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PRION PROTEIN GENE SEQUENCES ANALYSIS IN TWELVE SHEEP BREEDS OF PAKISTAN

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ABSTRACT

Prions are considered the only agents of transmissible spongiform encephalopathies (TSEs) and are harmful pathogens of mammals. These infectious agents of host are made up through aggregation of conformational isomers (PrPSc) and encode glycoprotein (PrPC) of 33-35 kDa. TSEs are the fatal group of diseases which are neurodegenerative and include chronic wasting disease in deer and elk, Creutzfeldt-Jakob disease (CJD) and transmissible mink encephalopathy (TME) in humans and scrapie in goats and sheep. The accumulation of abnormal form of the normal protein (PrP) is common in all diseases related TSE. This abnormal form of PrP called PrPSc is resistant to proteolysis as well as infectious. Present study was conducted in order to do sequence analysis of prion protein gene in twelve breeds of the sheep. We studied this gene to elucidate 12 of Pakistani sheep breeds and to compare gene order with other mammalian species. PCR amplification of 771 bp fragment was done on selected samples from all twelve breeds followed by sequencing. Sequence analysis was done and some sites were found to be heterozygous. These findings on prion protein gene in sheep will provide assistance for further studies on pathogenesis, cross-species transmission, breeding programs, resistance and susceptibility to scrapie.

Key words: Prions, TSEs, scrapie, neurodegenerative, sheep

INTRODUCTION

Prion diseases are a group of rapidly progressive, and fatal neurologic diseases which are commonly known as transmissible spongiform encephalopathies (TSEs). Infectious agent of these diseases, a protein as suggested by (Griffith, 1967; Bolton et al., 1982), was isolated as a protease-resistant sialoglycoprotein through the use of progressive enrichment of brain homogenates for infectivity. This protein is major component in the infective brain fractions and was shown to accumulate in the affected brains (Griffith, 1967; Bolton et al, 1982; Prusiner et al, 1982). The term prion was coined by Prusiner in 1982 in order to differentiate these proteinaceous infectious particles from viroids or viruses. The protein component of these particles was then recognized as Prion protein (PrP) and for this work

Prusiner was awarded Nobel Prize (et al., 2003). Prions, the smallest and simplest infectious pathogens, have no nucleic acid. While all other infectious agents like fungi, bacteria and viruses have genomes composed of RNA or DNA that direct the synthesis of their progeny (Pramood et al., 2003). The unique feature of prion diseases is the abnormal metabolism of the prion protein (PrP), as this protein is in two conformational states with various physicochemical properties. PrPC is the normal form of protein and is cell surface protein attached through glycophosphatidyl inositol anchor. This protein is highly conserved and expressed in different types of cells, specifically in neuronal cells. The molecular weight of PrPC is 33 to 35 kDa with high content of β-helical secondary structure, and it is soluble in detergents and sensitive to protease treatment. In the early embryogenesis, the PrPC is expressed while in adults it occurs in spinal cord and in neurons of the brain at the highest level (McKintosh et al. 2003). PrPC is also found in number of peripheral cell types and in glial cells of the CNS at lower levels (Moser et al, 1995; Ford et al, 2002). PrPC molecules normally present on the surface of cell attached to lipid bilayer through C-terminal, glycosyl-phosphatidylinositol (GPI) anchor (Stahl et al., 1987). The biosynthetic pathway of PrPC is exactly the same as of other membrane and secreted proteins which includes synthesis on rough ER, transfer to the Golgi, and then delivery to surface of the cell (Harris et al., 2004). PrPC is considered to be important protein in the context of biological functions due to its highly conservation in evolution. Therefore, the investigation of the biological activity of PrPC is very important for understanding the pathogenesis of prion diseases as change in its function could play a crucial role in the process of disease (Westergard et al., 2007). PrPSc is the disease associated isoform and is only present in the brains as an aggregated form. It has high content of β-sheet secondary structure and partially resistant to protease treatment and is not soluble in detergents (McKintosh et al., 2003). Diseases related prion occur due to the conversion of normal, cell surface glycoprotein (PrPC) into conformationally altered isoform (PrPSc) (Westergard et al., 2007). This conversion of PrPC to PrPSc is the basic reason of prion diseases (David et al., 2011). It is very important to develop novel diagnostic tools, due to the lackability of prion-directed immune response, for early prion disease diagnosis (Hussein and Al-Mufarrej, 2004).

