RESEARCH NOTE

Genetic variability at seven codons of the prion protein gene in nine Pakistani sheep breeds

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Introduction

Scrapie is a fatal neurodegenerative disease of sheep and goats, which is strongly believed to be caused by the accumulation of improperly folded forms of host-encoded cellular prion protein (PrP^c) in the central nervous system (CNS). The different genotypes of prion protein gene have been shown to make animals variably susceptible to this disease. We determined the genotypes of 284 sheep from six native (Buchi, Kachi, Kajli, Lohi, Sipli and Thalli), two crossbred (Hissardale and Pak-Karakul) and one imported (Awassi) breeds of sheep in the Punjab province of Pakistan at seven codons of the prion protein (*PrP*) gene, to assess the susceptibility/resistance of these breeds to natural scrapie. These breeds were polymorphic at codons 112 (M,T), 154 (R,H), 171 (Q,R,H) and 231 (nucleotide A or C), and monomorphic at codons 136 (A), 141 (L) and 241 (P). Six haplotypes and 18 genotypes were detected. M_{112} A_{136} L_{141} R_{154} Q_{171} Ra_{231} P_{241}, which is likely the ancestral haplotype of the *PrP* gene, was present in all breeds, and had high frequencies in the native breeds, ranging from 0.69 in Kajli to 0.95 in Kachi. Two rare haplotypes were detected, M_{112} R_{154} H_{171} Rc_{231} and M_{112} H_{154} Q_{171} Rc_{231}, each of which is the result of two mutations (H_{171} or H_{154} in combination with Rc_{231}) on the ancestral haplotype background. The former was present in all nine breeds at rather high frequencies. The $M_{112}A_{136}R_{154}R_{171}Ra_{231}$ haplotype, that confers resistance to the typical scrapie agent, was absent in the Buchi, Kachi and Sipli breeds but was present in other local breeds at low frequencies. Although the V_{136} allele, associated with the highest susceptibility to scrapie, was not present in any of the breeds, the low frequency of the highly resistant R_{171} allele puts the native breeds of Punjab at a moderate risk of infection by typical scrapie agents. As a result, strict import regulations need to be implemented to prevent scrapie outbreaks in Pakistan.

Scrapie, which is endemic to many countries, is a form of transmissible spongiform encephalopathy (TSE) in sheep and goats. Although scrapie is an animal health issue, its presence has not been investigated in Pakistan, where sheep and goats are major livestock species. Approximately 27-million sheep provide almost 33% of total red meat consumed in Pakistan. Although scrapie is not transmissible to human, there is evidence that bovine spongiform encephalopathy infects goats (Eloit *et al*. 2005) and humans (Bruce *et al*. 1997), making TSE a food safety issue as well. Surveillance of the disease and protecting sheep populations against scrapie introduction and outbreak are essential for the country's food security. There is no treatment or vaccine currently available for this disease, and there is no approved test to detect scrapie in living animals.

At present, selection for scrapie-resistant haplotypes and safety of animals from food infected with any of the PrP^{Sc} strains are considered possible strategies for avoiding this fatal neurodegenerative disease. The association of scrapie with the presence of abnormal forms of the prion protein (PrP) has been established, for example, variations in codons 136, 154 and 171 of the *PrP* gene are associated with animal's susceptibility to typical and experimental strains of scrapie agent (Baylis and Goldmann 2004). The objective of this study is to determine polymorphisms of these three codons of the *PrP* gene in major sheep breeds of Punjab (Pakistan), regarding which no information is available. The genotype of the sheep at these three codons would determine the degree of natural resistance of the local breeds to scrapie infection. Codons 112, 141, 231 and 241, were also included in this study because they are polymorphic in some European and Asiatic breeds (Ikeda *et al*. 1995; Billinis *et al*. 2004; Goldmann *et al*. 2005). The degree of polymorphism of these unimproved breeds at these codons would be useful to determining the origin of the *PrP* haplotypes.

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Keywords. prion protein; genetic variability; sheep; Pakistan.

