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PrP associated resistance to scrapie in five highly infected goat herds

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Summary

The PrP gene polymorphisms at codons 142 (I/M), 154 (R/H), 211 (R/Q), 222 (Q/K) and 240 (S/P) and their association with susceptibility to classical scrapie infection were investigated in five French goat herds displaying a high disease prevalence (>10%). On the basis of PrP^{Sc} detection in central nervous system and in various lymphoid tissues, 301 out of the 1343 goats were found to be scrapie infected. The statistical analyses indicated that while P_{240} mutation had no direct impact on scrapie infection risk, the H_{154}, Q_{211} and K_{222} mutations were associated with high resistance to scrapie. The M_{142} mutated allele was associated with a limited protection level against the disease.

These results further reinforce the view that, like in sheep, the control and eradication of classical scrapie through the selection of certain PrP alleles could be envisaged in commercial goat population.

Text

In sheep, the susceptibility to Transmissible Spongiform Encephalopathies (TSE) is strongly modulated by polymorphisms of the prion protein (*PrP*) gene and the nature of the prion disease agent (strain) (Baylis & Goldmann, 2004). The $A_{136}R_{154}R_{171}$ allele is associated with a highly protective effect against natural or experimental infection with classical scrapie and BSE agents, while the $V_{136}R_{154}Q_{171}$ or $A_{136}R_{154}Q_{171}$ alleles are associated with susceptibility (Elsen *et al.*, 1999; Hunter, 1997). However, in sheep, the ARR allele does not provide any particular protection against atypical scrapie, whereas the R154H or L141F amino acid substitutions are associated with an increased risk of occurrence of this TSE (Fediaevsky *et al.*, 2010; Goldmann, 2008; Moreno *et al.*, 2007 ; Moum *et al.*, 2005).

At the European level, the selection of the ARR allele carriers was successfully applied for controlling and eradicating classical scrapie in infected sheep flocks (Nodelijk *et al.*, 2011). At the population level, large scale selection programs were also implemented. They aimed at increasing the frequency of ARR allele in the general population making it less favorable for TSE agent circulation and spreading. This 'breeding for resistance policy' in combination with the other eradication measures, resulted in a significant reduction of the classical scrapie prevalence in populations where it was comprehensively applied (Dawson *et al.*, 2008; Fediaevsky *et al.*, 2008; Hagenaars *et al.*, 2010).

In goats, several field studies have identified coding mutations of the PrP gene that are associated with lower risk of developing classical scrapie; namely the I/M_{142}, N/D_{146} and S_{146}, R/Q_{211} and Q/K_{222} (Acutis *et al.*, 2006; Barillet *et al.*, 2009; Bouzalas *et al.*, 2010; Goldmann *et al.*, 1996; Goldmann *et al.*, 2011; Gonzalez *et al.*, 2009; Papasavva-Stylianou *et al.*, 2007; Papasavva-Stylianou *et al.*, 2011; Vaccari *et al.*, 2006).

The development of a PrP genotype selection program is now considered by the EU authorities as a potential tool for the control and eradication of scrapie in the commercial goat population. However, the available data related to PrP polymorphisms associated with TSE resistance in goat are still considered insufficient to recommend a breeding for resistance policy (EFSA, 2009). Indeed, because (i) the low frequency of certain alleles in goat populations and (ii) the relatively limited number of scrapie cases usually involved in certain studies, it still remains difficult to estimate the real level of resistance associated with the different goat PrP genotypes (Acutis *et al.*, 2006; Baylis & Goldmann, 2004; EFSA, 2009; Vaccari *et al.*, 2006).