Prion infections have been able to cross-species transmission to have infected human beings. Therefore, there is an urgent need to develop safe and effective vaccines against these fatal and currently incurable diseases (Mabbott, 2014). Although the molecular mechanisms of prion disease pathogenesis remains unclear, single-nucleotide polymorphisms (SNPs) of PrP were found to be associated with the incubation period, susceptibility, and species barrier to the scrapie disease in sheep (wang 2009). Sheep is an important meat animal of Pakistan and prion disease if present could be a public health threat. There were no reports on prion gene sequence and polymorphism analysis in Pakistani sheep breeds. In the present study, blood samples were collected from different sheep breads all over Pakistan, and PrP gene sequencing analysis was carried out. The

findings on sequence analysis could assist in breeding programs and TSE pathogenesis and cross species transmission studies.

MATERIALS AND METHODS

Sample collection:

Five mL blood was collected in falcon tubes containing 200 µL anticoagulant i.e. Ethylenediamine tetra-acetic acid (0.5 M EDTA) from all four provinces of Pakistan, Azad Jammu and Kashmir region (AJK) from different sheep breeds (Table 1). Proper record was maintained containing the information regarding the breed, animal ID, age, sex and location of animal. Field blood samples were placed on ice immediately after their collection and brought to the Molecular Biology and Genomic Laboratory and stored temporarily in freezer at -20 °C before DNA extraction.

DNA isolation:

Genomic DNA was extracted from blood samples using standard protocol, which involved RBCs lysis, protein digestion, and precipitation followed by DNA isolation and purification. The DNA samples were dissolved in Tris-EDTA(TE) buffer (pH 8.0) and stored at -20 °C for further use. Quantification of the DNA of the collected samples was done with the help of agarose gel electrophoresis (0.8 %). Standard DNA/ DNA ladder was used for simultaneous and accurate determination of amplified DNA fragment size. All samples were brought to same concentration level i.e. Approx. 50 ng/ µL.

Primer designing:

For the amplification of Prion protein gene (PrP); specific primers were designed from already reported sequences (Acession No. DQ346682) available on GenBank, National Centre for Biotechnology Information (NCBI) using Primer 3 software and in-silico PCR web facility (Rozen and Skaletsky, 2000) using the reference sequences of this gene.

PCR amplification and sequencing:

The PCR amplification of 876 bp containing the entire coding region in exon 3 of the PrP gene was carried out using forward (5'-1CTTTAAGTGATTTTTACGTGG21-3') and reverse (5'-854TGGCAAAGATTAAGAA GATAATG876-3') primers. Sequencing was done using ABI Genetic Analyzer 3130 XL (Applied Biosystems, USA).

RESULTS AND DISCUSSION

Sequence alignment and analysis:

Sequence alignment was done by using CodonCode Aligner software. 771 bp portion of PrP gene was aligned in all sheep breed and SNPs (single nucleotide polymorphisms) identification were done. 670 bp portion was selected for phylogenetic tree construction with other mammalian species (listed in table 2) by MEGA6 using UPGMA method with 1000 bootstrap value.

Table 1. SNPs Identified in the PrP gene in Sheep breeds.

