Caprine PRNP polymorphisms at codons 171, 211, 222 and 240 in a Greek herd and their association with classical scrapie

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The association between PRNP variation and scrapie incidence was investigated in a highly affected Greek goat herd. Four mutations were identified at codons 171Q/R, 211R/Q, 222Q/K and 240P/S. Lysine at codon 222 was found to be associated with the protection from natural scrapie ($P = 0.0111$). Glutamine at codon 211 was observed in eight animals, all of them being scrapie-negative, indicating a possible protective role of this polymorphism although statistical analysis failed to support it ($P = 0.1074$). A positive association ($P = 0.0457$) between scrapie-affected goats and the wild-type Q 171 R 211 Q 222 S 240 allele is presented for the first time. In addition, a novel R 171 RQS allele, which is identical to the A 136 R 154 R 171 allele that has been associated with resistance to classical scrapie in sheep, was observed in low frequency.

Scrapie is a transmissible spongiform encephalopathy (TSE) that affects sheep and goats. TSEs are characterized by spongiform lesions and deposition of an abnormal isoform (PrPSc) of the normal host-encoded cellular prion protein (PrPC) in the central nervous system (CNS) (Prusiner & De Armond, 1994; Prusiner, 1998). In scrapie, PrPSc also accumulates in lymphoid tissues (van Keulen et al., 1996). Besides the classical type of scrapie, cases of so-called atypical scrapie (Seuberlich et al., 2007) and bovine spongiform encephalopathy (BSE) have also been identified in goats (Eloit et al., 2005; Jeffrey et al., 2006).

Polymorphisms of the prion protein encoding gene (PRNP) are the most important determinants of host susceptibility to TSEs. In sheep, polymorphisms at PRNP codons 136, 154 and 171 strongly modulate the susceptibility to scrapie (Belt et al., 1995; Bossers et al., 1996; Hunter et al., 1996, 1997). In an effort to decrease the prevalence of TSEs in sheep populations, EU member states established eradication strategies based on the selection of the resistant A136R154R171 allele as an alternative approach to stamping-out policies (EU Decision, 2003). Similar to sheep, the goat PRNP displays high polymorphic variation. So far, 29 aa substitutions are described for domesticated European and Asian goat breeds (Vaccari et al., 2009) among which the following have been associated with scrapie resistance or susceptibility: I142M, H143R, R154H, N146S or N146D, R211Q and Q222K. In 1996, Goldmann et al. reported that goats encoding M142 showed increased incubation periods after experimental challenge with different scrapie strains and BSE. Studies on scrapie in goat herds from Greece and Cyprus suggested that alleles encoding R143, S146, D146 and H154 may offer protection against the disease (Billinis et al., 2002; Papasavva-Stylianou et al., 2007). In Italian scrapie-affect ed goat herds, K222 was strongly associated with resistance to preclinical and clinical scrapie (Vaccari et al., 2006; Acutis et al., 2006). More recent data from infected French Alpine and Saanen breeds revealed that natural scrapie infection was less frequent in animals with alleles encoding H154,
Q211 and K222 (Barillet et al., 2009). Despite these association studies and due to the lack of experimental data, a clear genetic determinant for scrapie resistance in goats has not yet been identified.

The Greek dairy goat population is approximately 5.4 million accounting for 30.1% of the European population (FAO, 2007). The majority of them belongs to the indigenous Skopelos and Hellenic breeds with the remainder being primarily crosses of these local goats with foreign milking breeds. As in other Mediterranean countries, it is also common practice in Greece to hold goats and sheep in mixed flocks. Although current efforts of genetic selection for scrapie resistance in sheep are successful, the goat population can provide a substantial reservoir for TSEs. Thus, the implementation of genetic selection programmes for the reduction of scrapie prevalence in goats would be of major importance.

In 2007, a massive outbreak of scrapie occurred in a goat herd in Northern Greece that included 250 animals. In the present study, we investigated the TSE status of 98 selected animals of this holding and the effects of breeds and PRNP polymorphisms to identify candidate PRNP haplotypes and genotypes for resistance to classical scrapie in goats.