In this study, five naturally scrapie infected herds (A to E) were selected on the basis of their high infection prevalence (table 1). Index cases were identified through the EU active TSE surveillance system either at the rendering plant or slaughter house. According to the EU regulation (EU999/2001) those TSE affected goat herds were destroyed. In more than 99.4% of the animals (1343 out of 1350), the ileum, the mesenteric lymph node, the tonsil and the posterior brain stem were sampled at the rendering plant. For each sample, PrP^{Sc} detection was carried out by immunohistochemistry using the 8G8 antibody (epitope amino acid sequence 95-108 of the human PrP), as previously described (Lacroux *et al.*, 2007). An animal was considered scrapie positive when at least one of its tissues was found positive. From the 1343 sampled goats a total of 301 scrapie cases were identified (table 1).

The five herds comprised Alpine, Saanen and Alpine/Saanen cross-bred goats. The PrP haplotype diversity in French Saanen and Alpine breeds has already been described, showing that PrP gene coding polymorphisms in this population are restricted to positions 142, 154, 211, 222 and 240. In particular, no polymorphism was reported at codon 146 of the PrP gene in the French goat population (Barillet *et al.*, 2009). Therefore, in this study genotypes were

89 established at codons 142, 154, 211, 222 and 240 by snapshot PCR (Labogena, Jouy en Josas,
90 France) (table 1). In scrapie cases, the *PrP* gene open reading frame (ORF) was sequenced
91 (Barillet *et al.*, 2009). Sequencing confirmed that no coding mutations additional to those
92 observed at codons 142, 154, 211, 222 and 240 were present. Genotyping and sequencing
93 were established using blood samples that had been collected in the herds several weeks
94 before stamping out.
95 In this study, we made the deliberate choice to restrict genetic / scrapie infection risk analysis
96 only to those animals for which traceable and complete information (PrP genotype, scrapie
97 infection status and age) was available. In four out of the five herds (A-D), such information
98 was established in more than 93% of the individuals. In the fifth herd (E), traceable
99 information was only available in 60% of the animals. Finally, 259 scrapie infected goats and
100 868 apparently healthy controls were considered. To check if a potential bias could have been
101 introduced to the analysis by excluding animals with incomplete information, a sensitivity
102 analysis was performed based on multiple imputations (Meng & Rubin, 1992). Assuming that
103 these data were missing at random, unknown information were randomly imputed based on
104 the within flock distributions of age and PrP haplotypes/ genotypes derived from animals with
105 complete data. This was done separately for scrapie positive and negative animals, to take into
106 account the distribution disequilibrium between these two groups. Statistical analyses were
107 performed on 100 imputed datasets as described below. Results were not different from those
108 reported here (not shown). We therefore concluded that no strong bias in the estimation of PrP
109 haplotypes / genotypes effect could be evidenced.
110 Six different haplotypes were identified; four ($IH_{154}RQS$, $IRQ_{211}QS$, $IRRK_{222}S$, $IRRQP_{240}$)
111 derived from the archetype $I_{142}R_{154}R_{211}Q_{222}S_{240}$ (subsequently noted as IRRQS) allele by a
112 single codon mutation, and one ($M_{142}RRQP_{240}$) by a double mutation. Strong differences in
113 allele frequencies were observed between herds and apart from the $IRRQP_{240}$ the PrP mutated

alleles were represented at a low level (Table 1). These results are consistent with those previously reported in the French Saanen and Alpine breeds (Barillet *et al.*, 2009; Vaccari *et al.*, 2009) and support the view that the five studied herds did not display any particularities in their PrP genetic structure.

Associations between PrP haplotypes / genotypes and the scrapie infection status were assessed using logistic regression models with a random "herd" effect to control potential clustering of data (Glimmix macro, SAS 9.2, SAS Institute). In order to consider the potential confounding effect of animals' age, models were adjusted for 3 age groups (younger than 2 years, 2 to 4 years old and older than 4 years). In the absence of scrapie cases, some haplotypes / genotypes could not be included in the mixed logistic model analysis and the Fisher exact test was used.