Breed _sample no	SNPs Position	SNP Nucleotide	Change with	Transition/ Transversion
Blochi_06	572	C	Y	Transition
	691	A	M	Heterozygous
	711	C	S	Heterozygous
Blochi_11	511	C	R	Transversion
Blochi_12	437	A	R	Transition
Balochi_21	512	A	G	Transition
Blochi_46	512	A	G	Transition
Blochi_61	691	A	M	Heterozygous
	711	C	S	Heterozygous
Blochi_83	379	G	A	Transition
Blochi_84	513	G	K	Heterozygous
	691	A	M	Heterozygous
	711	C	S	Heterozygous
Blochi_94	414	C	T	Transition
	428	A	G	Transition
	718	T	C	Transition
Balochi_98	404	G	R	Transition
	414	C	T	Transition
	718	T	C	Transition
Bulkhi_09	380	G	K	Heterozygous
Bulkhi_18	380	G	T	Transversion
Bulkhi_19	380	G	K	Heterozygous
Dumri_01	513	G	K	Heterozygous
	691	A	C	Transversion
	711	C	G	Transversion
	512	A	R	Transition
Dumri_02	512	A	G	Transition
Dumri_03	379	G	A	Transition
Dumri_07	691	A	M	Heterozygous
	711	C	S	Heterozygous
Dumri_11	513	G	K	Heterozygous
	691	A	M	Heterozygous
	711	C	S	Heterozygous
Dumri_10	513	G	K	Heterozygous
	711	C	S	Heterozygous
Dumri_12	379	G	R	Transition
	512	A	R	Transition
Dumri_16	512	A	G	Transition
Kaghani_01	379	G	R	Transition
	566	A	W	Heterozygous
Kaghani_04	566	A	W	Heterozygous
Kaghani_06	566	A	W	Heterozygous
Kaghani_10	414	C	T	Transition
	718	T	C	Transition
Kaghani_11	512	A	R	Transition
	566	A	W	Heterozygous
Kaghani_13	379	G	A	Transition
Kaghani_15	566	A	W	Heterozygous
Kaghani_16	379	G	R	Transition

	566	A	W	Heterozygous
Kaghani_17	437	A	G	Transition
Kaghani_19	437	A	R	Transition
Khail_106	512	A	R	Transition
	513	G	A	Transition
	691	A	M	Heterozygous
	711	C	S	Heterozygous
Khail_114	566	A	W	Heterozygous
Khail_123	691	A	C	Transversion
	711	C	G	Transversion
Khail_128	379	G	R	Transition
Koka_01	455	A	T	Transversion
	566	A	W	Heterozygous
Koka_02	566	A	W	Heterozygous
Koka_03	566	A	T	Transversion
Koka_04	566	A	W	Heterozygous
Koka_05	379	G	R	Transition
Koka_06	566	A	W	Heterozygous
Koka_08	513	G	K	Heterozygous
	566	A	W	Heterozygous
	691	A	M	Heterozygous
	711	C	G	Transversion
Koka_09	566	A	W	Heterozygous
Koka_10	566	A	W	Heterozygous
	691	A	M	Heterozygous
	711	C	S	Heterozygous
Koka_11	379	G	R	Transition
	566	A	W	Heterozygous
Koka_12	455	A	T	Transversion
Koka_14	566	A	W	-
Koka_15	566	A	T	Transversion
Mangli_05	512	A	R	Transition
Mangli_11	-	-	-	
Mangli_16	-	-	-	
Mangli_21	414	C	Y	Transition
	428	A	G	Transition
	718	T	Y	Transition
Mangli_38	379	G	R	Transition
	691	A	M	Heterozygous
	711	C	S	Heterozygous
Mangli_54	379	G	R	Transition
	511	C	M	Heterozygous
Mangli_55	437	A	R	Transition
Mangli_57	379	G	R	Transition
	572	C	Y	Transition
Mangli_58	-	-	-	-
Mangli_64	512	A	R	Transition
	566	A	W	Heterozygous
Mangli_65	566	A	W	Heterozygous
Poonchi_05	566	A	T	Transversion
Poonchi_06	513	G	K	Heterozygous
	691	A	C	Transversion
	711	C	G	Transversion
Poonchi_08	512	A	R	Transition