Initially, three goats of this flock exhibited clinical signs indicative of scrapie and finally died. They were confirmed by the Greek reference laboratory as being affected by classical scrapie. Based on further clinical examinations 14 additional animals were classified as suspected classical scrapie. As a consequence the flock was culled. In addition to the 17 clinically suspect animals, sampling material was collected from 81 randomly selected clinically non-suspect adult (age >12 month) goats. These included medulla oblongata, brain and palatine tonsil. Samples from the obex region were examined by the TeSeE screening test (Bio-Rad; hereafter referred to as ELISA) and by a Western blot (WB) procedure as developed by Stack et al. (2002). All available lymphoid tissues along with ELISA-positive brainstem tissues were tested for PrPSc depositions by immunohistochemistry (IHC). An animal was considered to be scrapie-positive when at least one tissue sample was found to be PrPSc-positive by WB or IHC.

Genomic DNA was extracted from frozen brain tissue and the PRNP open reading frame was amplified by PCR and both strands were sequenced. The PRNP haplotypes in genotypes with double heterozygosity were determined by molecular cloning in the pCR2.1 vector (Invitrogen) and sequencing of five individual clones per amplicon. Primer sequences are available upon request. Chi-squared and Fisher’s exact tests (SAS version 9.1; SAS Institute, 1999) were used to assess allele and haplotype frequency differences between scrapie-positive and -negative animals. A generalized linear model was applied to quantify haplotype and genotype effects on scrapie incidence and clinical manifestation, including the effects of breed, age and either genotype or haplotype of the animal. The analyses for scrapie incidence and clinical signs were conducted separately. A confidence threshold of 95% (P≤0.05) was considered to be statistically significant.

In total 27 of the 98 adult goats were found to be scrapie-positive. In particular, 19 goats (11 clinically affected, 8 clinically healthy) that were found to be PrPSc-positive in the obex by ELISA were also confirmed by WB, revealing a profile identical to classical scrapie and clearly distinguishable from BSE based on differential binding of the monoclonal antibodies (mAbs) P4 and 6H4 (Fig. 1). In addition, five animals that scored negative in the ELISA, gave the same positive profile in WB examination. PrPSc depositions were detected by IHC in brains of all ELISA-positive animals, of which fixed nervous tissues were available (n=18) (Fig. 1). PrPSc depositions were also detected in palatine tonsils of three additional animals that scored negative in the obex samples by ELISA and WB (Fig. 1).

Seven different PRNP polymorphisms were identified (Table 1). Three were silent mutations at codons 42 (g→a), 138 (c→t) and 231 (a→c), whereas four resulted in amino acid substitutions at positions 171 (Q→R), 211 (R→Q), 222 (Q→K) and 240 (P→S). Mutations of Q211 and K222 had not been reported before in Greek goats. Interestingly, R171 was detected for the first time in goats. It resulted from a CAG to CGG codon transition and was present only in one scrapie-negative, heterozygous animal. The Q211 and K222 polymorphisms were observed only among scrapie-negative goats. The Fisher’s statistical test revealed a protective role for K222 (P=0.0111) but not for Q211 (P=0.1074). No statistical association between S/P240 and the goats’ scrapie status was observed.

Fourteen animals harboured double heterozygosity and alleles were determined by cloning. In total, five haplotypes were detected (Table 2) giving rise to nine different PRNP genotypes. The allele with the highest frequency (60.99%) encoded Q171R211Q222P240 whereas the predominant genotype was QRQP240/QRQS. The difference in allelic distribution between healthy and positive animals was significant for QRK222S (P=0.0111) and QRQS (P=0.0457) (Table 2). A putative effect of S at codon 240 is also supported by statistical analysis of genotype distributions. QRQS homozygous goats were associated with disease (P=0.0424), whereas QRQP240 homozygous ones were not (P=0.8080). In QRP240/QRQS heterozygous animals, no association to scrapie positivity was supported (P=0.8881). No association could be established for the other genotypes also.