Materials and methods

Source of animals and blood collection

Blood samples from 284 sheep were collected into vacutainers containing EDTA, then immediately transferred to an ice-box stored at −20°C until further processing. Samples from six native (Buchi, Kachi, Kajli, Lohi, Sipli and Thalli), two crossbred (Hissardale and Pak-Karakul) and the imported Awassi breeds from Punjab province of Pakistan were collected in the year 2005. Samples of Buchi, Kajli, Lohi, Sipli and Thalli breeds were mostly from government livestock farms. In 2004, these farms purchased purebred sheep from many villages to expand the genetic base of their existing breeds. Purchased animals sampled for this study were chosen based on their phenotypes and were representatives of the breeds. Few animals from these breeds were sampled from village flocks.

Lohi and Kajli are the most popular and wide spread native breeds of Punjab. They are large-size sheep, mostly reared in irrigated farmlands. Lohi is mainly reared in the central area of Punjab, while Kajli whose lean meat is favoured in the region, is mainly distributed in the north-western part of the province. Buchi and Sipli occupy the southern part of Punjab and Thalli a small-size breed that is native to the Thall, a subdesert region of southern Punjab.

Kachi a native breed of the Sindh province, was imported to Punjab due to its relatively high milk production. This breed has been reared in Kheriwala farm at Layyah district of Punjab with a flock size of 300 to 400 heads.

The breed Awassi was imported from Lebanon in 1970s for crossbreeding with the local Lohi breed, but the project was terminated, as a result of which little improvement in the crossbred progeny was made. This breed has been maintained in the government livestock farms at Bahadurnagar in Okara district and at Jahangirabad in Khanewal district, with a flock size of approximately 400 heads each.

The Hissardale breed, is a cross between Merino and Kachi of India, and has the finest wool among the sheep breeds of Pakistan (27 μm fibre diameter). This breed was established in India, and 80 breeding animals were transferred to Pakistan in 1947. This breed has been kept as one flock in Jahangirabad government farm in Punjab. Pak-Karakul, is a cross between Karakul breed of the Afghanistan with the Kachi, and is maintained in Kheriwala farm with a flock size of approximately 800 sheep. The Kachi, Awassi, Hissardale and Pak-Karakul are maintained only by government livestock farms and are possibly inbred to some extent. Some of the characteristics of these breeds are described in table 1 of the electronic supplementary material at http://www.ias.ac.in/jgenet/.

Laboratory analysis

Genomic DNA was extracted from frozen peripheral whole blood, according to the protocol of Grimberg *et al*. (1989), and was dissolved in TE buffer, pH 8.0. Eight single nucleotide polymorphisms (SNPs) at codons 112, 136, 141, 154, 171, 231 and 241 of the *PrP* gene were determined using the single-base extension method as described by Benkel *et al*. (2007). In brief, a 1126-bp long segment of the *PrP* gene, that includes the entire coding region of the gene, was amplified by the PCR using a forward (5′ - GAG GAA GAG TTG TGT TAC TAC T) and a reverse (5′ - GTC TGC TTG TCA TTT CCC AGT G) primer. PCR products were purified by Exo-SAP-IT (Amersham Pharmacia Biotech, NJ, USA) according to the manufacturer's instructions. Eight SNP interrogating primers were used in the second PCR reaction to add a single nucleotide to the 3′ end of each of the primers. A mixture of cleaned single-based extended primers and a size standard was resolved on an ABI 3130 capillary DNA analyser (Applied Biosystems, Foster City, CA). The results were analysed using the GeneMapper (Applied Biosystems, Foster city, CA) software.

Results

The breeds studied were polymorphic at codons 112 (M,T), 154 (R,H), 171 (Q,R,H) and 231 (nucleotides A or C, which will be referred as Rc and Ra, respectively), and monomorphic at codons 136 (A), 141 (L) and 241 (P). Eighteen genotypes were detected (table 1) and six were homozygous, indicating that at least six haplotypes were present in these breeds (table 2).

All the observed heterozygotes were the combination of these six haplotypes suggesting that it is very likely that only these six haplotypes were segregating in these breeds. Table 3 shows the frequency of the haplotypes at codons 136, 154 and 171, which are those with known effects on scrapie resistance.

