French Alpine and Saanen breeds

Eight single nucleotide polymorphisms were observed in the *PrP* gene ORF of the 404 Saanen and Alpine bucks. Two were associated with silent mutations (codons 42 and 138) and six were responsible for amino acid substitutions (G127S, I142M, R154H, R211Q, Q222K and S240P) (Table 1). These amino acid substitutions were little represented in our data (1.1–18.5 %) except for codon 240 (49 %). All amino acid substitutions were observed in both French Saanen and Alpine breeds, but, apart from position 222, significant differences in allelic variant frequencies were evidenced between the two breeds (Table 1). S127 and H154 variant frequencies were very low in the French Saanen breed (1.1 and 0.5 %, respectively) compared with the French Alpine breed (9.8 and 5.4 %, respectively). Conversely, M142 and Q211 variant frequencies were about two times higher in the Saanen breed (8.7 and 18.5 %, respectively) than in the Alpine breed (3.9 and 7.1 %, respectively).

To compare the allelic frequencies of the 264 goats included in the case–control study to those of the Alpine and Saanen bucks, a theoretical herd was simulated with the same breeding structure as the affected herd (16 % Alpine, 5 % Saanen and 79 % Alpine × Saanen crosses) accounting for the allelic frequencies of the reference Alpine and Saanen bucks (Table 1) or frequency averages of both Alpine and Saanen bucks, respectively, for Alpine, Saanen and crossed animals of the simulated herd. Except for codon 127, no difference was evidenced between the observed and simulated frequencies of allelic variants (Supplementary Table S1 available in JGV Online). Such results suggest that the 264 goats of the affected herd were representative of the *PrP* genetic structure described in the French Alpine and Saanen breeds.

Table 1. Frequencies of allelic variants in 220 French Alpine and 184 Saanen AI bucks

Codon	Variant (allelic frequency)	Frequencies of allelic variants		χ^2	*P*-value
		Alpine bucks	Saanen bucks		
42*	a (0.518)	232 (55.2 %)	169 (47.7 %)	4.32	0.0375
	g (0.482)	188 (44.8 %)	185 (52.3 %)		
127†	G (0.942)	397 (90.2 %)	364 (98.9 %)	27.60	<0.0001
	S (0.058)	43 (9.8 %)	4 (1.1 %)		
138*	c (0.506)	242 (55.0 %)	167 (45.4 %)	7.42	0.0065
	t (0.494)	198 (45.0 %)	201 (54.6 %)		
142†	I (0.939)	423 (96.1 %)	336 (91.3 %)	8.21	0.0042
	M (0.061)	17 (3.9 %)	32 (8.7 %)		
154†	R (0.968)	416 (94.6 %)	366 (99.5 %)	15.52	<0.0001
	H (0.032)	24 (5.4 %)	2 (0.5 %)		
211†	R (0.878)	409 (92.9 %)	300 (81.5 %)	24.36	<0.0001
	Q (0.122)	31 (7.1 %)	68 (18.5 %)		
222†	Q (0.937)	407 (92.5 %)	350 (95.1 %)	2.3	0.1289
	K (0.063)	33 (7.5 %)	18 (4.9 %)		
240†	S (0.505)	242 (55.0 %)	166 (45.1 %)	7.84	0.0051
	P (0.495)	198 (45.0 %)	202 (54.9 %)		

*Silent nucleotide mutation.
†Amino acid polymorphism.

Association of codon polymorphisms with scrapie susceptibility

Association analyses based on the case–control groups in the affected herd showed that H154, Q211 and K222 mutations were present at significant lower frequencies in PrP^{Sc}-positive goats (Table 2). Conversely, no association was observed between polymorphisms at codons 127, 142 and 240 of the *PrP* gene and the goats' scrapie status.