Scrapie cases were mainly observed in the IRRQS or the $IRRQP_{240}$ haplotypes carriers and using IRRQS as a baseline, the $IRRQP_{240}$ and $M_{142}RRQP_{240}$ alleles were associated with a low to moderate decrease in scrapie infection risk (OR=0.79, p=0.036 and OR=0.47, p=0.003, respectively) (Table 2). Conversely, the H_{154}, Q_{211} and K_{222} mutated alleles were associated with a strong protection against scrapie ($OR<0.1$, $p<10^{-4}$).

Twenty-one different PrP genotypes were observed in the studied goat population (table 3). The $IRRQP_{240}$/IRRQS (n=238, 21.1 %) and $IRRQP_{240}$/$IRRQP_{240}$ (n=182, 16.1 %) genotypes were the most frequent and no difference in scrapie susceptibility was observed between these genotypes and the wild type IRRQS homozygote animals.

All the other genotypes were represented at substantially lower frequencies and for some genotypes, like homozygotes K_{222} and homozygotes $H_{154,}$ the low number of individuals precluded any statistical comparison.

However, still using IRRQS homozygote animals as a baseline, all the K_{222}, H_{154}, Q_{211} heterozygote genotypes (for which sufficient data was available) and the Q211 homozygote animals displayed a strongly reduced risk of scrapie infection. In M_{142} allele carriers, the situation was more complex. While homozygotes M_{142} and heterozygotes $IM_{142}RQP_{240}$/ $IRRQP_{240}$ displayed a reduced risk of scrapie infection, such a risk reduction was not significant in the $IM_{142}RQP_{240}$/IRRQS genotype (p=0.07).

The R/H_{154} PrP gene mutation was also associated with a strong protective effect against classical scrapie infection in heterozygotes animals (table 3). However, the R/H_{154} mutation has been demonstrated in goats, like in sheep, to be associated with higher risk of atypical scrapie occurrence (Colussi *et al.*, 2008). In our opinion, this precludes the use of this allele for genetic selection against TSE in goats.

In this study some scrapie cases, although only few, were identified in heterozygote Q_{211} and K_{222} goats. These findings could suggest a limited protective effect of Q_{211} and K_{222} alleles against scrapie. In sheep, whereas homozygote ARR sheep display a high resistance, some classical scrapie cases have also been reported in heterozygote ARR sheep (Baylis *et al.*, 2004). Therefore, a clear assessment of the level of resistance to scrapie infection in homozygote Q_{211} and K_{222} animals appears to be crucial before 'a breeding for resistance' program to control and eradicate scrapie in goats could be envisaged. However, despite the large number of scrapie infected / total goats involved in this study, the final number of goats with homozygous mutation at codons 211 (n=23) and 222 (n=2) remained too limited to address this point. Considering the low frequencies of such genotypes in goats, it is unlikely that any observational studies might provide sufficient information to answer this question.

In this context, the experimental challenge of Q_{211} and K_{222} homozygote animals with a panel of TSE isolates (different classical scrapie isolates and BSE) is probably the most suitable way to answer this question. Such experimental challenges have already been initiated and the

first results obtained in K_{222} heterozygote goats inoculated with a classical scrapie isolate are consistent with a high level resistance to infection in those animals (Acutis *et al.*, 2012)

