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Low frequency of the scrapie resistance-associated allele and presence of lysine-171 allele of the prion protein gene in Italian Biellese ovine breed

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Frequencies of polymorphisms at codons 136, 154 and 171 of the prion protein (*PrP*) gene were studied in 1207 pure-bred and cross-bred Italian Biellese rams, a small ovine breed of about 65 000 head in Italy. Aside from the five most common alleles (VRQ, ARQ, ARR, AHQ and ARH), the rare ARK allele was also found, with the highest frequency reported so far in an ovine breed (2·5 %). ARK/--- genotypes had a total frequency of 4·9 %. The resistance-associated ARR allele was seen at a low frequency (8·3 %). Only 1·4 % of animals examined had a resistant ARR/ARR *PrP* genotype. Semi-resistant (ARR/ARQ, ARR/ARH and ARR/AHQ) *PrP* genotypes had a total frequency of 12·6 % and *PrP* genotypes that are associated with high scrapie susceptibility (e.g. VRQ/VRQ and ARQ/ARQ) had a total frequency of 81·1 %. Statistical analysis comparing *PrP* allele frequencies between pure-bred and cross-bred animals showed that the ARR allele occurred at a significantly lower frequency in pure-bred rams. Furthermore, comparison of *PrP* allele frequencies between pure-bred rams over 18 months of age and those below 18 months of age showed a significant decrease in the ARR allele in breeding rams over 18 months of age. Based on these results, breeding for scrapie resistance in the Biellese breed will have to take into account the low frequency of the ARR allele, which also seems to be subject to negative selection by farmers. Further investigation is required to understand whether the ARK allele is also associated with resistance to transmissible spongiform encephalopathies.

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INTRODUCTION

Scrapie is a fatal, neurodegenerative disease that affects sheep and goats and belongs to the group of transmissible spongiform encephalopathies (TSEs). TSEs, or prion diseases, include bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt–Jakob disease (CJD) in humans. These diseases are associated with the accumulation of an abnormal isoform (PrP^{Sc}) of the host-encoded cellular prion protein (PrP^{C}) in the central nervous system. PrP^{C} is essential for the expression of prion diseases, e.g. PrP^{C} knockout mice do not develop clinical signs after experimental scrapie challenge (Büeler *et al.*, 1993).

In sheep, the cellular prion protein is 256 aa in length and is encoded by the prion protein gene (*PRNP* or *PrP*), which contains the entire ORF within exon 3 (Goldmann *et al.*, 1990; Goldmann, 1993; Westaway *et al.*, 1994). Although scrapie is an infectious disease, susceptibility to the disorder in sheep is influenced strongly by genotypes of the *PrP* gene (Hunter *et al.*, 1997a). Twenty-six *PrP* alleles, giving rise to twenty-five mutually exclusive amino acid polymorphisms, have been described: M112T, A116P, G127A/V/S, A136V/T, M137T, S138N, L141F, H143R, R151C, R154H, R167S, Q171R/H/K, N176K, H180Y, G189L/R, T195S, T196S, R211Q and P241S (Goldmann *et al.*, 1990, 1991; Laplanche *et al.*, 1993; Belt *et al.*, 1995, 1996; Clouscard *et al.*, 1995; Bossers *et al.*, 1996; Thorgeirsdottir *et al.*, 1999; Tranulis *et al.*, 1999; O'Rourke *et al.*, 2000; DeSilva *et al.*, 2003; Gombojav *et al.*, 2003; Guo *et al.*, 2003; Heaton *et al.*, 2003; Seabury & Derr, 2003; Billinis *et al.*, 2004). Only amino acid changes at codons 136, 154 and 171 have been shown to be associated with susceptibility to scrapie. These three polymorphic positions encode seven alleles: $A_{136}R_{154}Q_{171}$ (hereafter referred to as ARQ), VRQ, TRQ, ARR, AHQ, ARH and ARK (Goldmann *et al.*, 1990; Laplanche *et al.*, 1994; Belt *et al.*, 1995; Gombojav *et al.*,

2003; Billinis *et al.*, 2004). The VRQ allele is associated with high susceptibility (Belt *et al.*, 1995; Hunter *et al.*, 1996). ARQ allele susceptibility varies with sheep breed. In Suffolk sheep, in which the VRQ allele is rare or absent, ARQ/ARQ is the *PrP* genotype that is most susceptible to scrapie, although with an incomplete penetrance (Hunter *et al.*, 1997b). The AHQ allele is associated with resistance in some (Thorgeirsdottir *et al.*, 1999), but not all, breeds (Dawson *et al.*, 1998; Elsen *et al.*, 1999). In breeds in which the VRQ allele has primarily been associated with high scrapie susceptibility, the AHQ allele is associated with reduced scrapie susceptibility. A recently identified novel scrapie type, designated Nor98, seems to preferentially target sheep that carry at least one AHQ allele, irrespective of the breed (Benestad *et al.*, 2003). The ARH allele seems to be neutral (Dawson *et al.*, 1998). In a study on the efficiency of the *in vitro* conversion of PrP^{C} into protease-resistant PrP of different sheep *PrP* allelic variants, a reduced conversion efficiency of PrP^{C_ARH} and PrP^{C_AHQ}, in addition to PrP^{C_ARR}, was observed (Bossers *et al.*, 2000). Overall, the ARK allele has not been associated with scrapie resistance or susceptibility, as it is too rare. Similarly, the rare TRQ allele does not seem to affect scrapie susceptibility (Billinis *et al.*, 2004). The ARR allele has been linked to resistance to natural scrapie, with only a few cases in ARR heterozygotes (van Keulen *et al.*, 1996; Hunter *et al.*, 1997a) and one unconfirmed case in an ARR/ARR genotype (Ikeda *et al.*, 1995) reported so far.