Haplotypes of the goat *PrP* gene at codons 127, 142, 154, 211, 222 and 240

Results from the maximum-likelihood estimation showed that only seven haplotypes were possible in our population. The $G_{127}I_{142}R_{154}R_{211}Q_{222}S_{240}$ (subsequently noted GIRRQS) allele appeared as the wild-type allele (Table 3). Four mutated alleles were derived from the GIRRQS allele by a single-codon mutation ($GIH_{154}RQS$, $GIRQ_{211}QS$, $GIRRK_{222}S$ and $GIRRQP_{240}$). The last two alleles ($S_{127}IRRQP_{240}$ and $GM_{142}RRQP_{240}$) simultaneously resulted from two mutations S127 and P240, or M142 and P240, showing a complete linkage disequilibrium at these codons. Full *PrP* ORF from cloned goats harbouring double-heterozygote loci confirmed these haplotypes.

As expected from the single nucleotide polymorphism frequencies, mutated alleles, except the $GIRRQP_{240}$ haplotype, remained little represented in our reference population of AI bucks, with frequencies ranging from 0.039 to 0.098 in the Alpine breed, and from 0.005 to 0.185 in the Saanen breed (Table 3). Comparison of haplotype frequencies between French Alpine and Saanen breeds indicated significant differences ($\chi^2_{06}=95.62$; $P<10^{-3}$): the frequencies of the GIRRQS, $S_{127}IRRQP_{240}$ and $GIH_{154}RQS$ alleles were higher in the Alpine breed than in the Saanen breed ($P<10^{-3}$ in all cases). Conversely, $GM_{142}RRQP_{240}$ and $GIRQ_{211}QS$ alleles were observed less in the Alpine breed than in the Saanen breed ($P=0.0042$ and $P<10^{-3}$, respectively).

Association of *PrP* genotypes with scrapie susceptibility

Association analysis between *PrP* genotypes and scrapie susceptibility was first performed considering the genotypes at the three codons 154, 211 and 222 that were found to have a significant effect on scrapie status in the preliminary single polymorphism analyses. It confirmed that RH_{154}, RQ_{211} and QK_{222} heterozygous animals were significantly at lower risk of being infected (results not shown).

However, the S127 and M142 mutations were always associated with the P240 mutation (Table 3). These complete linkage disequilibriums could have biased the estimates of the influence of single polymorphisms. Moreover, the M142 mutation has already been described to influence the TSE incubation period in goats (Goldmann *et al.*, 1996). Thus in order to properly assess the effect of the double S127 P240 and M142 P240 mutations, an analysis was performed considering genotypes at codons 127, 142, 154, 211, 222 and 240. No effect of the G127S dimorphism was observed (results not shown) and therefore only the results based on genotypes at codons 142, 154, 211, 222 and 240 are presented in Table 4. Six haplotypes and 16 genotypes were observed in the

Table 2. Frequencies of allelic variants in scrapie-affected ($n=90$) and control goats ($n=174$) included in the case–control study

Codon	Variant (allelic frequency)	Frequencies of allelic variants		*P*-value Fisher's exact test
		Scrapie goats	Control goats	
42*	a (0.517)	88 (48.9 %)	185 (53.2 %)	0.3598
	g (0.483)	92 (51.1 %)	163 (46.8 %)	
127†	G (0.890)	162 (90.0 %)	308 (88.5 %)	0.6614
	S (0.110)	18 (10.0 %)	40 (11.5 %)	
138*	c (0.500)	85 (47.2 %)	179 (51.4 %)	0.4087
	t (0.500)	95 (52.8 %)	169 (48.6 %)	
142†	I (0.947)	173 (96.1 %)	327 (94.0 %)	0.4127
	M (0.053)	7 (3.9 %)	21 (6.0 %)	
154†	R (0.968)	180 (100 %)	331 (95.1 %)	0.0011
	H (0.032)	0 (0.0 %)	17 (4.9 %)	
211†	R (0.896)	173 (96.1 %)	300 (86.2 %)	0.0002
	Q (0.104)	7 (3.9 %)	48 (13.8 %)	
222†	Q (0.922)	177 (98.3 %)	310 (89.1 %)	0.0005
	K (0.078)	3 (1.7 %)	38 (10.9 %)	
240†	S (0.496)	99 (55.0 %)	181 (52.0 %)	0.1419
	P (0.504)	81 (45.0 %)	167 (48.0 %)	

*Silent nucleotide mutation.
†Amino acid polymorphism.