In the infected goats, 121 out of the 259 animals displayed PrP^{Sc} accumulation in one or several investigated lymphoid tissues but were negative in the Central nervous system (obex). Conversely to what has been reported in an infected goat herd in the UK (Gonzalez *et al.*, 2009) none of the infected animals showed PrP^{Sc} in theCNS while being negative in the lymphoreticular system (LRS). This discrepancy could be the consequence of the involvement of different scrapie agents (strains) in the studied populations. Alternatively it might reflect the possibility of different dissemination pathways of the TSE agent in infected individuals.
In our infected goat population no significant difference was observed in the proportion of the PrP^{Sc} LRS positive / CNS negative animals between the different age groups of IRRQS and IRRQP homozygotes and IRRQS/IRRQP heterozygote infected animals (Fisher exact test, all p values > 0.3) (supplementary Table 1). This observation supports the view that the S/P_{240} mutation has no strong influence on the kinetics of the scrapie agent's dissemination from peripheral tissues to the CNS.
In the M_{142}, Q_{211} and K_{222} allele carriers, a lower proportion of CNS positive goats (3 out of 16) was observed in animals younger than 48 months than in IRRQS and IRRQP homozygote and IRRQS/IRRQP heterozygous goats (70 out of 130) (Fisher exact test, p=0.015). This result suggests that the expression of the M_{142}, Q_{211} and K_{222} PrP alleles might slow the scrapie agent dissemination in the body of infected animals. While these results are in line with those reported in M_{142} allele carriers goat experimentally infected with BSE (Goldmann *et al.*, 1996), it is our opinion that the number of infected animals with appropriate genotype involved in our study remains too low to draw definitive conclusions on that question.

Therefore experimental oral challenges with scrapie in animals carrying appropriate genotypes is certainly the most suitable approach to definitively clarify the impact of the M_{142}, Q_{211} and K_{222} PrP alleles on scrapie agents' dissemination in the tissues of goats.

As in sheep naturally infected with scrapie (Gomez *et al.*, 2007; Reckzeh *et al.*, 2007), the substantial proportion of LRS positive/ CNS negative infected adult goats that we observed in our study probably has some consequences for the sensitivity of the EU TSE active surveillance program (PrP^{Sc} detection in the obex on a random sample of animals more than 18 months old). We are currently developing a simulation model that takes into account both the characteristics of the French commercial goat population (zootechnic management, demography, etc…) and the sensitivity of the PrP^{Sc} detection in the CNS as a tool for identifying scrapie infected animals to quantitatively estimate the impact of this phenomenon on the efficiency of the scrapie active surveillance program.

Beyond these epidemiological considerations, it should also be noticed that according to the EU regulation 746/2008[1] modifying the regulation 999/2001[2], EU member states can decide to put for human consumption carcasses prepared from animals born and/or raised in scrapie infected flocks providing that they have been tested and are negative for PrP^{Sc} in the CNS. In that perspective, the relatively high proportion of scrapie infected but CNS negative animals that can be found in infected herds is also likely to have consequence in terms of the consumer's dietary exposure to scrapie agents.

Acknowledgements

[1] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:202:0011:0019:EN:PDF
[2] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:2001R0999:20110318:EN:PDF

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Table 1: PrP haplotypes frequencies and scrapie prevalence in five naturally infected goat herds

Herd	Number of goats	Number of positive / sampled	Prevalence (%)	Genotyped goats		PrP allele frequencies (%)					
				scrapie infected	healthy	IRRQS	$M_{142}RRQP_{240}$	$IH_{154}RQS$	$IRQ_{211}QS$	$IRRK_{222}S$	$IRRQP_{240}$
A	290	38 / 290	13.1	38	247	33.4	16.3	0.7	12.8	1.2	35.6
B	247	42 / 245	17.1	39	193	24.6	3.0	1.3	26.1	5.8	39.2
C	162	37 / 162	22.8	32	120	38.2	1.6	14.8	11.5	2.3	31.6
D	208	64 / 208	30.8	60	134	24.2	0.5	2.8	15.2	10.6	46.7
E	443	120 / 438	27.4	90	174	28.2	5.3	3.2	10.4	7.8	45.1
Overall	1350*	301 / 1343	22.4	259	868	29.4	6.3	3.7	15.2	5.5	39.9

*Of these 1350 animals 223 were not fully traceable (PrP genotype, scrapie infection status, age) and therefore not used to assess the effect of the PrP haplotype (Table 2) and PrP genotype (Table 3) on the risk of infection with scrapie.