Based on the generalized linear model results, scrapie incidence was significantly affected by the haplotype QRQS (P=0.02), each copy of which increased the scrapie probability by 19.08%. The effect of all other haplotypes and all genotypes on scrapie incidence was non-significant (P>0.05).

Regarding the presence of clinical scrapie, each copy of the QRQS increased their probability by 21.96% (P=0.0111), while a copy of QRQP240 decreased this probability by 19.08% (P=0.0111). A significant protection role for QRQP240/QRQS was identified (P=0.05). No statistical association between Q211 and the goats’ scrapie status was observed.
12.11% (P=0.03). This trait was also significantly influenced (P=0.04) by genotype QRQS/QRQS that had the highest frequency among clinically affected goats.

A significant difference (P=0.0111) was found in scrapie incidence among breeds, with linear model predictions being calculated at 0.20 (SE=0.11), 0.25 (SE=0.15) and 0.32 (SE=0.07) for the Skopelos breed, Hellenic breed and crosses with foreign animals, respectively. This result suggests that among goats in the present study, those of the Skopelos breed are most resistant to scrapie. This could be due to the high prevalence of the QRK222S allele in this breed (10/22 animals had this haplotype). The breed had a non-significant effect on the presence of clinical signs, whereas the age of the animal had a non-significant effect on both, scrapie positivity and the presence of clinical signs (P>0.10).

Our results indicated a massive outbreak of classical scrapie in this Greek pure goat herd. Compared with the results from the ELISA, the WB using mAb P4 revealed the presence of PrPSc in five additional animals. A similar observation was reported recently from the UK, in which the same ELISA missed a large proportion of IHC-positive goats (González et al., 2009). It was speculated by the authors that this finding may be related to the sampling procedure applied. However, in our study the ELISA and the WB analysis sample was derived from the same brainstem homogenate and, therefore, this reflects a higher diagnostic sensitivity of the WB for classical scrapie in goats.

Among the three PRNP silent mutations found in our study, the one at codon 231, has been detected so far, in Chinese breeds only (Zhang et al., 2004), indicating a multi-variable phylogeographical origin in the Greek goat population. The PRNP polymorphisms, M142, R143, S146, D146 and H154, that had been associated with resistance to scrapie in other studies were not identified in our investigation (Goldmann et al., 1996; Billinis et al., 2002; Papasavva-Stylianou et al., 2007). The lack of these polymorphisms may at least partially explain the high prevalence of infection in this herd.

Our results revealed that the change of Q to K at codon 222 had a significant negative effect on scrapie incidence. This is in agreement with studies in other goat breeds from Italy (Acutis et al., 2006; Vaccari et al., 2006) and France (Barillet et al., 2009). A similar effect for R to Q changes at codon 211 has been published recently (Barillet et al., 2009). Our findings indicate a possible protective role for Q211, since all eight animals that carried this polymorphism

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**Fig. 1.** Determination of scrapie-positive animals. (a) Multifocal to coalescing PrPSc deposits in the neuropil of the caudal brainstem of animal 5 and (b) intrafollicular PrPSc deposits in the tonsillar lymphoid tissue of animal 59, which was negative in the CNS examination. (c) Representative comparative results of the ELISA and the discriminatory WB of positive goats indicated by their individual number. PrPSc was detected by mAbs 6H4 and P4, indicated on the left. The WB signal intensities were in agreement with the ELISA absorbance values, except in the case of samples 6, 32 and 75 that were negative in ELISA. NT, Not tested; C, control. The cut-off value for the TeSeE ELISA was at absorbance value 0.219.

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were scrapie-negative. Though the P-value (0.1074) was close to being significant, the effect of Q211 in resistance to scrapie could not be statistically supported probably due to the low frequency (n=8, 4.4%) of animals carrying this polymorphism (Table 1).