On this basis, the European Union (EU) has looked to genetics as a tool to control scrapie in the sheep population. To eradicate TSEs in sheep, EU legislation has introduced the killing and complete destruction of all animals in an ovine TSE outbreak, except for ARR/ARR breeding rams and sheep with at least one ARR and no VRQ allele. Furthermore, following the Commission Decision 2003/100/EC, each Member State has introduced a breeding programme to select for resistance to TSEs in its sheep breeds in order to increase the frequency of the ARR allele and to reduce susceptible alleles. Derogations from some requirements are foreseen in the case of breeds that are in danger of being lost to farming or display an ARR allele level below 25 %. In breeds with an ARR level below 10 %, a derogation from the entire breeding programme may be granted, under the condition that alternative scrapie control programmes are implemented.

In Italy, scrapie was first described in Piedmont in 1976 (Cravero *et al.*, 1976), in the Biellese breed. Since 1991, when scrapie became a compulsory notified disease in Italy, five other ovine TSE outbreaks have been detected in this breed. The Biellese breed accounts for about 50 % of the Piedmont sheep population, with an estimated population of 55 000 animals distributed in 350 flocks. It is also present in Veneto (7000 heads), Emilia-Romagna (2000) and Lombardy (1000). Mainly bred for meat production, this breed displays desirable features such as hardiness and prolificacy.

So far, no extensive studies on *PrP* genotypes of the Biellese breed have been carried out. Within the framework of a regional control programme, this study compared the frequencies of polymorphisms at codon 136, 154 and 171 of the *PrP* gene in a large number of pure-bred and cross-bred rams. We also examined the *PrP* genotypes of scrapie-affected Biellese sheep from outbreaks detected in Piedmont in the last 3 years.

METHODS

Rams. In this study, 1207 rams of the Biellese breed were included, 251 of which were classified as not being of pure pedigree, but crossed with other breeds. The animals came from 113 flocks throughout Piedmont: 64 flocks consisted of only pure Biellese breed, 40 consisted of only cross-bred rams and nine included both pure and crossed animals. In 47 flocks, the one ram present was sampled, whereas in the other flocks, more rams were available for sampling, as were uncastrated lambs that were intended for slaughter (maximum number of rams from the same flock, 281). Most of the animals were relatively young: 46 % were <1 year of age, 40 % were 1–2 years of age and 1 % were >6 years of age.

Scrapie-affected sheep. Thirty-four scrapie-affected sheep (33 ewes and 1 ram) from four different outbreaks in Piedmont since 2002 were examined. All four cases that led to the detection of the outbreaks and the remaining positive animals that were culled during the eradication procedures were tested by the rapid Prionics-check Western method (Prionics AG). Scrapie positivity was confirmed in all cases by histopathology, immunohistochemistry (IHC) and Western blot (Wb) according to the protocols described by Caramelli *et al.* (2001), using the antibodies F99/97.6.1 (O'Rourke *et al.*, 2000) for IHC and P4 (Harmeyer *et al.*, 1998) for Wb.

DNA extraction. Blood was collected in EDTA-treated vacutainers and frozen at −20 °C. Genomic DNA was isolated by using manual Qiagen kits or Thermo Labsystems KingFisher kits.

PCR. PCR amplification of part of the *PrP* ORF (approx. 351 bp) was performed in a 50 µl reaction volume that contained 0·5–1 µg genomic DNA, 200 µM dNTPs, 1·5 mM $MgCl_2$, 1 U *Taq* DNA polymerase (HotStarTaq; Qiagen) and 1 µM each primer, p61 (+) (5′-AACCAACATGAAGCATGTGG-3′) (Belt *et al.*, 1995) and p143 (−) (5′-CTGGGATTCTCTCTGGTACT-3′) (Bossers *et al.*, 1996). P61 and p143 hybridize on the target *PrP* DNA at codons 104–110 and 226–220, respectively. The amplification reaction was performed in a GeneAmp PCR system 9700 (Applied Biosystems) for 41 cycles of 1 min at 94 °C, 1·5 min at 58 °C and 1 min at 72 °C, with a final extension period of 7 min at 72 °C.

***PrP* genotype analysis.** *PrP* polymorphisms were detected by automated DNA sequencing on both strands by using dye terminator cycle sequencing and analysis by capillary electrophoresis on an ABI Prism 310 genetic analyser (Applied Biosystems). Sequencing primers were p61 and p143. *PrP* alleles are indicated by using the commonly accepted nomenclature of amino acids at codons 136 (A/V/T), 154 (R/H) and 171 (Q/R/H/K). These *PrP* alleles were formed on the basis of the assumption that all described *PrP* polymorphisms in sheep are thus far mutually exclusive.

