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Single nucleotide polymorphism in the PrP allele (A_{136}, R_{154}, Q_{171}) in Malpura sheep breed

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ABSTRACT

PCR-SSCP method was used to identify polymorphisms in the 136 (A/V), 154 (R/H), and 171 (R/H/Q) codons of the prion protein gene (PrP) in 50 animals of the Malpura sheep breed from its native tract in and around Malpura *Tehsil* in Tonk District and parts of adjoining Jaipur district. PCR amplicons of approximate size 358 bp were generated for PrP gene exon-3 partial CD's covering 352 to 700 nucleotide positions. Alanine (A) at codon 136, arginine (R) at codon 154, and arginine (R) or glutamine (Q) at codon 171, responsible for the presence of 2 alleles (ARR and ARQ) and genotype ARQ/ARQ was found in the analyzed population with the 100% frequency. SNP at codon 114, 127, 184 and 189 are HH/RR, GG/GS/SS, NN/NH and QQ/QL/LL with frequencies of 88.89/11.11, 55.56/11.11/33.33, 88.89/11.11 and 66.67/11.11/22.22 respectively. In all flocks, ARQ/ARQ genotype was the dominant haplotype regardless of sheep sex, age and health. However, the generated data will help for the detection of SNP markers related to disease resistance or susceptibility in India.

Key words: Allele, Malpura Sheep, PrP gene, Scrapie, Single nucleotide polymorphism

The fatal neurodegenerative prion disease is both genetic and infectious and can inflict on humans beings and animals. They are characterized by the accumulation of an abnormal isoform of the prion protein (PrPSc) in the central nervous system and show characteristic pathomorphological presentation of brain and cerebral cortex lesions resulting from neuron atrophy and gliosis. Animal TSE include scrapie in sheep and goats. A number of polymorphisms that cause a change in the amino acid chain were observed in the coding portion of the PrP gene encoding sheep prion protein (Babar *et al*. 2009, Piestrzyñska-Kajtoch *et al*. 2006 and Wiœniewska *et al*. 2006). However, a significant relationship with susceptibility of sheep to scrapie was observed for polymorphisms at codons 136, 154, and 171 (Baylis and Goldman 2004). Various PCR-based approaches have been used to determine ovine PrP sequences at codons 136, 154, and 171. These include direct DNA sequencing, RFLP, allele-specific oligonucleotide hybridization and primer extension assay. None of these methods allows for accurate determination of the PrP haplotype, as they either do not profile more than a fragment of the gene or require sequencing, which can be confounded in heterozygous sheep. PCR-SSCP allows for the accurate identification of *ovine* PrP genotypic variants, the resultant SSCP profiles are uncomplicated and can be easily interpreted either manually or automatically with a gel reader. This technique scans the whole sequence of the gene region amplified and has the potential to identify unknown or new allelic variations.

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MATERIALS AND METHODS

Sample collection and DNA extraction: Blood samples (50) were collected from Malpura sheep in its native tract in and around Malpura Tehsil in Tonk District and parts of adjoining Jaipur district at random. 10 ml of blood was collected aseptically by jugular vein puncture in a sterile vacutainer. Genomic DNA was isolated using standard procedure (Sambrook *et al*. 1989).

PCR and single strand conformational polymorphism analysis: PCR was carried out on about 50 ng genomic DNA. Amplifications were performed in a MJ PTC 100 thermal cycler.The PCR products were denatured at 85°C, followed by rapid cooling. The samples were loaded on 12% polyacrylamide gels containing 0.2% glycerol. Electrophoresis was performed using protean-II xi cells. The gels were stained in silver nitrate using a standard protocol (Bassam *et al.* 1991) and were dried on gel dryer.

PCR cleanup and sequencing: The PCR-SSCP amplicons were identified for sequencing based on different haplotypes observed on gels for genomic DNA products. PCR products were treated with shrimp alkaline phosphatase and Exonuclease III in 96 well plates, incubated at 37°C for 30 min followed by 80°C for 10 min. Out of this 1–5 mL PCR product was used in sequencing. PCR products were loaded into a 96 well format and analyzed on ABI 370 DNA analyzer. All sequences were analyzed using DNA Star version 4.0. The SNPs were identified in assembled sequences and codon identification was done using online sequin software. The SSCP alleles were identified using the nomenclature followed by Zhou *et al*. (2005) and compared with amino acid of the codon.