Table 3. Haplotype frequencies for the goat *PrP* gene at codons 127, 142, 154, 211, 222 and 240 in 404 AI bucks and 264 goats from the scrapie-affected herd

Amino acid position							Haplotype frequency		
Haplotype	127	142	154	211	222	240	Alpine bucks	Saanen bucks	Affected herd
1	G	I	R	R	Q	S	0.350	0.212	0.282
2	–	–	–	–	–	P	0.314	0.451	0.341
3	S	–	–	–	–	P	0.098	0.011	0.110
4	–	M	–	–	–	P	0.039	0.087	0.053
5	–	–	H	–	–	–	0.054	0.005	0.032
6	–	–	–	Q	–	–	0.071	0.185	0.104
7	–	–	–	–	K	–	0.074	0.049	0.078

affected herd. The six haplotypes corresponded to the wild-type $I_{142}R_{154}R_{211}Q_{222}S_{240}$ allele or IRRQS allele and to five mutated alleles, i.e. $IRRQP_{240}$, $M_{142}RRQP_{240}$, $IH_{154}RQS$, $IRQ_{211}QS$ and $IRRK_{222}S$. The IRRQS/IRRQS genotype was first considered as the baseline in the analysis. No significant differences were found between the IRRQS/IRRQS, $IRRQP_{240}$/IRRQS and $IRRQP_{240}$/$IRRQP_{240}$ genotypes (results not shown).

We then considered the IRRQS/IRRQS, $IRRQP_{240}$/IRRQS and $IRRQP_{240}$/$IRRQP_{240}$ genotypes together as the baseline to reduce the confidence intervals (CI) of the estimated odds ratio (OR) (Table 4), given the frequencies of the IRRQS and $IRRQP_{240}$ haplotypes in the affected herd (0.282 and 0.451, respectively). Four of the 16 genotypes ($IH_{154}RQS$/$IRRK_{222}S$, $IRQ_{211}QS$/$IRQ_{211}QS$, $M_{142}RRQP_{240}$/$IH_{154}RQS$ and $M_{142}RRQP_{240}$/$IRRK_{222}S$) were excluded from the analysis because of insufficient data (1, 2, 1 and 1 goats, respectively). The OR of scrapie-positive cases in $M_{142}RRQP_{240}$/$IRRQP_{240}$ heterozygous goats was significantly four times lower compared with the baseline group (OR=0.243, P=0.0186), while no difference was shown for the $M_{142}RRQP_{240}$/IRRQS heterozygous goats. The small number of animals carrying this genotype (n=9) may, however, reduce the power in the analysis. Considering mutation Q211, the OR in $IRQ_{211}QS$/IRRQS and $IRQ_{211}QS$/$IRRQP_{240}$ heterozygous goats were significantly decreased, respectively, by five and 10 times compared with the baseline group (OR=0.190, P=0.0126; and OR=0.102, P=0.0004, respectively), without any difference between the two heterozygote QR_{211} genotypes (P=0.4849). Further, for mutation K222, the OR in $IRRK_{222}S$/IRRQS and $IRRK_{222}S$/$IRRQP_{240}$ goats were 17 and 24 times lower,

Table 4. Association between PrP genotypes at codons 142, 154, 211, 222 and 240 and individual scrapie infectious status, with reference to IRRQS/IRRQS, $IRRQP_{240}$/IRRQS and $IRRQP_{240}$/$IRRQP_{240}$ genotypes, in the affected herd