Statistical analysis. Exact binomial 95 % confidence intervals (CIs) were obtained to compare the differences in the allele frequencies of pure-bred and cross-bred animals and, in the pure-bred animal group, the differences between rams below and those over 18 months of age. According to local use, the rams are selected for breeding purposes at 18 months of age. The STATA 8.2 package (College Station, TX, USA) was used.

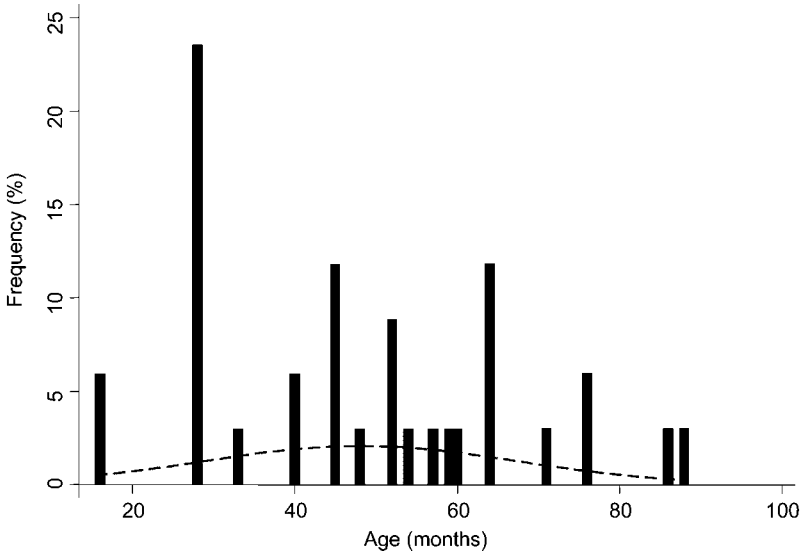

Fig. 1. Age distribution of the 34 scrapie-affected Biellese sheep (a normal density curve has been added as a dashed line).

RESULTS

In total, 1207 Biellese rams and 34 scrapie-affected Biellese sheep were studied with regard to *PrP* gene polymorphisms at codons 136, 154 and 171.

In the scrapie-affected sheep group, only two *PrP* genotypes occurred: ARQ/ARQ ($n=32$) and ARQ/AHQ ($n=2$), with no other associated mutations. No positive animals with the VRQ/VRQ or VRQ/--- *PrP* genotypes were found. Fig. 1 shows the age distribution; the ARQ/AHQ cases were 16 and 64 months old, respectively.

In the ram group, aside from the five most common *PrP* alleles (VRQ, ARQ, ARR, AHQ and ARH), the rare ARK allele was also found. *PrP* allele frequencies are shown in Table 1. Interestingly, the data displayed a low frequency of the resistance-associated ARR allele (8·3 %) and a remarkably high frequency of the ARK allele (2·5 %). The high-risk VRQ and ARQ alleles were also found at a high frequency. In the sequenced region of the *PrP* gene, aside from codons 136, 154 and 171, three other mutations, which were not linked to scrapie resistance or susceptibility (Bossers *et al.*, 1996; O'Rourke *et al.*, 2000; DeSilva *et al.*, 2003), were

Table 1. *PrP* alleles detected in Biellese rams and their frequencies

PrP allele	*n*	Frequency (%)
ARR	201	8·3
ARQ	1797	74·4
AHQ	91	3·8
ARH	99	4·1
VRQ	165	6·8
ARK	61	2·5
Total	**2414**	**100·0**

Table 2. *PrP* genotypes detected in Biellese rams and their frequencies

Risk groups are classified according to the UK National Scrapie Plan. NC, Not classified.

PrP genotype	*n*	Frequency (%)	Risk group
ARR/ARR	17	1·4	1*
ARR/ARQ	137	11·4	2†
ARR/ARH	6	0·5	2
ARR/AHQ	8	0·7	2
ARQ/ARQ	680	56·3	3‡
ARQ/AHQ	66	5·5	3
ARQ/ARH	69	5·7	3
AHQ/AHQ	2	0·2	3
AHQ/ARH	5	0·4	3
ARH/ARH	3	0·2	3
ARR/VRQ	15	1·2	4§
ARQ/VRQ	119	9·9	5‖
AHQ/VRQ	4	0·3	5
ARH/VRQ	9	0·7	5
VRQ/VRQ	8	0·7	5
AHQ/ARK	4	0·3	NC
ARH/ARK	4	0·3	NC
ARQ/ARK	46	3·8	NC
ARR/ARK	1	0·1	NC
ARK/VRQ	2	0·2	NC
ARK/ARK	2	0·2	NC
Total	**1207**	**100·0**	–

*Sheep that are genetically most resistant to scrapie.

†Sheep that are genetically resistant to scrapie, but need careful selection when used for further breeding.

‡Sheep that genetically have little resistance to scrapie.

§Sheep that are genetically susceptible to scrapie and should not be used for breeding.

‖Sheep that are highly susceptible to scrapie and should not be used for breeding.