RESULTS AND DISCUSSION

To know the incidences of scrapie, the farmers/flock owners of Malpura sheep were interviewed. All the farmers under study informed that they have never observed any symptoms detailed to them for the incidence of the scrapie. Further the veterinary staff of the respective areas also confirmed that neither of the sheep in the sampled flocks showed any symptom of scrapie nor they have received any case of clinical scrapie alive or carcass for post-mortem showing any prevalence of the disease in sheep of these areas.

PCR-SSCP Profile: PCR product amplicons developed by using PrP forward primers (5'-caa ggt ggt agc cac agt ca-3') and reverse primers (5'-cca cca ctc gct cca tta tc-3') of approximate size of 358 bp were generated for PrP gene exon-3 partial CD's covering 352 to 700 nucleotide positions. The SSCP amplimers were resolved on non-denaturing gel. Polyacrylamide gels of Malpura sheep breed are shown in Fig. 1. A total of 8 variants were designated as A, B, C, D, E, F, G and H (Table 1). The classification of SSCP variants was given based on conformational changes in the band patterns. Variant 'A' showed the highest frequency (40.42%). Variant 'F' and 'H' were observed in one animal each with frequency of 2.12%.

Table 1. Frequency (%) of PCR-SSCP variants

Sr. no.	1	2	3	4	5	6	7	8
Variants	A	B	C	D	E	F	G	H
Variant name	Mal-1, Mal-9	Mal-2	Mal-3	Mal-4	Mal-5	Mal-6	Mal-7	Mal-8
Frequency (%)	40.42	25.53	8.51	8.51	8.51	2.12	4.42	2.12

DNA sequence analysis of PrP gene: The sequences of Malpura sheep were aligned with the gene bank sequences (AJ000734, AJ000736, AJ000737, A000738, AJ000739, AY488861 and AY488862) from nucleotide positions 352 to 700. The sequence alignment of Malpura sheep is given in Table 2. These sequences of '9' variants showed complete alignment with gene bank sequences shown by dots. However, the differed nucleotides are represented by the actual base substitutions. Fig. 2(B) shows the phylogenic tree of PrP partial CD's of exon-3 in comparison with known gene bank sequences. Our sequences showed distinct alignment within the breed and all sequences of a breed form separate clusters from that of reported gene bank sequences. PCR-SSCP products generated by forward primer and reverse primer were sequenced separately. After checking the PCR amplification, purified products were sequenced on

Table 2. Comparison of nucleotide sequence in PrP gene.

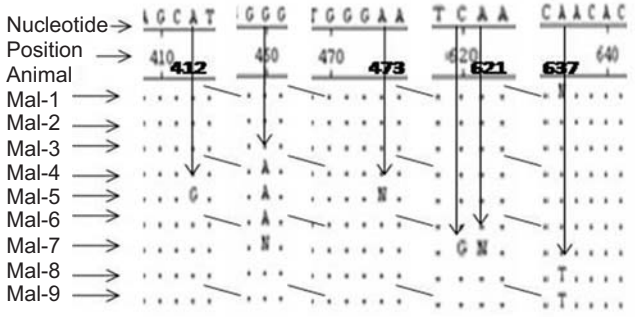
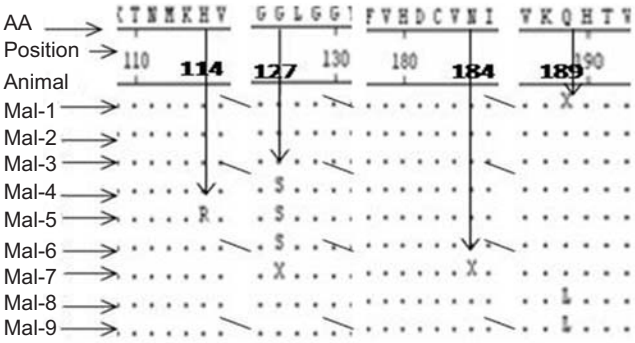