PrP genotype	No. positive/no. genotyped (%)	OR (CI 95 %)*	*P*-value
$IH_{154}RQS$†	0/15 (0 %)	ND‡	<0.0001
$IH_{154}RQS/IRRK_{222}S$	0/1	Insufficient data	
$IRQ_{211}QS/IRQ_{211}QS$	0/2	Insufficient data	
$IRQ_{211}QS/IRRK_{222}S$	1/6 (16.7 %)	0.158 (0.018–1.414)	0.0989
$IRQ_{211}QS/IRRQP_{240}$	3/28 (10.7 %)	0.102 (0.029–0.358)	0.0004
$IRQ_{211}QS$/IRRQS	3/17 (17.6 %)	0.190 (0.052–0.701)	0.0126
$IRRK_{222}S/IRRQP_{240}$	1/19 (5.3 %)	0.041 (0.005–0.319)	0.0023
$IRRK_{222}S$/IRRQS	1/14 (7.1 %)	0.058 (0.007–0.461)	0.0071
$M_{142}RRQP_{240}/IH_{154}RQS$	0/1	Insufficient data	
$M_{142}RRQP_{240}/IRRK_{222}S$	0/1	Insufficient data	
$M_{142}RRQP_{240}/IRRQP_{240}$	4/17 (23.5 %)	0.243 (0.075–0.790)	0.0186
$M_{142}RRQP_{240}$/IRRQS	3/9 (33.3 %)	0.363 (0.086–1.529)	0.1672
$IRRQP_{240}$/$IRRQP_{240}$ and $IRRQP_{240}$/IRRQS and IRRQS/IRRQS	74/134 (55.2 %)	1.000	–

*OR (95 % CI): IRRQS/IRRQS, $IRRQP_{240}$/IRRQS and $IRRQP_{240}$/$IRRQP_{240}$ genotypes used as the baseline.

†The $IH_{154}RQS$ group includes $IH_{154}RQS/IRRQP_{240}$ (n=8) and $IH_{154}RQS$/IRRQS (n=7) genotypes.

‡For genotypes with sufficient data but without any PrP^{Sc}-positive case, OR could not be determined (ND) and the comparison was performed using the Fisher's exact test.

respectively, compared with the baseline (OR=0.058, P=0.0071; and OR=0.041, P=0.0023, respectively), without a significant difference between the two KQ_{222} heterozygous genotypes (P=0.8133). Thus, gathering together $IRQ_{211}QS$/IRRQS and $IRQ_{211}QS$/$IRRQP_{240}$ goats also yielded a reduced OR (OR=0.133, 95 % CI: 0.052–0.338, P<0.0001). Similarly, the OR of PrP^{Sc}-positive cases in $IRRK_{222}S$/IRRQS and $IRRK_{222}S$/$IRRQP_{240}$ goats considered together was significantly lower (OR=0.048, 95 % C: 0.011–0.211, P<0.0001). Thus the OR of scrapie cases in QR_{211} and KQ_{222} heterozygous goats were 8 and 20 times lower, respectively, than in the baseline genotypes.

No PrP^{Sc}-positive case was found in the 15 goats carrying the IH_{154}RQS/IRRQS or IH_{154}RQS/$IRRQP_{240}$ genotypes (Table 4). In these groups, the prevalence of scrapie was significantly lower than in the baseline group (Fisher's exact test, P<0.0001). Finally, double $IRQ_{211}QS$/$IRRK_{222}S$ heterozygous goats were too few (six goats) to find a significant difference with the baseline (Table 4).

DISCUSSION

PrP gene polymorphisms and haplotypes in French Alpine and Saanen breeds

Alpine and Saanen breeds are the two most important dairy goat breeds in France. They compose about 90 % of the national goat livestock (about 1 million goats). The Alpine breed originated from the French and Swiss Alps, and the Saanen breed was imported from the Saanen valley in Switzerland. They have now been bred everywhere in France and efficiently selected for milk production traits for the past 50 years. Gene migration has occurred between these two populations due to the importance of cross-breeding in France. Thus we now call them French Alpine and Saanen breeds.

The 404 AI Alpine or Saanen bucks born between 1998 and 2005 represented 82 % of all the AI buck progeny tested in France between 1999 and 2006. They were born from 98 sires and 200 maternal grandsires, and were representative of the French Alpine and Saanen breeds: indeed half of the goats of the Alpine and Saanen herd books are born from AI bucks and natural mating bucks are always sons of AI sires in the herd books (149 000 and 125 000 goats recorded each year in Alpine and Saanen breeds, respectively). Moreover, in commercial herds, natural mating bucks are also often sons of AI bucks. Thus the 404 AI Alpine or Saanen bucks were a representative sample of these two French breeds. Eight of the 30 polymorphisms of the *PrP* gene described so far in goats were observed in the French Alpine and Saanen breeds, of which six were amino acid substitutions already described in the literature. It is usual to observe only a part of the global goat species polymorphisms of the *PrP* gene when looking at a given breed: for instance, results in the Italian Saanen breed (Acutis *et al.* 2008) also showed six polymorphic codons (110, 127, 142, 211, 220 and 240), of which five are common with the French Saanen breed (127, 142, 211, 222 and 240), and two are observed either only in Italian (codon 110) or French Saanen (codon 154) with very low allelic frequencies (0.007 and 0.005, respectively). These Italian and French Saanen results are in fact very similar, remembering that the Saanen breed was imported from Switzerland with possible initial founder effects combined with crossbreeding effects with local goat breeds.

