RESEARCH NOTE

Genetic evidence for susceptibility and resistance against scrapie in Indian sheep

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Introduction

Scrapie is a fatal, neurodegenerative disease of sheep and goats (Prusiner 1982). The cause behind the disease is infection by prion proteins (Prusiner 1982). The progression of scrapie is known to be influenced by the amino acid polymorphism of the host prion protein (*PRNP*) gene (Belt *et al*. 1995). The present study was carried out on Indian sheep breeds i.e., Karnah, Mandya, Malpura and Garole to identify polymorphisms of the *PRNP* gene at codons (136, 154 and 171) responsible for the susceptibility and resistance of the scrapie disease in sheep. The known risky allele (ARQ) was found to be the most frequent (77.75%). The most resistant (ARR) and most susceptible (VRQ) alleles were found equally distributed with a frequency of 1.75%. Although, most of the alleles were known to confer susceptibility or partial susceptibility to scrapie, yet so far none of the Indian sheep breeds showed any clinical symptoms of the disease. The possible reason could be genotype–environment interaction, management practices and/or resistance of Indian sheep to scrapie via some other mechanism.

Fatal neurodegenerative prion diseases are both genetic and infectious to humans and other mammals (Prusiner 1991). Scrapie is considered as a prototype of various forms of prion diseases that include bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt–Jakob disease in human (Prusiner 1991). It is characterized by the accumulation of an abnormal isoform of the *PRNP* in the central nervous system (Prusiner 1991). Scrapie is a rare, contagious, slowly progressive, endemic brain disease in sheep and goats (Prusiner 1998; Hadlow 1999). The likelihood of progression to disease and the incubation period are strongly linked to at least three polymorphisms in the *PRNP* gene at positions 136,154 and 171 (Hunter 1997).

Several studies have investigated the occurrence of scrapie in European (McGowan 1922) and American (APHIS 2001) sheep breeds. Although, sheep play an important role in livelihood of large proportion of small and marginal farmers and landless labourers in India, yet there is no comprehensive study about the occurrence of scrapie in indigenous sheep breeds. India ranks the fourth in the world with 50.8 million sheep which is 4.57% of the world sheep population (Kumar *et al.* 2007). Sheep in India are the source of about 169 million kg of meat, 42.7 million kg of wool and 40 million kg of skin production annually (Kumar *et al.* 2007). The diversity of geo-climate over the ages has manifested into diversity of sheep breeds (Acharya 1982). The aims of this study are i) to identify susceptible and resistant alleles with regard to scrapie; and ii) to ascertain the allelic frequencies of *PRNP* gene in four representative Indian sheep breeds; from different climatic and geographical regions.

Materials and methods

Sample collection and DNA extraction

Blood samples of 200 animals belonging to four different Indian sheep breeds (Karnah, Mandya, Malpura and Garole) were collected from their native breeding tracts. For avoiding narrow genetic relationships among the sampled animals, only one sample was collected per small flock (<20). Approximately 10 mL of blood was obtained by jugular vein puncture and stored in vacutainers (vacuum tubes) treated

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with 15% ethylenediaminetetraacetic acid (EDTA) as an anti-coagulant. DNA was extracted from blood as described by Sambrook *et al.* (1989) with minor modifications. The final working dilutions were prepared of 50 ng/μL concentration after checking the DNA yield for quality and quantity.

Primer designing

In silico data mining was performed and all *PRNP* sequences (GenBank accession number AJ000736, AJ000737 and AJ000738) were aligned to obtain longest sequence, that was used further as a template for primer designing. Three single-strand conformation polymorphism (SSCP) primer pairs were designed (5′-TCT TAC GTG GGC ATT TGA TG-3′, 5′-TGA CTG TGG CTA CCA CCT TG-3′, 5′-CAA GGT GGT AGC CAC AGT CA-3′, 5′-CCA CCA CTC GCT CCA TTA TC- 3′, 5′-GAT AAT GGA GCG AGT GGT GG-3′, 5′-GCT TGT CAT TTC CCA GTG CT-3′) to amplify three fragments (480, 358 and 310 bp) of exon 3 of the ovine *PRNP* gene using Primer3 (Razen and Skaletsky 2000). The primer pairs were covering a part of intron 2, complete exon 3 and a part of intron 3. Primers were checked for their thermodynamic properties and secondary structures using Primer select (DNA Star-Lasergene® ver. 7.2 software). The designed primers were reanalysed by basic local alignment search tool (BLAST) analysis (http://www.ncbi.nlm.nih.gov/BLAST) to ensure their respective homology over ovine genome.