	566	A	W	Heterozygous
Poonchi_09	379	G	R	Transition
Poonchi_12	407	C	Y	Heterozygous
	461	G	R	Transition
Poonchi_15	512	A	R	Transition
	691	A	M	Heterozygous
	711	C	G	Transversion
Poonchi_16	512	A	G	Transition
Rakhshani_05	711	C	S	Heterozygous
Rakhshani_07	511	C	R	Transversion
	512	A	R	Transition
Rakhshani_10	513	G	K	Heterozygous
	691	A	M	Heterozygous
	711	C	S	Heterozygous
Rakhshani_13	513	G	K	Heterozygous
	691	A	M	Heterozygous
	711	C	G	Transversion
Rakhshani_17	-	-	-	-
Rakhshani_18	512	A	R	Transition
	566	A	W	Heterozygous
Ramboullet_01	512	A	G	Transition
Ramboullet_23	511	C	M	Heterozygous
Ramboullet_33	512	A	R	Heterozygous
Ramboullet_37	512	A	R	Transition
	566	A	W	Heterozygous
Ramboullet_44	461	G	R	Transition
	691	A	M	Heterozygous
	711	C	G	Transversion
Ramboullet_54	566	A	W	Heterozygous
	691	A	M	Heterozygous
Salt Range_01	379	G	R	Transition
Salt Range_02	512	A	R	Transition
	691	A	M	Heterozygous
	711	C	S	Heterozygous
Salt Range_03	437	A	R	Transition
Salt Range_04	513	G	T	Transversion
Salt Range_05	566	A	W	Heterozygous
Salt Range_06	-	-	-	-
Salt Range_07	513	G	K	
	691	A	M	Heterozygous
	711	C	S	Heterozygous
Salt Range_09	437	A	R	Transition
Salt Range_10	379	G	R	Transition
	566	A	W	Heterozygous
Salt Range_13	379	G	R	Transition
	513	G	K	Heterozygous
	691	A	M	Heterozygous
	711	C	S	Heterozygous
Salt Range_14	566	A	W	Heterozygous
	691	A	M	Heterozygous
	711	C	S	Heterozygous
Salt Range_15	513	G	T	Transversion
	691	A	M	Heterozygous
	711	C	G	Transversion

Shenwari_04	-	-	-	-
Shenwari_07	566	A	W	Heterozygous
	691	A	M	Heterozygous
	711	C	S	Heterozygous
Shenwari_09	566	A	W	Heterozygous
Shenwari_11	-	-	-	-
Shenwari_21	379	G	R	Heterozygous
Shenwari_22	-	-	-	-
Shenwari_24	512	A	R	Heterozygous
Shenwari_33	566	A	W	Heterozygous
	572	C	Y	Heterozygous
	691	A	M	Heterozygous
	711	C	S	Heterozygous
Shenwari_32	512	A	R	Transition
	566	A	W	Heterozygous

Phylogenetic analysis:

A Phylogenetic tree using MEGA6 software (Tamura et al., 2011) was constructed from prion protein gene in twelve sheep breeds of Pakistan and some species were taken as an outgroup. The tree showed different clades with branching and re branching pattern.

Table 2. Samples collected from different sheep breeds at different locations

Breeds	**Province**	**Purpose**	**Samples(n)**
Salt Range	Punjab	Meat and wool	12
Bulkhi	Khyber Pakhtunkhwa	Meat and wool	11
Kaghani	Khyber Pakhtunkhwa	Meat and wool	10
Koka	Sindh	Meat and wool	13
Balochi	Balochistan	Meat and wool	11
Dumri	Balochistan	Meat and wool	10
Mangli	Balochistan	Meat and wool	11
Rakhshani	Balochistan	Meat and wool	6
Shenwari	Balochistan	Meat and wool	9
Kail	AJK	Meat and wool	12
Poonchi	AJK	Meat and wool	11
Ramboullet	AJK	Meat and wool	13
			129