Table 3. *PrP* genotype frequencies in pure-bred and cross-bred Biellese rams

PrP genotype	Pure-bred		Cross-bred	
	n	Frequency (%)	*n*	Frequency (%)
ARR/ARR	10	1·0	7	2·8
ARR/ARQ	97	10·1	40	15·9
ARR/ARH	5	0·5	1	0·4
ARR/AHQ	3	0·3	5	2·0
ARQ/ARQ	564	59·0	116	46·2
ARQ/AHQ	36	3·8	30	12·0
ARQ/ARH	59	6·2	10	4·0
AHQ/AHQ	1	0·1	1	0·4
AHQ/ARH	4	0·4	1	0·4
ARH/ARH	3	0·3	–	0·0
ARR/VRQ	11	1·2	4	1·6
ARQ/VRQ	94	9·8	25	10·0
AHQ/VRQ	3	0·3	1	0·4
ARH/VRQ	9	0·9	–	0·0
VRQ/VRQ	6	0·6	2	0·8
AHQ/ARK	4	0·4	–	0·0
ARH/ARK	4	0·4	–	0·0
ARQ/ARK	38	4·0	8	3·2
ARR/ARK	1	0·1	–	0·0
ARK/VRQ	2	0·2	–	0·0
ARK/ARK	2	0·2	–	0·0
Total	**956**	**100·0**	**251**	**100·0**

found: L141F (3·3 %), H143R (0·2 %) and H180Y (0·7 %), associated with the ARQ/ARQ or the ARQ/--- genotypes.

All of the 21 possible *PrP* genotypes (from the six detected *PrP* alleles) were found. Table 2 lists the *PrP* genotypes with their frequency and the risk group as classified by the UK National Scrapie Plan (http://www.defra.gov.uk/animalh/bse/bse-science/scrapie/nsp/nsp.html). ARR/ARR resistant animals (risk group 1) were present at a very low frequency (1·4 %). Semi-resistant (ARR/ARQ, ARR/ARH and ARR/AHQ) *PrP* genotypes (risk group 2) had a total frequency of 12·6 % and *PrP* genotypes that are associated with high scrapie susceptibility (risk groups 3, 4 and 5) had a total frequency of 81·1 %.

The ARK allele was found in all the six possible *PrP* genotypes combined, including homozygous ARK/ARK, at a total ARK/--- genotype frequency of 4·9 %. No risk classification was possible, due to the rarity of this allele and the consequent lack of data about its resistance or susceptibility to scrapie.

The *PrP* genotype and allele frequencies of pure-bred and cross-bred rams are reported in Table 3 and Fig. 2. The ARQ allele was present significantly more often among pure-bred rams, whereas the ARR and AHQ alleles were associated more often with cross-bred rams. No differences in VRQ, ARH or ARK allele frequencies were found between the two groups. Comparison of *PrP* allele frequencies between the pure-bred rams over and those below

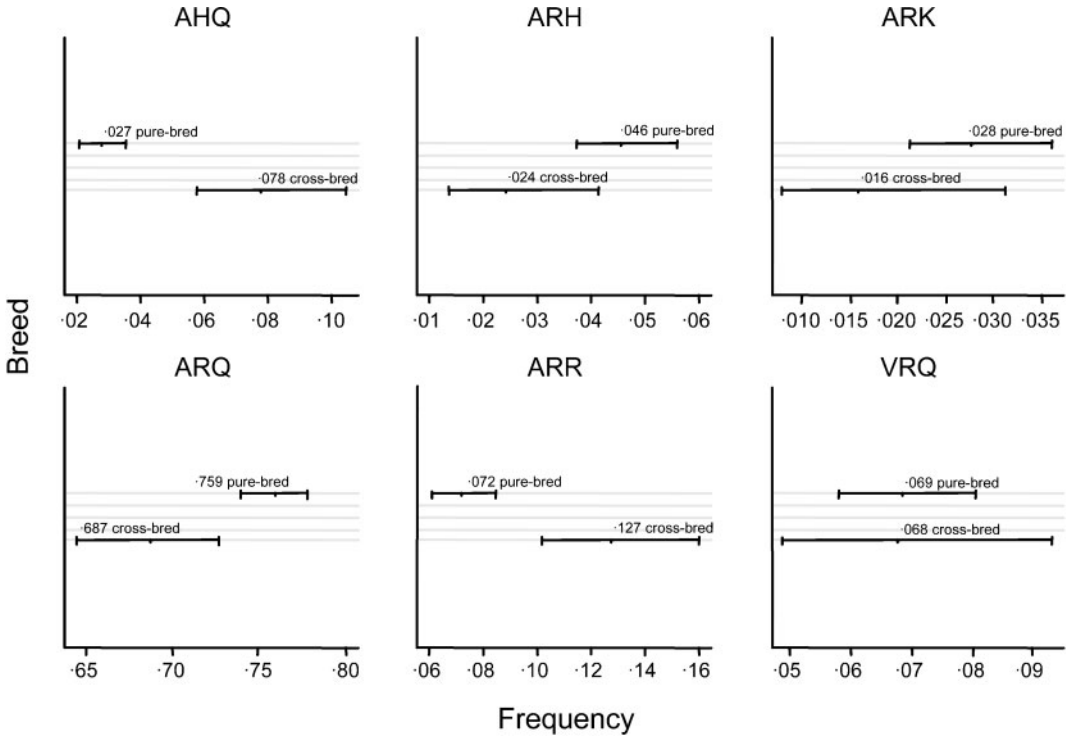

Fig. 2. Comparison of *PrP* allele frequencies in pure-bred ($n=1912$) vs cross-bred ($n=502$) rams. Values are frequencies ± 95 % CI.