PCR-SSCP analysis

PCR amplifications were carried out using 50 ng of genomic DNA template in MJ PTC 100 thermal cycler (MJ Research, Waltham, USA). Amplifications were verified on agarose gel (2%) electrophoresis (w/v) with 1x Tris acetate EDTA (TAE) buffer using a 100 bp ladder as a molecular weight marker. For SSCP, the PCR products were denatured at 85°C, followed by immediate snap-cooling. The PCR products were resolved by SSCP analysis. Several factors were tested for each fragment to optimize conditions, such as amount of PCR product, denaturing solution, acrylamide concentration, percentage cross-linking, glycerol, voltage, running time and temperature as described by Jain *et al.* (2009). The SSCP analysis was carried out as follows: each PCR product was diluted in denaturing solution (95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue, 20 mM EDTA), denatured at 95°C for 5 min, chilled on ice and resolved (10 h, 375 V, 4°C) on 12% acrylamide : bisacrylamide gels (29:1). Electrophoresis was carried out in a Biorad Protean II xi vertical electrophoreses unit using 1× Tris-borate-EDTA (TBE) buffer. Gels were silver-stained (Sambrook and Russell 2001) and dried on cellophane paper sheet using a Biorad (Hercules, California, USA) gel dryer (model 583). The gel phenotypes were identified and scored manually to obtain PCR-SSCP allele frequency.

PCR cleanup and sequencing

The PCR-SSCP amplicons were identified for sequencing based on different band patterns/haplotypes observed on gels. Sequencing was done in duplicates using another primer pair (forward 5′-CAA GGT GGT AGC CAC AGT CA-3′ and reverse 5′-CCA CCA CTC GCT CCA TTA TC-3′). PCR products were treated with shrimp alkaline phosphatase (1 U/mL) and exonuclease III (10 U/mL) in the duplicates, incubated at 37°C for 30 min followed by 80°C for 10 min. Purified PCR, 1–5 μL, product was used in sequencing. The sequencing PCR products were loaded into a 96 well-format and analysed on ABI 370 DNA analyser (Applied Biosystems, Foster City, USA). The quality of the sequence was assured by the PHRED (Ewing and Green 1998; Ewing *et al.* 1998).

In silico sequence analysis and allele mining

All sequences were analysed using DNA star (DNA Star-Lasergene® v7.2 software) for SNP identification (http://www.dnastar.com/). BLAST analysis was also performed to check the identity of sequences. The amino acid codons were also identified using online sequin software. Sequences have been submitted to GenBank, (NCBI, USA) with accession numbers KF188784–KF188802 and KF207876–KF207879. Extensive allele mining of *PRNP* gene was done to search nucleotide variations between species and within breeds of sheep. The sequences of ovine, caprine, bovine, bubaline and human were compared for homology.

Results

In this study, we selected four different Indian sheep breeds; belonging to different geographic areas and climatic conditions. We selected Karnah sheep from Jammu and Kashmir, Mandya sheep from Karnataka in southern peninsular region, Malpura sheep from western hot arid and semiarid region of Rajasthan and Garole sheep from West Bengal, the eastern part of India. Farmers and flock owners of sheep were interviewed while collecting blood samples to record incidences of scrapie. 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 the sheep in the sampled flocks showed any symptom of scrapie nor they have received any case of clinical scrapie alive or carcass for postmortem.

PCR-SSCP profile

Genetic variability in prion protein genes were assessed by PCR-SSCP. SSCP banding pattern for *PRNP* gene were found to be of 5, 6, 8 and 7 kinds in Karnah, Mandya, Malpura and Garole sheep breeds, respectively.