The $M_{112}R_{154}Q_{171}Ra_{231}$ was present in all nine breeds, and its frequency was the highest in six native breeds, ranging from 0.69 in Kajli to 0.95 in Kachi. This haplotype was also the most common in the Hissardale breed with the frequency of 0.55. The $M_{112}R_{154}H_{171}Rc_{231}$ was also present in all the nine breeds, and was the most abundant haplotype in the Awassi and the second most abundant haplotype in the native Buchi, Kajli, Lohi and Thalli breeds. This haplotype differs from the ancestral type by two nucleotides: H_{171} and Rc_{231}. The $M_{112}H_{154}Q_{171}Rc_{231}$ haplotype was present only in three native breeds: Kajli, Lohi and Sipli. This haplotype is also the result of two nucleotide substitutions in the ancestral type (H_{154} and Rc_{231}), and it is the only haplotype that includes H_{154}. Since both H_{171} and H_{154} appeared only with Rc_{231}, it seems logical to hypothesize that these two nucleotide substitutions occurred on the $M_{112}R_{154}Q_{171}Rc_{231}$ background, which differs from the ancestral haplotype by only one nucleotide (Ra_{231} to Rc_{231}). Among the native breeds, however, $M_{112}R_{154}Q_{171}Rc_{231}$ was present only in Buchi and Kachi with very low frequencies (0.02 and 0.03), but had moderate frequencies in crossbreds and Awassi (0.11–0.19).

Table 1. Genotype frequency distribution of the *PrP* gene at codons 112, 154, 171 and 231 in nine breeds.

	Native breeds						Crossbreed		Foreign
Genotype	Buchi (n = 32)	Kachi (n = 20)	Kajli (n = 50)	Lohi (n = 45)	Sipli (n = 41)	Thalli (n = 36)	Hissardale (n = 20)	Pak-Karakul (n = 19)	Awassi (n = 21)
MRQRa/MRQRa	0.719	0.900	0.520	0.667	0.585	0.583	0.300	0.053	0.048
MHQRa/MHQRa					0.024				
MRQRa/MHHRa			0.020						
MRQRa/MHQRc			0.020		0.341				
MRRRa/MHQRc				0.022					
MRHRC/MRHRc	0.063		0.060	0.044		0.083		0.105	0.095
MRQRa/MRHRc	0.189	0.050	0.260	0.156	0.024	0.278		0.053	0.333
MRQRc/MRHRc							0.050	0.053	0.190
MRQRa/MRQRc	0.031	0.050					0.100	0.053	0.190
MRQRc/MRQRc							0.050	0.053	
MRRRa/MRQRa			0.060	0.111		0.056	0.300	0.053	
MRRRa/MRQRc			0.040					0.158	0.095
MRRRa/MRRRa			0.020				0.050		
TRQRa/MRHRc								0.105	0.048
TRQRa/MRQRa					0.024		0.100		
TRQRa/MRQRc							0.050		
TRRRa/MRQRa								0.263	
TRQRa/TRQRa								0.053	

Table 2. Haplotype frequency distribution of the *PrP* gene at codons 112, 154, 171 and 231 nine breeds.

	Native breeds						Crossbreeds		Foreign	
Haplotype	Buchi (n = 32)	Kachi (n = 20)	Kajli (n = 50)	Lohi (n = 45)	Sipli (n = 41)	Thalli (n = 36)	Hissardale (n = 20)	Pak-Karakul (n = 19)	Awassi (n = 21)	Total
MRQRa	0.83	0.95	0.69	0.80	0.78	0.75	0.55	0.13	0.31	0.69
MRHRc	0.16	0.02	0.22	0.12	0.01	0.22	0.03	0.29	0.43	0.16
MRQRc	0.02	0.03					0.15	0.11	0.19	0.04
MHQRc			0.02	0.01	0.20					0.03
MRRRa			0.07	0.07		0.03	0.20	0.24	0.05	0.06
TRQRa					0.01		0.08	0.24	0.02	0.03