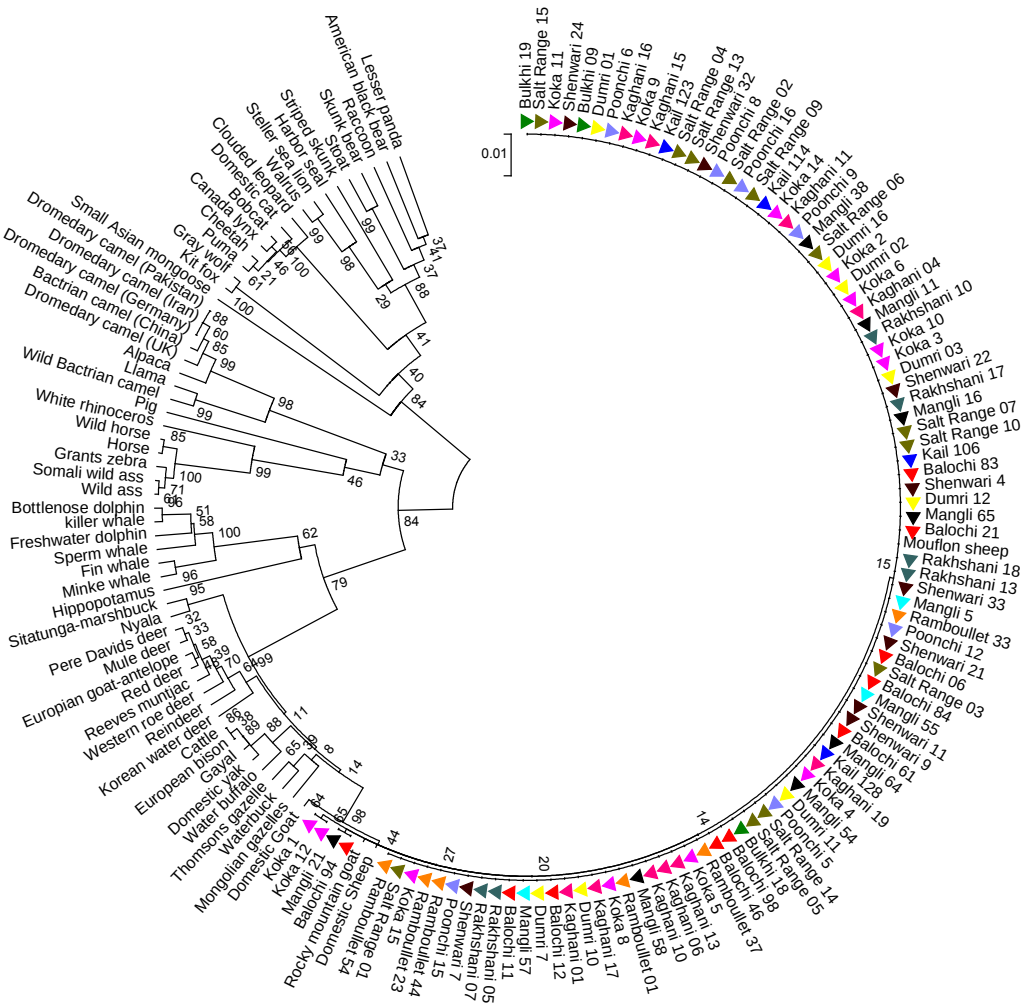

Figure 1: Neighbor Joining (circular) Phylogenetic Tree for Prion protein gene (MEGA6) of twelve Pakistani sheep breeds with other mammalian PrP sequences available on GenBank NCBI.

