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The association between 171 K polymorphism and resistance against scrapie affection in Greek dairy sheep

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ABSTRACT

Polymorphisms at codon 171 of the sheep prion protein gene (*PRNP*) are known to be genetically associated with scrapie (Hunter et al., 1993, 1997). Glutamine (Q) and histidine (H) at codon 171 contribute to scrapie susceptibility, while arginine (R) is associated with resistance. In some breeds, lysine (K) occurs at codon 171 (171 K), but its effect on scrapie resistance has not been fully determined yet. To investigate the role of 171 K in dairy sheep in Greece, we initially analyzed blood samples from 2368 sheep with no clinical signs of the disease, from thirteen scrapie-affected flocks that were then monitored for 5 years for scrapie clinical signs. During this period 177 sheep resulted positive in classical scrapie, after obex testing. The 177 scrapie-affected brain samples *PRNP* polymorphisms were analyzed using a Real Time-PCR protocol while the 2368 sheep blood samples were analyzed by a micro-array method based on primer extension (Homogenous Mass Extend Assay – SEQUENOM, Genaissance Pharmaceuticals) in MALDI-TOF-MS. Single Nucleotide Polymorphisms (SNPs) present at *PRNP* codons 136A/V (Alanine/Valine), 154R/H and 171Q/R/H/K were detected. The ARQ haplotype as well as the ARQ/ARQ genotype predominated. In the studied population, ARK haplotype was found at the highest frequency reported so far (6.98%) whereas, ARK/-genotypes had a total frequency of 12.81%. Relative risk (RR) and the percent relative effect were calculated to assess the association between ARK/-genotypes and scrapie affection. The results showed that carriers of K allele at codon 171 compared to Q and H alleles had *ca.* 0.25 and 0.40 times the risk of scrapie affection, respectively, indicating a possible association of K allele at codon 171 with scrapie resistance.

1. Introduction

PRNP gene encodes the prion protein, a highly conserved 32-kDa glycophosphatidylinositol (GPI)-anchored sialoglycoprotein, which is mainly expressed in brain and the central nervous system, but also occurs in other organs' tissues such as lymphoid organs, lungs, heart, kidneys, gastrointestinal tract, muscles, and mammary glands (Zomosa-Signoret et al., 2008). Prion protein has been associated mainly with prion diseases and nervous degenerative disorders. The basis in the pathogenesis of prion disease is the conversion of normal cellular PrP (PrP^{C}) to PrP^{Sc} (Prusiner, 1982), an alternate isoform characterized by increased beta-sheet content, resistance to proteases and detergent insolubility (McKinley et al., 1983). In farm animals, the best known forms of the disease are scrapie in sheep and goats and bovine spongiform encephalopathy in cattle. The incubation period of the disease is long and the transmission may be either horizontal or vertical (Goldmann et al., 1990). The transmission and the incubation period of the disease depend on the exposure to the infectious agent, the scrapie strain and the genetic background of the host (O'Rourke et al., 1997; Houston et al., 2015).

According to Meydan et al. (2013) more than 40 amino acid polymorphisms have been described in the ovine PRNP open-reading frame but only the polymorphisms at codons 136, 154 and 171 are known to be closely linked to resistance or susceptibility to natural and experimental scrapie affection (Clouscard et al., 1995; Hunter et al., 1996; Dawson et al., 1998; Elsen et al., 1999; Thorgeirsdottir et al., 1999;

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Tranulis, 2002; Houston et al., 2015). The three polymorphic positions encode nine different haplotypes; ARR, AHR, ARH, AHQ, ARQ, AHH, VRQ, VRH and ARK. The ARR/ARR genotype according to numerous studies is correlated with resistance to scrapie (Baylis et al., 2002; Hunter et al., 1994; Goldmann et al., 1994) while the presence of VRQ haplotype is strictly associated with susceptibility both in homozygosis and in heterozygosis (Belt et al., 1995; Hunter et al., 1996). The other haplotypes may modify the degree of resistance or susceptibility to scrapie depending on the sheep breeds. For example, the haplotype AHQ may be associated with increased resistance and incubation time in some breeds such as Icelandic sheep breeds, Texel, Galway, Belclare and Cheviot (Dawson et al., 1998; Elsen et al., 1999; Hunter et al., 1996; O' Doherty et al., 2002; Thorgeirsdottir et al., 1999), while it is associated with high susceptibility in purebred and crossbred German Merinoland sheep (Luhken et al., 2004), as well as, in Romanov breed (Diaz et al., 2005). In Greece, with a dairy sheep population of about 9 million, there is no much information available about PrP alleles' distribution in sheep. According to Leontides et al. (2000), scrapie was firstly diagnosed in 1986; later Billinis et al. (2004) reported some polymorphisms in healthy and scrapie-affected sheep and Ekateriniadou et al. (2007a) described the alleles and genotype frequencies of healthy sheep from 13 rare breeds, as well as, from healthy and scrapie-affected sheep during 2003–2005 (Ekateriniadou et al., 2007b). In the later study, histidine in codon 154 was found at a significantly high frequency in the Chios crossbred scrapie-affected sheep population, suggesting that probably: (a) this allele is associated with increased susceptibility, at least in Chios breed, (b) there is a local scrapie strain strongly correlated with histidine in codon 154 or (c) there is a combination of the allele susceptibility and the scrapie strain tropism (Ekateriniadou et al., 2007b). The ARQ haplotype's susceptibility varies from sheep breed, while the ARH and TRQ haplotypes seem to be rather neutral (Billinis et al., 2004; Dawson et al., 2003). Little is known about ARK haplotype and it has not been clearly associated with scrapie resistance or susceptibility yet (Acutis et al., 2004; Alexander et al., 2005; Guo et al., 2003; Pongolini et al., 2009). According to Greenlee et al. (2012), while a single K allele did not confer resistance to scrapie, it did significantly increase the incubation period to the onset of clinical signs. According to Acutis et al. (2006), ARK allele does not confer full resistance against scrapie and needs to be studied further before it can be considered for breeding purposes.