18 months of age showed no significant differences for the AHQ, ARK or VRQ alleles, whereas there was a significant increase in the ARQ allele and a significant decrease in the ARR and ARH alleles in breeding rams over 18 months of age (Fig. 3).

DISCUSSION

In this study, *PrP* gene polymorphism frequencies of the Italian Biellese ovine breed are reported. Some remarkable features were noted in the *PrP* genotypes of this breed. Not only were the five well-known alleles (VRQ, ARQ, ARR, AHQ and ARH) with all their fifteen *PrP* genotypes found, but the rare ARK allele (2·5 %) was also detected, indicating the highest ARK frequency ever found in a single ovine breed. Other recent studies have reported a frequency of this allele: in Mongolian sheep (0·6 %; Gombojav *et al.*, 2003), in Oklahoma sheep (0·35 %; DeSilva *et al.*, 2003) and in various pure or crossed Greek milking breeds (1·6 %; Billinis *et al.*, 2004). Furthermore, the large sample size of our study allowed us to detect all the six possible *PrP* genotypes with the ARK allele, including ARK/ARK homozygotes. The reason why this allele is not rare in the Biellese breed, where it shows a frequency comparable to that of the AHQ and ARH alleles, is unknown. Although there is presently no evidence, it could have been selected because of a link to an unknown quantitative trait. In addition, its frequency in young animals is not significantly different from that seen in adult rams that have been kept for breeding, showing that the ARK allele is not negatively selected by farmers. On the other hand, one could speculate that the ARK allele might also be present in other sheep breeds, but that it is not detected by some routinely used, high-throughput *PrP* genotyping methods (e.g. methods that are based on the use of allele-specific probes or single-base extensions). Thus far, no data about the linkage of the ARK allele to differential scrapie susceptibility are available. It should be noted that none of the 34 scrapie-affected Biellese sheep showed the presence of the ARK allele. Recently, moreover, Billinis *et al.* (2004) reported the presence of the ARK allele restricted to healthy Greek sheep.

Another characteristic of the Biellese breed is that it may be classified as a so-called 'valine breed', i.e. a breed, like the Cheviot, that encodes the VRQ allele with the amino acid valine at codon 136. The VRQ allele showed a frequency (6·8 %) comparable to frequencies reported in other breeds carrying this allele in Iceland (7·3–10·0 %; Thorgeirsdottir *et al.*, 1999), Spain (3·3 %; Hurtado *et al.*, 2002), Austria (4·2 %; Sipos *et al.*, 2002) and Italy (4·4–9·9 %; Agrimi *et al.*, 2003). *PrP* genotypes with the VRQ allele, classified in the UK National Scrapie Plan as risk groups 4 and 5 (susceptible

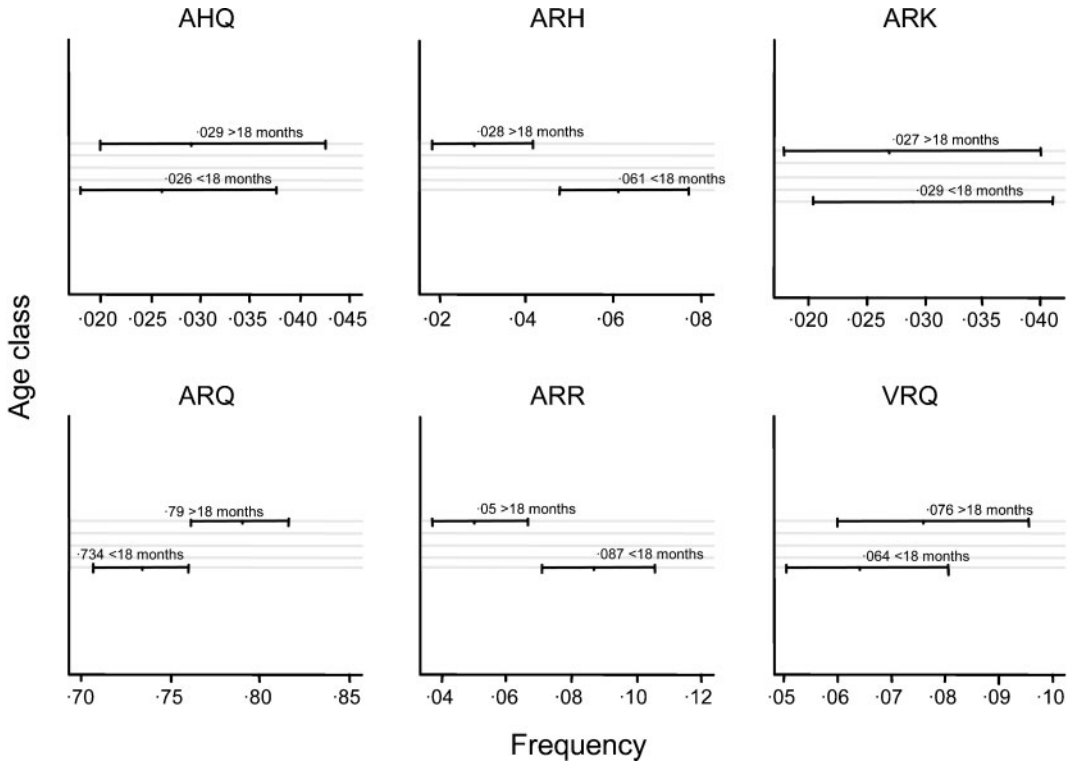

Fig. 3. Comparison of *PrP* allele frequencies in pure-bred Biellese rams over ($n = 858$) and below ($n = 1036$) 18 months of age. Values are frequencies ± 95 % CI.