Based on the EM algorithm and direct cloning and sequencing, only seven haplotypes were found when considering codons 127, 142, 154, 211, 222 and 240 (Table 3). Two main *PrP* alleles (GIRRQS and GIRRQP) were observed in French Alpine and Saanen breeds, differing by the presence of serine (S) or proline (P) at codon 240. According to our results, the $G_{127}I_{142}R_{154}R_{211}Q_{222}S_{240}$ haplotype corresponds to the wild-type allele, in agreement with the fact that the S_{240} allele is homologous to the wild-type allele of the ovine *PrP* gene (Baylis & Goldmann, 2004). The six other alleles are derived either by a single-codon mutation ($GIH_{154}RQS$, $GIRQ_{211}QS$, $GIRRK_{222}S$ or $GIRRQP_{240}$), or by a double-codon mutation ($S_{127}IRRQP_{240}$ and $GM_{142}RRQP_{240}$) due to a complete linkage disequilibrium at codons 127 and 240 or at codons 142 and 240. As in the Italian Valdostana breed (Acutis *et al.*, 2008), the wild-type GIRRQS allele is the most frequent in the French Alpine breed (frequency 0.350). Conversely, in the French Saanen breed, the GIRRQP allele is mostly present (frequency 0.451) as also described in other Italian (Vaccari *et al.*, 2006; Acutis *et al.*, 2008) and Greek goats (Billinis *et al.*, 2002).

Finally our specific results relate to the estimated frequencies of the seven alleles at codons 127, 142, 154, 211, 222 and 240 for the so-called French Alpine and Saanen breeds. Comparable trends were observed for the two main alleles (GIRRQS and GIRRQP) considered together (frequencies of 0.664 and 0.663, respectively). The five other mutated alleles were present at low to very low frequencies (between 0.185 and 0.005) with significant differences between the two breeds (Table 3). So low haplotype frequencies are often observed when exploring *PrP* gene goat polymorphism, so that a representative sample of at least 100 animals is recommended to accurately estimate allele frequencies of the *PrP* gene for a given goat breed.

PrP gene polymorphisms and scrapie susceptibility

A case–control study accounting for the age of animals was implemented in a highly affected herd. We first checked the absence of association between scrapie susceptibility and mutations at codon S127. Consequently we focused only on codons 142, 154, 211, 222 and 240. Mutations at codon M142 in MI_{142} heterozygous animals showed a protective effect only in goats that were also PP_{240} homozygous at codon 240 ($M_{142}RRQP_{240}$/$IRRQP_{240}$ goats; OR=0.243, P=0.0186). No other effect of the S240P dimorphism

was observed. Compared with IRRQS/IRRQS, $IRRQP_{240}$/IRRQS and $IRRQP_{240}$/$IRRQP_{240}$ genotypes as the baseline, the OR of scrapie cases in QR_{211} and KQ_{222} heterozygous animals were significantly lower (OR=0.133, $P<0.0001$; and OR=0.0484, $P<0.0001$, respectively) whatever the genotype at codon 240. These results support and reinforce previous results regarding the favourable influence of mutations at codon M142 on incubation length (Goldmann *et al.*, 1996) and of mutations at codon K222 on resistance towards classical scrapie (Acutis *et al.*, 2006; Vaccari *et al.*, 2006). On the contrary this is the first demonstration of a lower susceptibility for the QR_{211} heterozygous goats.