A noteworthy result is that haplotype QRQS seems to predispose to classical scrapie (P<0.04) and its clinical manifestation (P<0.001). Conversely, the alteration of this allele from S to P240 appeared to partially protect from clinical disease (P<0.03), but did not affect the resistance to infection (R=0.72). P240 was present at a high frequency in mature sub-clinical goats. Considering that scrapie incidence and clinical manifestation in this herd were not affected by the animal age, we speculate that the low probability of clinical disease in animals with P240 was due to elongation of disease incubation time. This was also supported by statistical analysis of the genotype effect. Previous publications reported contradictory results for the role of P240, indicating either a positive (Acutis et al., 2006) or no association with scrapie (Goldmann et al., 1996; Billinis et al., 2002; Vaccari et al., 2006; Barillet et al., 2009). Considering that the C terminus of PrPC, including amino acid 240 is removed by post-translational cleavage (Stahl et al., 1987), it is not expected that this polymorphism directly affects scrapie susceptibility. This could give a

Table 1. Comparison of the allelic polymorphism frequencies between 27 scrapie-positive and 64 -negative goats in the affected herd

To ensure that scrapie-positive and -negative goats were within the same birth and breed cohorts seven of 71 scrapie-negative animals were removed from statistical analyses data. The nucleotide silent mutations are presented with lower case letters and the amino acid substitutions with upper case letters. Statistical differences that are significant are indicated in bold. NA, Not applied.

<table>
<thead>
<tr>
<th>Codon</th>
<th>Polymorphism (allelic frequency)</th>
<th>Frequencies of allelic variants</th>
<th>P-value of chi-squared test</th>
<th>P-value of Fisher’s exact test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive goats</td>
<td>Negative goats</td>
<td></td>
</tr>
<tr>
<td>42 (126)*</td>
<td>g (0.929)</td>
<td>48 (88.9%)</td>
<td>121 (94.5%)</td>
<td>0.1770</td>
</tr>
<tr>
<td></td>
<td>a (0.077)</td>
<td>6 (11.1%)</td>
<td>7 (5.5%)</td>
<td></td>
</tr>
<tr>
<td>138 (414)*</td>
<td>c (0.885)</td>
<td>47 (87%)</td>
<td>114 (89.1%)</td>
<td>0.6960†</td>
</tr>
<tr>
<td></td>
<td>t (0.115)</td>
<td>7 (13%)</td>
<td>14 (10.9%)</td>
<td></td>
</tr>
<tr>
<td>171</td>
<td>Q (0.995)</td>
<td>54 (100%)</td>
<td>127 (99.2%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R (0.005)</td>
<td>0 (0%)</td>
<td>1 (0.8%)</td>
<td>NA</td>
</tr>
<tr>
<td>211</td>
<td>R (0.956)</td>
<td>54 (100%)</td>
<td>120 (93.8%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Q (0.044)</td>
<td>0 (0%)</td>
<td>8 (6.2%)</td>
<td>0.0603</td>
</tr>
<tr>
<td>222</td>
<td>Q (0.929)</td>
<td>54 (100%)</td>
<td>115 (89.8%)</td>
<td>0.0151</td>
</tr>
<tr>
<td></td>
<td>K (0.071)</td>
<td>0 (0%)</td>
<td>13 (10.2%)</td>
<td></td>
</tr>
<tr>
<td>231 (691)*</td>
<td>a (0.873)</td>
<td>48 (88.9%)</td>
<td>111 (86.7%)</td>
<td>0.6870†</td>
</tr>
<tr>
<td></td>
<td>c (0.126)</td>
<td>6 (11.1%)</td>
<td>17 (13.3%)</td>
<td></td>
</tr>
<tr>
<td>240</td>
<td>S (0.390)</td>
<td>20 (37%)</td>
<td>51 (39.8%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P (0.610)</td>
<td>34 (62%)</td>
<td>77 (60.2%)</td>
<td>0.7229†</td>
</tr>
</tbody>
</table>

*Position of silent nucleotide mutation.
†Indicates the valid P-value for each polymorphic codon.