and highly susceptible to scrapie, respectively), had a total frequency of 12·8 %. *PrP* genotypes also associated with scrapie susceptibility (risk group 3) showed a total frequency of 68·3 %. The scrapie-affected Biellese sheep analysed in this study carried the *PrP* genotypes ARQ/ARQ ($n=32$) or ARQ/AHQ ($n=2$), both belonging to risk group 3, and no scrapie-positive animals with VRQ/--- genotypes were found. These data are intriguing because the genetic susceptibility to scrapie of the Biellese breed appears to be different from that of other 'valine breeds', in which scrapie is detected most often in the VRQ/VRQ and VRQ/ARQ genotypes and in which the AHQ allele appears to be linked to low risk (Dawson *et al.*, 1998). Mutinelli *et al.* (2003) reported a similar situation in the Italian Massese breed; this may suggest a peculiar genetic target of the scrapie strains circulating in Italy. It therefore appears that the Biellese breed population is formed largely by TSE-susceptible animals (81·1 %, excluding *PrP* genotypes with the ARK allele).

Sheep with semi-resistant *PrP* genotypes (risk group 2) were found with a frequency of only 12·6 % (the ARR allele frequency was 8·3 %). Furthermore, the resistance-associated ARR/ARR genotype (risk group 1) had an unexpectedly low frequency of 1·4 %. Much higher frequencies of semi-resistant (ARR/---) and resistant (ARR/ARR) *PrP* genotypes were reported in Irish Suffolk sheep (ARR/---, 48·2 %; ARR/ARR, 39·1 %; O'Doherty *et al.*, 2000), in Austrian breeds (ARR/---, 15–40 %; ARR/ARR, 2·9–11·5 %; ARR allele, 14·8–25·8 %; Sipos *et al.*, 2002), in the Spanish Latxa breed (ARR/---, 48·8 %; ARR/ARR, 5·9 %; ARR allele, 25·4 %; Hurtado *et al.*, 2002), in the Italian Sarda (ARR/---, 57 %; ARR/ARR, 20 %; Vaccari *et al.*, 2001; ARR allele, 39·0 %; Agrimi *et al.*, 2003), in the Comisana and Massese breeds (ARR allele, 41·4 and 45·9 %, respectively; Agrimi *et al.*, 2003) and in some breeds from the Netherlands (e.g. Ryeland sheep: ARR/---, >95 %; ARR/ARR, >78 %) (A. Bossers, unpublished data).

Situations similar to that of the Biellese breed have been reported in the Icelandic sheep breed, in which the ARR allele was completely absent (Thorgeirsdottir *et al.*, 1999), and in German East Friesian milk sheep, in which only the ARR/ARQ genotype was found with a frequency of 6 %, albeit only 93 animals were analysed for this breed (Junghans *et al.*, 1998).

The low level of the ARR allele in the Biellese breed is even more striking when pure-bred and cross-bred animals are considered separately and the ARR allele frequency in the two groups is compared. In cross-bred animals, the ARR allele increased to more usual values (12·7 %), but was significantly lower in the pure-bred rams. Why so few Biellese sheep carry the ARR allele is difficult to explain. One possible explanation is that in this breed, the scrapie-resistant allele is linked to some undesirable feature and is therefore (knowingly or unknowingly) negatively selected by the breeders. An interesting piece of indirect evidence for this negative selective pressure can be drawn from the statistical analysis comparing the ARR allele frequencies between rams older than 18 months, i.e. those kept for breeding, and rams below 18 months of age. The significant decrease in the ARR allele in the breeding rams suggests that the animals with this allele are preferentially not used for breeding purposes. This is the first report of a possible link between *PrP* alleles and other quantitative traits in a sheep breed. However, we could equally hypothesize a linkage with another unfavourable gene or random choice, rather than a direct influence of or a linkage with the *PrP* gene itself.

In view of the future TSE-resistance breeding programme that is being planned by the EU, several considerations can be advanced, based on the results of this work. Accurate knowledge of the genetic characteristics of a breed is an essential requisite for designing a suitable selection programme and the best approach to genotyping a less well-known breed is by using methods that can also detect rare *PrP* alleles (i.e. sequencing-based methodologies). In our study, we found the ARK allele, for which the Biellese breed has the highest frequency ever reported. Hence, methods that recognize this allele will need to be included in the selective-breeding programme for this breed, in order to avoid methodological mistakes, such as *PrP* genotyping an animal as ARR/ARR that should have been genotyped as ARR/ARK.