The Sipli breed is the only native breed of Punjab that carried the $T_{112}R_{154}Q_{171}Ra_{231}$ haplotype with a low frequency (0.01), but the frequency of this haplotype ranged from 0.02 to 0.24 in Awassi and crossbred breeds. This was the second most abundant haplotype in Pak-Karakul (0.24). The R_{171} allele ($M_{112}A_{136}R_{154}R_{171}Ra_{231}$), the most resistant haplotype against typical scrapie strains, was absent in Buchi, Kachi and Sipli breeds and had low frequencies (less than 0.08) in all the other breeds, except the crossbred Hissardale (0.20) and Pak-Karakul (0.24). The V_{136} allele ($M_{112}V_{136}R_{154}Q_{171}Ra_{231}$), which is the most susceptible allele to typical scrapie strains, was absent in all the breeds.

$MM_{112}RR_{154}QQ_{171}RaRa_{231}$ was the only genotype that was present in all the breeds, with high frequencies in the local breeds, ranging from 0.52 in Kajli to 0.90 in Kachi. Its frequency was low in Awassi and crossbreds. Six of the genotypes were present only in one breed, mostly with low frequency, and all contained either T_{112} or H_{154}. The only exception was $TM_{112}RR_{154}RQ_{171}RaRa_{231}$, which was present only in Pak-Karakul, with a moderate frequency (0.26).

Discussion

The presence of $M_{112}R_{154}Q_{171}Ra_{231}$ in all the native breeds with high frequencies, supports the hypothesis that $A_{136}R_{154}Q_{171}$ and thus, $M_{112}A_{136}L_{141}R_{154}Q_{171}Ra_{231}P_{241}$ is the ancestral haplotype of the *PrP* gene, and is the most abundant haplotype in unimproved breeds of Asia (Gombojav *et al*. 2004). Other haplotypes are assumed to be derived from this ancestral type by single, double or triple nucleotide substitutions. The $M_{112}A_{136}R_{154}R_{171}Ra_{231}$ is a rare haplotype in native breeds of Punjab, and its moderate frequency in crossbred sheep has originated from Merino and Karakul, respectively. The findings also suggest that the native breeds of Punjab are vulnerable to infection by typical

Table 3. Haplotype frequency distribution of the *PrP* gene at codons 136, 154, and 171 in nine breeds of Punjab.

	Native breeds						Crossbreeds		Foreign	
Haplotype	Buchi	Kachi	Kajli	Lohi	Sipli	Thalli	Hissardale	Pak-Karakul	Awassi	Total
ARQ	0.84	0.97	0.69	0.80	0.79	0.75	0.77	0.47	0.52	0.75
ARH	0.16	0.03	0.22	0.12	0.01	0.22	0.03	0.29	0.43	0.16
AHQ			0.02	0.01	0.20					0.03
ARR			0.07	0.07		0.03	0.20	0.24	0.05	0.06

scrapie agents, and therefore, strict regulations need to be imposed on the importation of sheep from infected countries. The frequency of $A_{136}R_{154}R_{171}$ allele, which was infrequent in unimproved Pakistani sheep breeds, is higher than $A_{136}R_{154}Q_{171}$ allele in improved European breeds (Tkáčikova *et al*. 2003), but the reverse is true for local unimproved European breeds (Sipos *et al*. 2002; Goldmann *et al*. 2005).

Allele H_{154}, which is grouped among scrapie-resistant alleles (Dawson *et al*. 1998) and has been reported both in European (Tkáčikova *et al*. 2003; Eglin *et al*. 2005; Holko *et al*. 2005) and Asian sheep breeds (Gombojav *et al*. 2003; Lan *et al*. 2006), and was also found in three breeds in the present study. Allele T_{112}, which was almost absent in local breeds of Punjab, has been reported both in European (Goldmann *et al*. 2005) and Asian sheep (Ikeda *et al*. 1995; Gombojav *et al*. 2003, 2004). No association has been reported between this allele and scrapie. The source of moderate frequency of this allele in Pak-Karakul could be the Karakul breed of Afghanistan, for which no information is available regarding random genetic drift in this isolated population, or its selective advantage.