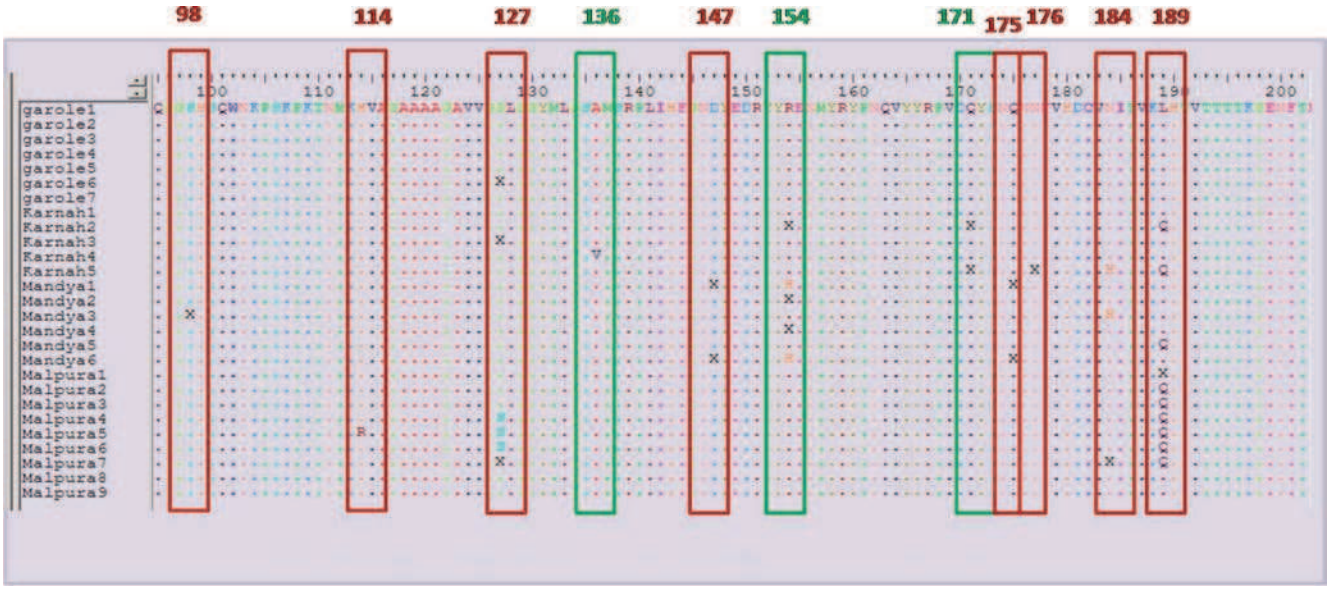

Figure 1. Amino acid sequences showing polymorphism at different codon numbers: amino acids are represented by dots which show alignment with each other. Different colours indicate different amino acids: green, principle scrapie codons; red, changes at new codon positions.

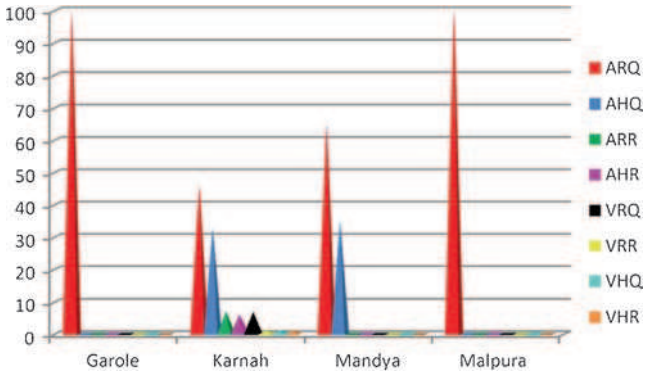

Figure 2. Allelic frequencies for the *PRNP* gene in Indian sheep breeds.

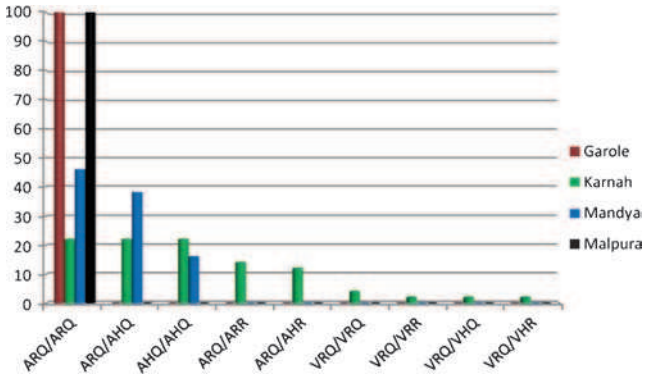

Figure 3. Genotypic frequencies for the *PRNP* gene in Indian sheep breeds.

Sequencing, SNP identification and protein translation

The DNA samples showed different patterns on SSCP gels were sequenced (26 samples) to screen the SNPs in this region having nucleotides at 136, 154 and 171 positions which are considered important for scrapie disease susceptibility/resistance. All mutations including deletions, insertions and substitutions were noted and analysed.

Polymorphism was observed at codons 114, 127, 136, 147, 154, 171, 175, 176, 184 and 189. Only Karnah and Mandya sheep breeds were showing polymorphism for principle *PRNP* locus. The deduced amino acid sequences based on Indian sheep *PRNP* gene exon 3 (partial CD's) are presented in figure 1.