The scrapie-control programme by selective breeding for the Biellese breed will have to take into account the current low frequency of the resistance-associated ARR allele, which in this breed also seems to be subject to negative selection by farmers. According to Commission Decision 2003/100/EC, this breed, which has an ARR allele frequency below 10 %, could be excluded from the breeding programme if it is submitted to a scrapie-control programme. As no scrapie cases carrying the ARK allele have been found, the possible inclusion of the ARK allele for breeding purposes should be contemplated. However, further investigation is required to understand whether the ARK allele is also associated with resistance to TSEs (like the ARR allele) and whether it could be additionally selected to improve the TSE-resistance status of the Biellese breed.

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REFERENCES

Agrimi, U., Conte, M., Morelli, L. & 9 other authors (2003). Animal transmissible spongiform encephalopathies and genetics. *Vet Res Commun* **27** (Suppl. 1), 31–38.

Belt, P. B. G. M., Muileman, I. H., Schreuder, B. E. C., Bos-de Ruijter, J., Gielkens, A. L. J. & Smits, M. A. (1995). Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie. *J Gen Virol* **76**, 509–517.

Belt, P. B. G. M., Bossers, A., Schreuder, B. E. C. & Smits, M. A. (1996). PrP allelic variants associated with natural scrapie. In *Bovine Spongiform Encephalopathy: the BSE Dilemma*, pp. 294–305. Edited by C. J. Gibbs, Jr. New York: Springer.

Benestad, S. L., Sarradin, P., Thu, B., Schonheit, J., Tranulis, M. A. & Bratberg, B. (2003). Cases of scrapie with unusual features in Norway and designation of a new type, Nor98. *Vet Rec* **153**, 202–208.

Billinis, C., Psychas, V., Leontides, L., Spyrou, V., Argyroudis, S., Vlemmas, I., Leontides, S., Sklaviadis, T. & Papadopoulos, O. (2004). Prion protein gene polymorphism in healthy and scrapie-affected sheep in Greece. *J Gen Virol* **85**, 547–554.

Bossers, A., Schreuder, B. E. C., Muileman, I. H., Belt, P. B. G. M. & Smits, M. A. (1996). PrP genotype contributes to determining survival times of sheep with natural scrapie. *J Gen Virol* **77**, 2669–2673.

Bossers, A., de Vries, R. & Smits, M. A. (2000). Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine naturally occurring variants of PrP. *J Virol* **74**, 1407–1414.

Büeler, H., Aguzzi, A., Sailer, A., Greiner, R.-A., Autenried, P., Aguet, M. & Weissman, C. (1993). Mice devoid of PrP are resistant to scrapie. *Cell* **73**, 1339–1347.

Caramelli, M., Ru, G., Casalone, C., Bozzetta, E., Acutis, P. L., Calella, A. & Forloni, G. (2001). Evidence for the transmission of scrapie to sheep and goats from a vaccine against *Mycoplasma agalactiae*. *Vet Rec* **148**, 531–536.

Clouscard, C., Beaudry, P., Elsen, J. M. & 7 other authors (1995). Different allelic effects of the codons 136 and 171 of the prion protein gene in sheep with natural scrapie. *J Gen Virol* **76**, 2097–2101.

Cravero, G., Guarda, F., Dotta, U. & Guglielmino, R. (1976). La scrapie in pecore di razza biellese. Prima segnalazione in Italia. *Clin Vet (Milano)* **100**, 1–14 (in Italian).

Dawson, M., Hoinville, L. J., Hosie, B. D. & Hunter, N. (1998). Guidance on the use of PrP genotyping as an aid to the control of clinical scrapie. Scrapie Information Group. *Vet Rec* **142**, 623–625.

DeSilva, U., Guo, X., Kupfer, D. M., Fernando, S. C., Pillai, A. T. V., Najar, F. Z., So, S., Fitch, G. Q. & Roe, B. A. (2003). Allelic variants of ovine prion protein gene (PRNP) in Oklahoma sheep. *Cytogenet Genome Res* **102**, 89–94.

Elsen, J.-M., Amigues, Y., Schelcher, F. & 7 other authors (1999). Genetic susceptibility and transmission factors in scrapie: detailed analysis of an epidemic in a closed flock of Romanov. *Arch Virol* **144**, 431–445.

Goldmann, W. (1993). PrP gene and its association with spongiform encephalopathies. *Br Med Bull* **49**, 839–859.

Goldmann, W., Hunter, N., Foster, J. D., Salbaum, M., Beyreuther, K. & Hope, J. (1990). Two alleles of a neural protein gene linked to scrapie in sheep. *Proc Natl Acad Sci U S A* **87**, 2476–2480.

Goldmann, W., Hunter, N., Benson, G., Foster, J. D. & Hope, J. (1991). Different scrapie-associated fibril proteins (PrP) are encoded by lines of sheep for different alleles of the Sip gene. *J Gen Virol* **72**, 2411–2417.

Gombojav, A., Ishiguro, N., Horiuchi, M., Serjmyadag, D., Byambaa, B. & Shinagawa, M. (2003). Amino acid polymorphisms of PrP gene in Mongolian sheep. *J Vet Med Sci* **65**, 75–81.