Comparisons between the $IRRK_{222}$ allele in goats and the $A_{136}R_{154}Q_{171}$ allele in sheep can be made on the basis of our results. In sheep, dominance between the resistant ARR allele and the susceptible ARQ is variable, but in most cases, ARR/ARQ heterozygous sheep are quite resistant to classical scrapie strains compared with ARQ/ARQ homozygous ones. In our study the OR of scrapie cases in $IRRK_{222}$/IRRQ heterozygous goats compared with susceptible IRRQ/IRRQ animals was in a similar range (OR=0.048, 95 % CI=0.011–0.211) than the OR in ARR/ARQ heterozygous sheep compared with ARQ/ARQ sheep (i.e. OR between 0.01 and 0.08; Barillet *et al.*, 2002; Tongue *et al.*, 2006; Corbière *et al.*, 2007). But the $IRRK_{222}$/$IRRK_{222}$ homozygous goats were not observed in natural exposure conditions, in contrast to ARR/ARR sheep, due to the low frequency of the $IRRK_{222}$ allele in the French Alpine and Saanen breeds. It is therefore important to study further the influence of Q222K dimorphism in other herds and breeds in natural exposure conditions, and also to implement experimental challenges with different TSE strains, to evaluate whether or not the goat $IRRK_{222}$ allele provides as high a protective effect as the ARR allele in sheep. Such an idea is also supported by the fact that the K mutation is also described in humans at position 219 (homologous to goat codon 222), and is protective against sporadic CJD (Shibuya *et al.*, 1998).

The frequency of scrapie PrP^{Sc}-positive cases in $IH_{154}RQ$/IRRQ heterozygous goats was significantly lower than in the IRRQ/IRRQ baseline group (Fisher's exact test, $P<10^{-4}$). H154R dimorphism is present in both the ovine and goat *PrP* gene. In sheep it has been described to be partially protective towards classical scrapie infection (Baylis *et al.*, 2004; Goldmann, 2008). The H154 mutation is, however, now recognized to be a risk factor for atypical scrapie in sheep (Moum *et al.*, 2005; Arsac *et al.*, 2007). Interestingly, the few atypical goat scrapie cases already reported ($n=3$) were H154-mutated animals (Seuberlich *et al.*, 2007), suggesting that it could also confer a high susceptibility to atypical scrapie in goats.

PrP gene polymorphism frequency impact

In different goat breeds or populations, six mutations associated with a lower risk towards classical scrapie (M142, D146, S146, H154, Q211 and K222) have now been identified but are usually present at low to very low frequencies. The first consequence is and will remain the difficulty or impossibility to assess the susceptibility of homozygote animals (as $IRRK_{222}$/$IRRK_{222}$ goats for instance) through field observational studies in natural exposure conditions. However, analysing the resistance level in MM_{142}, DD_{146}, SS_{146}, HH_{154}, QQ_{211} and KK_{222} homozygous goats appears to be a crucial point for an accurate evaluation of breeding strategies for resistance in goats. Therefore, in association with further epidemiological approaches in other herds, experimental challenges in goats of appropriate genotypes, specially procreated for these experiments, are needed. Such experiments, in progress at the European level (Bossers, 2006), will also permit us to answer the question of scrapie resistance to the BSE strain.

At this stage, preparing possible operating breeding tools to select for resistance towards classical scrapie in French Alpine and Saanen breeds means most likely to consider genotypes at codons 211 and 222 and surely to neglect codon 154. Two reasons justify not considering codon 154: (i) the very low frequency of the H154 mutation, especially in the Saanen breed, and (ii) the doubt that the H154 mutation could also confer a high susceptibility to atypical scrapie in goats as in sheep. At the moment a routine genotyping of all the AI Alpine and Saanen bucks has been implemented in France in the Labogena laboratory, on five codons (142, 154, 211, 222 and 240) using the snapshot technique: this allows inseminating with Q211 or K222 carrier bucks in the few scrapie-affected herds detected each year. Despite the initial low frequencies for Q211 and K222 alleles, an optimized genetic strategy selecting resistant bucks could be implemented if necessary, to minimize the genetic loss on production traits, as already done in France for instance in the Manech red faced dairy sheep breed for the ARR allele (Barillet, 2007).