Table 3. Comparison of amino acid sequence in PrP gene



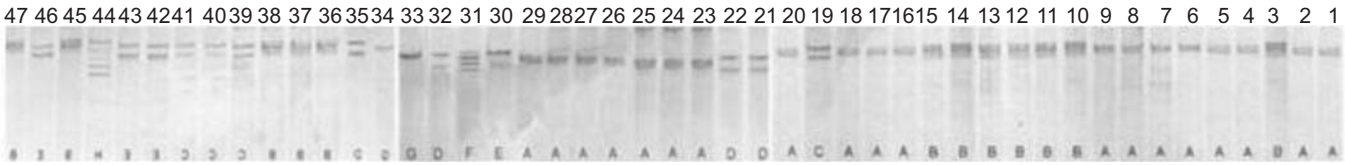


Fig. 1. Polyacrylamide gels (SSCP) showing different band pattern (variants).

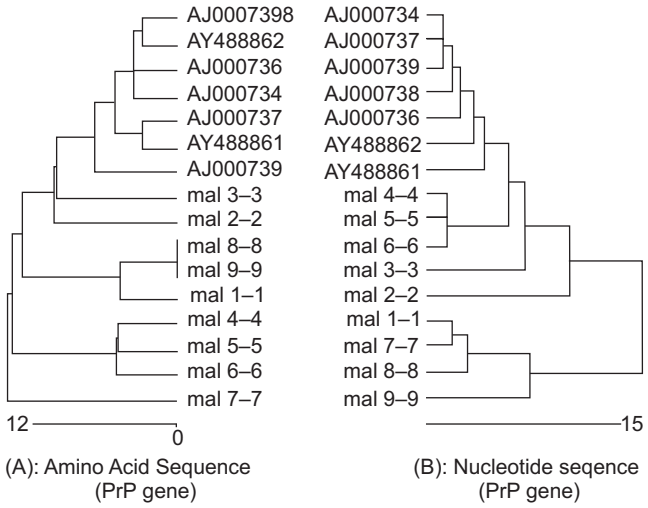


Fig. 2. Dendrogram showing comparison of (A) amino acid sequence, and (B) nucleotide sequence in Malpura sheep PrP gene with gene bank sequences.

automated sequencer using Hi-DiTM Formamide terminator chemistry.

Protein translation of PrP gene sequences: The projected translation of genetic codes formed by the triplet of PrP nucleotides were developed using edit sequence module of DNA Star Version 4.0 software. The amino acid sequence of DNA in these breeds were aligned with amino acid sequence of known gene bank accessions from codon number 95 to 210 of complete protein sequences of PrP gene exon-3. The align sequences with similar amino acid pattern has been depicted by dots while altered amino acid are shown by respective symbols (Table 3). Amino acid sequences of PrP gene in Malpura sheep breed are forming a separate cluster than gene bank DNA sequences of ovine PrP gene exon-3, Fig 2(A). Within Malpura breed amino acid sequences of variant 8 and 9 are at one node and related closely with that of sequence mal-1. Amino acids of DNA sequence of variant Malpura 4, 5 and 6 were closely related while sequence mal-7 is placed distinctly.

SNP in PrP gene: We resolved the DNA fragment of PrP gene exon-3 partial CDs that includes the important codons 136, 154 and 171 for the susceptibility/resistance against the scrapie disease in sheep. The SNP sites were identified in the DNA sequences of different sheep breeds, out of 750 nucleotide positions 6 showed changes in nucleotide sequences which were resulted into change in amino acids. Out of these 6 nucleotide positions, two (621and 637) were heterozygous in different breeds. One position (450) showing two types of results in three variants (mal-4, 5 and 6) it is homozygous and in mal-7 it is heterozygous. In rest of the nucleotide positions i.e. 412, 473 and 620 were homozygous (Table 4).