Guo, X., Kupfer, D. M., Fitch, G. Q., Roe, B. A. & DeSilva, U. (2003). Identification of a novel lysine-171 allele in the ovine prion protein (*PRNP*) gene. *Anim Genet* **34**, 303–305.

Harmeyer, S., Pfaff, E. & Groschup, M. H. (1998). Synthetic peptide vaccines yield monoclonal antibodies to cellular and pathological prion proteins of ruminants. *J Gen Virol* **79**, 937–945.

Heaton, M. P., Leymaster, K. A., Freking, B. A. & 7 other authors (2003). Prion gene sequence variation within diverse groups of U.S. sheep, beef cattle, and deer. *Mamm Genome* **14**, 765–777.

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.

Hunter, N., Goldmann, W., Foster, J. D., Cairns, D. & Smith, G. (1997a). Natural scrapie and PrP genotype: case-control studies in British sheep. *Vet Rec* **141**, 137–140.

Hunter, N., Moore, L., Hosie, B. D., Dingwall, W. S. & Greig, A. (1997b). Association between natural scrapie and PrP genotype in a flock of Suffolk sheep in Scotland. *Vet Rec* **140**, 59–63.

Hurtado, A., García-Pérez, A. L., Beltrán de Heredia, I., Barandika, J., Sanz-Parra, A., Berriatua, E. & Juste, R. A. (2002). Genetic susceptibility to scrapie in a population of Latxa breed sheep in the Basque Country, Spain. *Small Rumin Res* **45**, 255–259.

Ikeda, T., Horiuchi, M., Ishiguro, N., Muramatsu, Y., Kai-Uwe, G. D. & Shinagawa, M. (1995). Amino acid polymorphisms of PrP with reference to onset of scrapie in Suffolk and Corriedale sheep in Japan. *J Gen Virol* **76**, 2577–2581.

Junghans, F., Teufel, B., Buschmann, A., Steng, G. & Groschup, M. H. (1998). Genotyping of German sheep with respect to scrapie susceptibility. *Vet Rec* **143**, 340–341.

Laplanche, J. L., Chatelain, J., Westaway, D., Thomas, S., Dussaucy, M., Brugere-Picoux, J. & Launay, J. M. (1993). PrP polymorphisms associated with natural scrapie discovered by denaturing gradient gel electrophoresis. *Genomics* **15**, 30–37.

Laplanche, J. L., Delasnerie-Laupretre, N., Brandel, J. P., Chatelain, J., Beaudry, P., Alperovitch, A. & Launay, J. M. (1994). Molecular genetics of prion diseases in France. French Research Group on Epidemiology of Human Spongiform Encephalopathies. *Neurology* **44**, 2347–2351.

Mutinelli, F., Aufiero, G. M., Pozzato, N., Marangon, S., Agrimi, U., Vaccari, G. & Vincenzi, G. (2003). Eradication of scrapie in a Massese sheep flock by PrP allele selection. *Vet Rec* **152**, 60.

O'Doherty, E., Aherne, M., Ennis, S., Weavers, E., Hunter, N., Roche, J. F. & Sweeney, T. (2000). Detection of polymorphisms in the prion protein gene in a population of Irish Suffolk sheep. *Vet Rec* **146**, 335–338.

O'Rourke, K. I., Baszler, T. V., Besser, T. E. & 9 other authors (2000). Preclinical diagnosis of scrapie by immunohistochemistry of third eyelid lymphoid tissue. *J Clin Microbiol* **38**, 3254–3259.

Seabury, C. M. & Derr, J. N. (2003). Identification of a novel ovine PrP polymorphism and scrapie-resistant genotypes for St. Croix White and a related composite breed. *Cytogenet Genome Res* **102**, 85–88.

Sipos, W., Kraus, M., Schmoll, F., Achmann, R. & Baumgartner, W. (2002). PrP genotyping of Austrian sheep breeds. *J Vet Med A Physiol Pathol Clin Med* **49**, 415–418.

Thorgeirsdottir, S., Sigurdarson, S., Thorisson, H. M., Georgsson, G. & Palsdottir, A. (1999). PrP gene polymorphism and natural scrapie in Icelandic sheep. *J Gen Virol* **80**, 2527–2534.

Tranulis, M. A., Osland, A., Bratberg, B. & Ulvund, M. J. (1999). Prion protein gene polymorphisms in sheep with natural scrapie and healthy controls in Norway. *J Gen Virol* **80**, 1073–1077.

Vaccari, G., Petraroli, R., Agrimi, U. & 8 other authors (2001). PrP genotype in Sarda breed sheep and its relevance to scrapie. *Arch Virol* **146**, 2029–2037.

van Keulen, L. J. M., Schreuder, B. E. C., Meloen, R. H., Mooij-Harkes, G., Vromans, M. E. W. & Langeveld, J. P. M. (1996). Immunohistochemical detection of prion protein in lymphoid tissues of sheep with natural scrapie. *J Clin Microbiol* **34**, 1228–1231.

Westaway, D., Zuliani, V., Cooper, C. M., Da Costa, M., Neuman, S., Jenny, A. L., Detwiler, L. & Prusiner, S. B. (1994). Homozygosity for prion protein alleles encoding glutamine-171 renders sheep susceptible to natural scrapie. *Genes Dev* **8**, 959–969.