Discussion

Scrapie belongs to the most intriguing group of diseases. The study of scrapie is important due to its contagious nature from animals and humans. There is a well-established association between sheep *PRNP* genotype and the risk of death from scrapie (Baylis and Goldmann 2004). Certain genotypes are clearly associated with susceptibility to the disease and others to resistance. The association between scrapie susceptibility and the *PRNP* with 136(A/V), 154(R/H) and 171(Q/R/H/K) alleles are very strong (Baylis and Goldmann 2004). The greatest scrapie risk observed for the VRQ-encoding genotypes, ARQ/VRQ, ARH/VRQ and VRQ/VRQ. The next risky genotype was ARQ/ARQ. The ARR/ARR genotype was the only high resistance genotype to scrapie. Indian sheep breeds analysed for scrapie showed that the ARQ allele was the most frequent (100%) allele in Garole and Malpura. No polymorphism was observed for *PRNP* locus in these two breeds. The high risky allele VRQ and high resistance allele ARR were found in only Karnah sheep (figure 2). AHQ allele was present in Karnah

and Mandya with 33% and 35% frequencies, respectively. Other alleles ARR, AHR, VRQ, VRR, VHQ and VHR were reported in Karnah sheep only.

The ARQ/ARQ was the most frequent (77.75%) genotype (figure 3); which is experimentally challenged with either scrapie or BSE (Vaccari *et al.* 2007). Malpura and Garole were having only the high risky genotype of ARQ/ARQ whereas 22% animals of Karnah breed and 46% animals of Mandya breed were having this genotype. The ARQ/ARQ genotype appears to be the most susceptible to scrapie governing genotype reported in Suffolk sheep, French Romanov sheep (Elsen *et al.* 1999) and in Texel sheep in UK (Hunter *et al.* 1997; Baylis *et al.* 2002). The ARQ/ARQ genotype appears to be resistant to scrapie in the NPU flock of Cheviot sheep (Hunter *et al.* 1996).

Second genotype observed was AHQ/AHQ, which is considered as a little susceptible genotype for the scrapie (Arsac *et al.* 2007). The third genotype was ARQ/AHQ which is found to be partially resistant in Greece sheep (Billinis *et al*. 2004). Recently, a novel strain of scrapie was detected in Norway that resulted in a different pattern of PrP^{Sc} deposition and Western blot glycoprotein profile (Benestad *et al*. 2003). Natural scrapie in Norway has been reported previously to be strongly associated with the VRQ allele (Tranulis *et al.* 1999), while the new strain has so far affected only the AHQ/AHQ and ARQ/AHQ alleles. These two genotypes were present in only two breeds Karnah (22%) and Mandya (38% ARQ/ARH and 16% AHQ/AHQ), although no animal showed any symptom of scrapie. ARQ/ARR genotype was observed in 14% animals of Karnah sheep which was considered as genetically resistant to scrapie. The remaining genotypes were present in 0.50% to 3.00% frequencies.

Sheep breeds used in this study represent most of all geographic regions and climatic conditions of India. Karnah belongs to northern high altitude temperate Himalaya where mercury drops down to −40°C, Malpura is from hot arid and semiarid region where temperature is almost 50°C, Mandya from hot and humid southern peninsular region, and Garole has unique attributes of prolificacy and survive from plains of Bengal and Sundarbans delta. Despite having such harsh and diverse climatic conditions, occurrence of scrapie in these sheep breeds are not yet reported.

The prevalence of these genotypes in indigenous sheep is indicative of the susceptibility of indigenous sheep to scrapie; however no report has so far been published where any Indian sheep carrying these genotypes contracted the disease. The possible reasons could be, i) inherent resistance of the animals to scrapie, ii) some other polymorphism protecting the animals from prion infections, and iii) management practices of sheep rearing solely on range grazing without any concentrate supplements are preventing the animals from infections. 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.

These results have important bearing on deterrence of scrapie disease in sheep globally through traditional management of sheep on forage. It has been well-documented even in case of BSE outbreaks in cattle population of UK that the animals fed on animal protein only showed the symptoms of mad cow syndrome (Columbus 2004). In this case, this study has special significance and can have far-reaching implications in preventing scrapie disease symptoms in sheep globally. More detailed genetic survey in larger population is warranted by molecular markers using high throughput screening protocols to apply the concept widely.

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