Table 2: Association between PrP haplotypes and natural scrapie infection risk in goats

PrP Allele	Scrapie infected goats		Healthy goats		OR (95% CI)*	p value
	#	%	#	%		
IRRQS	215	41.5	448	25.8	1	-
$IRRQP_{240}$	266	51.3	634	36.5	0.79 (0.63 – 0.98)	0.036
$M_{142}RRQP_{240}$	21	4.1	121	7.0	0.47 (0.28 – 0.77)	0.003
$IH_{154}RQS$	0	0	83	4.8	ND†	$<10^{-4}$
$IRQ_{211}QS$	13	2.5	330	19.0	0.08 (0.04 - 0.14)	$<10^{-4}$
$IRRK_{222}S$	3	0.6	120	6.9	0.04 (0.01 – 0.11)	$<10^{-4}$

An animal was assigned to an allele group on a dose-effect basis (i.e., 0, 1 or 2 copies of the allele).
*Adjusted odd's ratio from the mixed logistic regression model with age and random herd effects.
† ND: not determined. In the absence of scrapie case, these genotypes could not be included in the mixed logistic model analysis. The Fisher exact test was used instead

Table 3: Association between PrP genotypes and natural scrapie infection risk in goats

PrP genotype	# scrapie positive / # scrapie negative	scrapie infected (%)	Odds ratio* (95 % CI)	p value
IRRQS / IRRQS	45 / 66	40.5	1	
IRRQS / IRRQP$_{240}$	108 / 130	45.4	0.97 [0.60 - 1.56]	0.900
IRRQP$_{240}$ / IRRQP$_{240}$	70 / 112	38.5	0.73 [0.42 - 1.16]	0.169
M$_{142}$RRQP$_{240}$ / IRRQS	11 / 36	23.4	0.47 [0.21 - 1.06]	0.070
M$_{142}$RRQP$_{240}$ / IRRQP$_{240}$	10 / 50	16.7	0.28 [0.13 – 0.63]	0.002
M$_{142}$RRQP$_{240}$ / M$_{142}$RRQP$_{240}$	0 / 9	0.0	ND†	0.029
IH$_{154}$RQS / IRRQS	0 / 18	0.0	ND	0.015
IH$_{154}$RQS / IRRQP$_{240}$	0 / 38	0.0	ND	<10-4
IRQ$_{211}$QS / IRRQS	5 / 95	5.0	0.06 [0.02 - 0.17]	<10-4
IRQ$_{211}$QS / IRRQP$_{240}$	7 / 140	4.8	0.06 [0.02 - 0.13]	<10-4
IRQ$_{211}$QS / IRQ$_{211}$QS	0 / 25	0.0	ND	<10-3
IRRK$_{222}$S / IRRQS	1 / 37	2.6	0.02 [0.00 – 0.16]	<10-3
IRRK$_{222}$S / IRRQP$_{240}$	1 / 52	1.9	0.01 [0.00 – 0.10]	<10-4
M$_{142}$RRQP$_{240}$ / IRQ$_{211}$QS	0 / 13	0.0	ND	0.009
IH$_{154}$RQS / IRQ$_{211}$QS	0 / 10	0.0	ND	0.028
IRQ$_{211}$QS / IRRK$_{222}$S	1 / 22	4.3	0.04 [0.00 – 0.31]	0.002

Because their too low number no statistical comparison was possible in M$_{142}$RRQP / IH$_{154}$RQS (n=3); M$_{142}$RRQP / IRRK$_{222}$S (n=1); IH$_{154}$RQS / IH$_{154}$RQS (n=5); IH$_{154}$RQS / IRRK$_{222}$S (n=4); IRRK$_{222}$S / IRRK$_{222}$S (n=2) genotype goats. No scrapie infected animals was found in animals caarying those genotypes.

*Adjusted odd's ratio from the mixed logistic regression model with age and random herd effects.
†ND: not determined. In the absence of scrapie case, these genotypes could not be included in the mixed logistic model analysis.