ACKNOWLEDGEMENTS

Financial support of this work was provided by French national contracts, 31B06134 from GIS PRION and AIP P00297 from INRA, by contracts from the Poitou-Charentes region, 04/RPC-A-103 and 05/RPC-A-13, and by an EU project CT-2006-36353 'Goat BSE'. The authors wish to thank the technical staff of CAPRI-IA, Pascal Boué and Pierre Martin, who provided us with DNA from the AI bucks of the Alpine and Saanen breeds. The authors are indebted to the technical assistance of Myriam Thomas, Isabelle Brémaud and Samuel Martin (AFSSA Niort), as well as Mrs W. Brand-Williams for the language correction.

REFERENCES

Acutis, P. L., Bossers, A., Priem, J., Riina, M. V., Peletto, S., Mazza, M., Casalone, C., Forlini, G., Ru, G. & Caramelli, M. (2006). Identification of prion protein gene polymorphisms in goats from Italian scrapie outbreaks. *J Gen Virol* **87**, 1029–1033.

Acutis, P. L., Colussi, S., Santagada, G., Laurenza, C., Maniaci, M. G., Riina, M. V., Peletto, S., Goldmann, W., Bossers, A. & other authors (2008). Genetic variability of the *PRNP* gene in goat breeds from Northern and Southern Italy. *J Appl Microbiol* **104**, 1782–1789.

Agrimi, U., Conte, M., Morelli, L., Di Bari, M. A., Di Guardo, G., Ligios, C., Antonucci, G., Aufiero, G. M., Pozzato, N. & other authors (2003). Animal transmissible spongiform encephalopathies and genetics. *Vet Res Commun* **27** (*Suppl. 1*), 31–38.

Andréolletti, O., Berthon, P., Levavasseur, E., Marc, D., Lantier, F., Monks, E., Elsen, J. M. & Schelcher, F. (2002). Phenotyping of protein–prion (PrPsc)-accumulating cells in lymphoid and neural tissues of naturally scrapie-affected sheep by double-labeling immunohistochemistry. *J Histochem Cytochem* **50**, 1357–1370.

Arsac, J. N., Andréoletti, O., Bilheude, J. M., Lacroux, C., Benestad, S. L. & Baron, T. (2007). Similar biochemical signatures and prion protein genotypes in atypical scrapie and Nor98 cases, France and Norway. *Emerg Infect Dis* **13**, 58–65.

Barillet, F. (2007). Genetic improvement for dairy production in sheep and goats. *Small Ruminant Research* **70**, 60–75.

Barillet, F., Andréoletti, O., Palhière, I., Aguerre, X., Arranz, J. M., Minery, S., Soulas, C., Belloc, J. P., Briois, M. & other authors (2002). Breeding for scrapie resistance using PrP genotyping in the French dairy sheep breeds. *Proc. 7th WCGALP*, Montpellier, France, Session 13-20.

Baylis, M. & Goldmann, W. (2004). The genetics of scrapie in sheep and goats. *Curr Mol Med* **4**, 385–396.

Baylis, M., Chihota, C., Stevenson, E., Goldmann, W., Smith, A., Sivam, K., Tougue, S. & Gravenor, B. (2004). Risk of scrapie in British sheep of different prion protein genotype. *J Gen Virol* **85**, 2735–2740.

Billinis, C., Panagiotidis, C. H., Psychas, V., Argyroudis, S., Nicolaou, A., Leontides, S., Papadopoulos, O. & Sklaviadis, T. (2002). Prion protein gene polymorphisms in natural goat scrapie. *J Gen Virol* **83**, 713–721.

Bossers, A. (2006). *Proposal of the STREP goat BSE, European Contract FOOD-CT-2006-36353: Proposal for Improvement of Goat TSE Discriminative Diagnosis and Susceptibility Based Assessment of BSE Infectivity in Goat Milk and Meat*, p. 94.

Corbière, F., Barillet, F., Andréoletti, O., Fidelle, F., Laphitz-Bordet, N., Schelcher, F. & Joly, P. (2007). Advanced survival models for risk-factor analysis in scrapie. *J Gen Virol* **88**, 696–705.