The V_{136} allele, which is commonly found in many European (Hurtado *et al*. 2002; Sipos *et al*. 2002; Tkáčikova *et al*. 2003; Eglin *et al*. 2005; Holko *et al*. 2005) and Asiatic (Gombojav *et al*. 2003, 2004) breeds, and confers susceptibility for typical scrapie strains, was not detected in any of the breeds.

The F_{141}, which is associated with a typical scrapie and has been reported in some European (Dawson *et al*. 1998; Buschmann *et al*. 2004; Goldmann *et al*. 2005; Saunders *et al*. 2006) and Asiatic (Lan *et al*. 2006) breeds, was not detected in populations of Pakistani sheep studied. Similarly, S_{241} was not present in any of the samples.

References

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

Benkel B. F., Valle E., Bissonnette N. and Farid A. H. 2007 Simultaneous detection of eight single nucleotide polymorphisms in the ovine prion protein gene. *Mol. Cell Prob.* **21**, 363–367.

Billinis C., Psychas V., Leontides L., Spyrou V., Argyroudis S., Vlemmas I. *et al*. 2004 Prion protein gene polymorphisms in healthy and scrapie-affected sheep in Greece. *J. Gen. Virol*. **85**, 547–554.

Bruce M. E., Will R. G., Ironside J. W., McConnell I., Drummond D., Suttie A. *et al*. 1997 Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature* **389**, 498–501.

Buschmann A., Biacabe A. G., Ziegler U., Bencsik A., Madee J. Y., Erhardt G. *et al*. 2004 A typical scrapie cases in Germany and France are identified by discrepant reaction patterns in BSE rapid tests. *J. Virol. Methods* **117**, 27–36.

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

Eglin R. D., Warner R., Gubbins S., Sivam S. K. and Dawson M. 2005 Frequencies of PrP genotypes in 38 breeds of sheep sampled in the National Scrapie Plan for Great Britain. *Vet. Rec*. **156**, 433–437.

Eloit M., Adjou K., Coulpier M., Fontaine J. J., Hamel R., Lilin T. *et al*. 2005 BSE agent signatures in a goat. *Vet. Rec*. **156**, 523–524.

Goldmann W., Baylis M., Chihota C., Stevenson E. and Hunter N. 2005 Frequencies of PrP gene haplotypes in British sheep flocks and the implications for breeding programmes. *J. Appl. Microbiol*. **98**, 1294–1302.

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

Gombojav A., Ishiguro N., Horiuchi M. and Shinagawa M. 2004 Unique amino acid polymorphisms of PrP genes in Mongolian sheep breeds. *J. Vet. Med. Sci*. **66**, 1293–1295.

Grimberg J., Nawoschik S., Belluscio L., Mckee R., Turk A. and Eiswenberg A. 1989 A simple and efficient non-organic procedure for the isolation of genomic DNA from blood. *Nucleic Acids Res*. **17**, 8390.

Holko I., Novackova A., Holkova T. and Kmet V. 2005. PrP genotypes in sheep breeds in Slovakia. *Vet. Rec*. **157**, 628–630.

Hurtado A., Garcia-Perez A. L., Beltran de Heredia I., Barandika J., Sanz-Parra A., Berriatua E. and Juste R. A. 2002 Genetic susceptibility to scrapie in a population of Latsa 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. and 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.

Lan Z., Wang Z. L., Liu Y. and Zhang X. 2006 Prion protein gene (PRNP) polymorphisms in Xinjiang local sheep breeds in China. *Arch. Virol*. **151**, 2095–2101.

Saunders G. C., Cawthraw S., Mountjoy S. J., Hope J. and Windl O. 2006 PrP genotypes of a typical scrapie cases in Great Britain. *J. Gen. Virol*. **87**, 3141–3149.

Sipos W., Kraus M., Schmoll F., Achmann R. and Baumgartner W. 2002 PrP Genotyping of Austrian Sheep Breeds. *J. Vet. Med*. **49**, 415–418.

Tkáčikova L., Hanusovska E., Novak M., Arvayova M. and Mikula I. 2003 The PrP genotype of sheep of the improved Valachian breed. *Folia Microbiol*. **48**, 269–276.

Received 7 February 2008, in revised form 18 February 2008; accepted 21 February 2008

Published on the Web: 30 June 2008