The aim of the present work was to study the polymorphisms which are related with lysine at codon 171 and their relationship with scrapie occurrence. Thus, 13 scrapie-affected Greek crossbred sheep flocks have been under a 5-year monitoring program in order to determine the association of the 171 K allele with clinical scrapie. SNPs present at codons 136, 154 and 171 of the PrP gene were detected in 2368 blood samples with no signs of the disease and in 177 obex samples of sheep that have been confirmed as scrapie-affected by the Greek TSEs Reference Laboratory.

2. Materials and methods

In total, 2368 sheep blood samples have been analyzed originating from thirteen scrapie-affected flocks from five different counties of Greece.

Blood sampling was performed from the jugular vein of each individual sheep using Vacutainer needles and blood collection tubes (containing K_2EDTA) within the first three months of the study in the framework of the Greek TSEs Surveillance and Control Programme. The above 2368 sheep have been monitored for five years. During these five years, according to the Greek National Surveillance Program, all slaughtered sheep have been examined for scrapie and 177 from them have been confirmed as scrapie-affected by the National TSEs Reference Laboratory. The remaining 2191 sheep, except for the VRQ carriers, were considered as unaffected till the end of their life. VRQ carriers were slaughtered within the first two years of the study. All the animals were crossbred of indigenous breeds mainly Chios and Karagouniko.

2.1. DNA extraction

DNA from sheep blood was isolated by using the sheep DNA Blood Kit (Magnetic-PolyVinol Alcohol carrier beads, M-PVA beads) in the Magnetic Separation Module (Chemagic).

Brain tissue samples were collected from the 177 scrapie-affected sheep and high molecular mass DNA was isolated using the PUREGENETM DNA Tissue Kit (Gentra SYSTEMS).

2.2. PrP genotype analysis by MALDI TOF MS

The 2191 blood samples were analyzed by the MALDI TOF MS. PCR amplification of a 236 bp fragment of the PrP ORF was performed in a 20 µl reaction volume containing 1 µl (50–70 ng) genomic DNA, 1 mM $MgCl_2$, 1xPCR Buffer (10xPCR Buffer QIAGEN), 0.2 mM dNTPs, 0.8U Taq DNA (HotStarTaq; QIAGEN) and 0.2 µM of each primer, OARPRNPDS1–F (5′-TGGTGGCTACATGCTG-3′) and OARPRNPDS1–R (5′-CTT CACCGAAACTGA-3′). The amplification reaction was performed in a PTC-200 Peltier Thermal Cycler (MJ Research), after an initial denaturation step of 10 min at 95 °C, for 55 cycles of denaturation for 20 sec at 95 °C, annealing for 30 s at 56 °C and extension for 1 min at 72 °C, with a final extension of 3 min at 72 °C. The alleles at codons 136 (A/V), 154 (R/H) and 171 (Q/R/H/K) were discriminated using the appropriate Homogenous Mass Extend (hME) tetraplex reactions based on the annealing of oligonucleotide primers (hME primers) adjacent to the SNPs of interest (procedures originally developed by (Heaton et al., 2003). The addition of a DNA polymerase along with a mixture of terminator nucleotides allows extension of each hME primer through the polymorphic site and generates allele-specific extension products with unique molecular mass. The resultant masses of the extension products are analyzed by a Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) and the genotypes are assigned in real time (SEQUENOM's MassARRAY™, Genaissance Pharmaceuticals).

2.3. Real time PCR

A Real Time PCR method was used to detect the eight polymorphisms: 171Q/R/H/K, 136A/V and 154R/H in 177 infected sheep. The analyses were performed by two tetraplex Real-Time PCR reactions. The first one was for the detection of the four polymorphisms of the 171 amino acid and the second one for the polymorphisms of the 136 and 154 amino acids. In both tetraplex reactions a DNA fragment of 180 bp was amplified using the primers: ScF-5′-GCC TTG GTG GCT ACA TG-3′and ScR-5′- CTG TGA TGT TGA CAC AGT CAT-3′. The labelled probes sequences are listed in Table 1. Amplification reaction mixtures were prepared at a final volume of 15 µl containing 1X KAPA PROBE FAST qPCR kit Master Mix Universal, 400 nM of each primer and probe and 40–50 ng of sample's DNA. qPCRs were performed in a Chromo4™ Real-Time Detector. The cycling conditions for all the reactions consisted of

Table 1
Taqman probes used for the detection of SNPs at codons 136, 154 and 171 by Real-Time PCR.

Oligo name	Sequence (5′-3′)	5′-Label	3′-Label
A136-P1	CTCATGGCACTTCCCA	6FAM	BHQ1
V136-P2	CTGCTCATGACACTTCCCAG	HEX	BHQ1
R154-P3	CCGTTACTATCGTGAAAACATGTAC	ROX	BHQ2
H154-P4	CCGTTACTATCATGAAAACATGTACC	Cy5	BHQ2
R171-P5	CCAGTGGATCGGTATAGTAACCA	6FAM	BHQ1
H171-P6	AGACCAGTGGATCATTATAGTAACCA	HEX	BHQ1
Q171-P7	CCAGTGGATCAGTATAGTAACCAGA	ROX	BHQ2
K171-P8	ACCAGTGGATAAGTATAGTAACCAGA	Cy5	BHQ2