SNPs at codons (136, 154 and 171) having association with scrapie disease and other codons: At codon 136 only

Table 4. Single nucleotide polymorphism in PrP gene

Codon	Substitutions	Breeds	
		Homozygous	Heterozygous
412	H→R (A/G)	Mal 5	-
450	G→S (G/A)	Mal 4, 5, 6	Mal 7
473	No alteration (A/N)	Mal 5	-
620	No alteration (C/G)	Mal 7	-
621	N→H (A/C)	-	Mal 7
637	Q→L (A/T)	-	Mal 1, 8, 9

AA genotypes were present. These data are consistent with the observations at V_{136} which is considered a rare allele in sheep population. The prevalence of this allele in indigenous sheep is indicative of the susceptibility of indigenous sheep to scrapie; however, no report has so far been published where any animal carrying this allele contracted the disease. This could have been either due to small sample size or due to the other management practices that could be important in prevention of onset of disease conditions. At codon 154 only RR genotypes were present. 'Q' allele at 171 loci alone has been reported to be positively correlated with naturally occurring incidence of scrapie in sheep (Hunter *et al.* 1994 and De Silva *et al*. 2003). In present study all the genotypes at locus 171 were of QQ type, in homozygous condition but none of the animal showed any clinical symptoms of scrapie so far. In a study it was documented by Zlotnik and Katiyar (1961), 4 animals died because of scrapie infection. However, their genotypes were not studied using molecular markers. These results suggested that either some mutation or polymorphism at other codons or loci is protecting the animals from getting infected with prion protein or it is the management practices of sheep rearing solely on range grazing without any concentrate supplements probably prevents the onset of scrapie disease symptoms. These results have important bearing on prevention of scrapie disease in sheep globally through traditional management of sheep on forages. It has been very well documented even in BSE outbreaks in cattle population of United Kingdom that the animals fed on animal protein only showed the symptoms of mad cow syndrome.

Two genotypes HH and RR were observed at codon 114 in Malpura sheep breed with genotypic frequencies 88.89 and 11.11% respectively. The frequencies of GG, GS and SS genotypes at codon 127 in Malpura sheep were 55.56, 11.11 and 33.33% respectively. The frequencies of NN and HH genotypes at codon 184 were 88.89 and 11.11% respectively. At codon 189 three genotypes QQ, LL and QL were present in Malpura sheep breed in the frequencies of 66.67, 22.22 and 11.11% respectively. These are new SNPs which were observed in this indigenous breed as no earlier studies reported this polymorphism. Our results are in confirmation with the earlier reports of PCR-SSCP variants in PrP gene at

exon-3 in other breeds (Zhou *et al.* 2005). Authors found that unlike earlier methods viz. PCR-RFLP which determine PrP genotype by detecting the nucleotide sequence at specific positions, this PCR-SSCP technique profiles an extended region of exon-3, the key exon in determining PrP genotype. This endows the PCR-SSCP method with technical advantage over the other methods; it is able to determine the A/V_{136}-H/R_{154}-H/Q/R/K_{171} haplotype, which is considered important in determining scrapie susceptibility (Baylis and Goldmann 2004). The PCR-SSCP approach is typically less expensive, particularly, when large numbers of samples require analysis.

In total 7 SNPs have been observed in this study including the principal SNPs at 136, 154 and 171 codons. The significance of the polymorphism at other codons in prevention of scrapie disease susceptibility in indigenous breed cannot be ruled out because sheep carrying known susceptible alleles at 136 AA, at 154 RR and at 171 QQ did not show any symptoms of naturally occurring scrapie. Similar views were also held by Westaway *et al.* (1994) and Hunter (1996). Further these results carry specific significance in confirming the earlier concept of Hunter *et al*. (1994) where it has been suggested that despite the positions of susceptible alleles in animal may not show the symptoms of naturally occurring scrapie because additional factors might be involved in control of scrapie including genotype×environment interactions. In this case, our results are of special significance and can have far reaching consequences in preventing of scrapie disease symptoms in sheep globally. Before, this concept is applied globally, more detailed genetic survey in larger population is warranted by molecular markers using high throughput screening protocols.

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