Elsen, J. M., Amigues, Y., Schelcher, F., Ducrocq, V., Andréoletti, O., Eychenne, F., Khang, J. V., Poivey, J. P., Lantier, F. & Laplanche, J. L. (1999). Genetic susceptibility and transmission factors in scrapie: detailed analysis of an epidemic in a closed flock of Romanov. *Arch Virol* **144**, 431–445.

Excoffier, L. & Slatkin, M. (1995). Maximum-likelihood estimation of molecular haplotype frequencies in a diploid population. *Mol Biol Evol* **12**, 921–927.

Excoffier, L., Laval, G. & Scheider, S. (2006). *Arlequin version 3.01: an Integrated Software Package for Population Genetics Data Analysis.* Switzerland: University of Berne. http://cmpg.unibe.ch/software/arlequin3/.

Goldmann, W. (2008). PrP genetics in ruminant transmissible spongiform encephalopathies. *Vet Res* **39**, 30.

Goldmann, W., Martin, T., Foster, J., Hugues, S., Smith, G., Hugues, K., Dawson, M. & Hunter, N. (1996). Novel polymorphism in the caprine PrP gene: a codon 142 mutation associated with scrapie incubation period. *J Gen Virol* **77**, 2885–2891.

Goldmann, W., Chong, A., Foster, J. H. & Hunter, N. (1998). The shortest known prion protein gene allele occurs in goats, has only three octapeptide repeats and is non-pathogenic. *J Gen Virol* **79**, 3173–3176.

Goldmann, W., Perucchini, M., Smith, A. & Hunter, H. (2004). Genetic variability of the PrP gene in goat herd in the UK. *Vet Rec* **155**, 177–178.

Hunter, N., Foster, J. D., Goldmann, W., Stear, M. J., Hope, J. & Bostock, C. (1996). Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes. *Arch Virol* **141**, 809–824.

Kurosaki, Y., Ishiguro, N., Horiuchi, M. & Shinagawa, M. (2005). Polymorphisms of caprine PrP gene detected in Japan. *J Vet Med Sci* **67**, 321–323.

Moum, T., Olsaker, I., Hopp, P., Moldal, T., Valheim, M. & Benestad, S. L. (2005). Polymorphisms at codons 141 and 154 in the ovine prion protein gene are associated with scrapie Nor98 cases. *J Gen Virol* **86**, 231–235.

Papasavva-Stylianou, P., Kleanthous, M., Toumazos, P., Mavrikiou, P. & Loucaides, P. (2007). Novel polymorphisms at codons 146 and 151 in the prion protein gene of Cyprus goats, and their association with natural scrapie. *Vet J* **173**, 459–462.

SAS Institute (1999). *SAS OnlineDoc, version 9.1.* Cary, NC: SAS Institute Inc.

Seuberlich, T., Botteron, C., Benestad, S. L., Brünisholz, H., Wyss, R., Kihm, U., Schwermer, H., Friess, M., Nicolier, A. & other authors (2007). Atypical scrapie in a swiss goat and implications for transmissible spongiform encephalopathy surveillance. *J Vet Diagn Invest* **19**, 2–8.

Shibuya, S., Higuchi, J., Shin, R. W., Tateischi, J. & Kitamoto, T. (1998). Codon 219 Lys allele of PRNP is not found in sporadic Creutzfeldt–Jacob disease. *Ann Neurol* **43**, 826–828.

Tongue, S. C., Pfeiffer, D. U., Warner, R., Elliot, H. & Del Rio Vilas, V. (2006). Estimation of the relative risk of developing clinical scrapie: the role of prion protein (PrP) genotype and selection bias. *Vet Rec* **158**, 43–50.

Vaccari, G., Di Bari, M. A., Morelli, L., Nonno, R., Chiappini, B., Antonucci, G., Marcon, S., Esposito, E., Fazzi, P. & other authors (2006). Identification of an allelic variant of the goat PrP gene associated with resistance to scrapie. *J Gen Virol* **87**, 1395–1402.

Zhang, L., Li, N., Fan, B., Fang, M. & Xu, W. (2004). PRNP polymorphisms in Chinese ovine, caprine and bovine breeds. *Anim Genet* **35**, 457–461.