Table 2. Frequencies of alleles detected in the analysed goats, by breed and association with scrapie incidence

Statistical differences that are significant are indicated in bold. NA, Not applied.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Codon</th>
<th>Frequency per breed</th>
<th>Frequency per case</th>
<th>P-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>171</td>
<td>211</td>
<td>222</td>
<td>240</td>
<td>Skopelos</td>
</tr>
<tr>
<td>1</td>
<td>Q</td>
<td>R</td>
<td>Q</td>
<td>S</td>
</tr>
<tr>
<td>2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>P</td>
</tr>
<tr>
<td>3</td>
<td>R</td>
<td>–</td>
<td>–</td>
<td>S</td>
</tr>
<tr>
<td>4</td>
<td>–</td>
<td>Q</td>
<td>–</td>
<td>S</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>–</td>
<td>K</td>
<td>S</td>
</tr>
</tbody>
</table>

*P-value was estimated primarily by chi-squared test and when this test was not valid, estimation was done by Fisher’s exact test.
reason for the controversial findings related to the role of this codon polymorphisms. It could also be hypothesized that a 240-codon polymorphism linkage with other loci may indirectly affect scrapie susceptibility and disease incubation time. Although S240 is associated with scrapie in the examined goats, all polymorphic goat haplotypes reported so far link this polymorphism with Q211 and K222. However, in our study, the impact of lysine at codon 222 on the QRK222S240 allele was found to be dominant. Interestingly, a novel dimorphism (Q171R) was found in one scrapie-negative 36-months-old goat of the indigenous Hellenic breed. This allele (Q171RQS) was identical to the ovine allele A136R154R171 which provides a high degree of protection to classical scrapie (Hunter et al., 1996, 1997). Unfortunately, due to the low frequency of this polymorphism it was insufficient to infer any statistical significance for its effect. There are indications that this polymorphism is not uncommon among Greek goats. We recently analysed a second scrapie-affected Greek herd consisting of crosses of Hellenic breed with Alpine and Saanen (n = 150), and our results indicated a frequency of approximately 8.6% for the Q171R dimorphism. All of these animals were scrapie-negative. However, the low prevalence of scrapie-positive animals (2%) did not allow for statistical analysis (data not shown).

So far, the high polymorphic variation of goat PRNP gave the opportunity for a number of polymorphisms to be considered as candidates in scrapie eradication programmes. Among them R143, S146 and D146 have been reported once. H154 has been repeatedly reported for its protective role; however, it is considered a risk factor for atypical scrapie (Colussi et al., 2008). On the other hand, three relevant previous studies suggest that, at least in specific goat breeds, K222 and Q211 polymorphisms may influence resistance to classical scrapie (Acutis et al., 2006; Vaccari et al., 2006; Barillet et al., 2009). Our results agree with this assumption, extend it to additional goat breeds and provide the basis for the development of a feasible breeding programme for scrapie eradication that includes candidate alleles for scrapie resistance. Both alleles containing K222 and Q211 polymorphisms are present in goats and absent or rare in sheep. Considering that the degree of PrP sequence similarity affects TSE transmissibility between species (Scott et al., 1989, 1993; Telling et al., 1994) we could hypothesize that selection for K222 and Q211 in goats could provide a possible inter-species transmission barrier between goat and sheep.

The Greek breeds, mainly Skopelos, appeared to be more resistant than their crosses with foreign milking breeds. This can be mainly attributed to the frequencies of polymorphisms at codons 211 and 222. These polymorphisms in Greek breeds are promising candidates that would allow the local population to be included in future scrapie resistance selection schemes. It would be worthwhile to investigate the effect of these polymorphisms on scrapie infection in an experimental setup.

Acknowledgements

The authors are indebted to the excellent technical assistance of Valerie Juillerat and Kostas Efthymiou. We would also like to thank Ms Olga Kanelli for language corrections as well as Dr Georgios Tsousis for the statistical support. I.G.B. thanks the Greek scholarship foundation for economic support. This work was partially financed by research project Pythagoras II 80823 funded by the European Union and the Greek Ministry of Education.

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