Our study leaded to some of great important findings and exemplifies the first screening made on prion gene sequence and polymorphism analysis in Pakistani sheep breeds. The most striking result to emerge from the data is that 6 single nucleotide polymorphism was observed at position 379, 380, 404, 407, 414, 428, 437, 455, 461, 511, 512, 513, 566, 572, 691, 711, 718 using software MEGA 6 for conducting sequence alignment. Further, some of these mutations were of transitional nature and some were transversional one. Furthermore, pylogenetic tree was constructed to infer the evolutionary relationships among prion protein gene sequence in different species. Interestingly, the more distant species doesn't share the same clade. By comparing PrP coding sequences of goats with those of sheep,

high genetic conservation was revealed. This result is in line with that of Goldmann et al.(1996).

The common name for transmissible spongiform encephalopathy (TSE) is scrapie that affects goats, mouflfons and sheeps of the world. As all prion diseases, scrapie is also fatal and neurodegenerative disease. This disease is associated with different signs which may be of neurological or behavioral type abnormalities. Immunodetection of PrPSc depositions in central nervous system using biochemical and immunohistochemical methods is primarily required in order to diagnose disease. The findings of this study have a number of important implications for developing diagnostic markers for prion diseases, and providing assistance for further studies on pathogenesis, cross-species transmission, breeding programs, resistance and susceptibility to scrapie.

Prion ailments are infrequent neurodegenerative conditions bringing about exceptionally variable clinical disorders, which regularly incorporate unmistakable neuropsychiatric manifestations. (Thompson et al) did a clinical investigation of behavioral and psychiatric indications in a substantial planned companion of patients with prion disease in the United Kingdom, permitting us to operationalise particular behavioral/psychiatric phenotypes as qualities in human prion infection. They particularly analyzed a determination of competitor SNPs that have indicated expansive relationship with psychiatric conditions in beforehand distributed studies, and the codon 129 polymorphism of the prion protein quality, which is known not different parts of the phenotype of prion sickness. No SNPs achieved all inclusive centrality and there was no confirmation of modified weight of known psychiatric danger alleles in applicable prion cases. SNPs demonstrating suggestive confirmation of affiliation incorporated a few lying close qualities already involved in affiliation investigations of other psychiatric and neurodegenerative maladies. These incorporate ANK3, SORL1 and a district of chromosome 6p containing a few qualities ensnared in schizophrenia and bipolar disorder.

Prion sicknesses are a differing gathering of neurodegenerative conditions, brought about by the templated misfolding of prion protein. Beside the strong genetic hazard presented by different variations of the prion protein quality (PrPn), a few different variations have been proposed to give hazard in the most well-known sort, sporadic Creutzfeldt-Jakob sickness (sCJD) or in the obtained prion maladies. Substantial and uncommon duplicate number variations (CNVs) are known not hazard in a few related issue including Alzheimer's malady (at APP), schizophrenia, epilepsy, mental hindrance, and a mental imbalance. Lukic et al. (2016), reported the main all inclusive investigation for CNV-related danger utilizing information got from a late universal synergistic affiliation study in sCJD (n = 1147 after quality control) and openly accessible controls (n = 5427). A cell-based prion contamination test did not give steady confirmation to a part for PARK2 in prion ailment defenselessness. This information are steady with an unobtrusive effect of CNVs on danger recently onset neurologic conditions and propose that, not at all like APP, PRNP duplication is not a causal high-hazard change.

A similar type of study was conducted by Hussain et al. [4] for finding novel polymorphisms in prp gene of two Pakistani sheep breeds (Damani and Hashtnagri) and two Pakistani goats breeds (kamori and local hairy). They broke down the PrP quality succession to decide the recurrence of polymorphisms in 56 sheep (28 each from Damani and Hashtnagri breeds) and 56 goats (28 each from Kamori and Local Hairy breeds). An aggregate of 7 amino corrosive polymorphisms were recognized in the PrP quality for sheep and 4 for goats. These amino corrosive polymorphisms were joined in 13 alleles and 15 genotypes in sheep and 5 alleles and 6 genotypes in goats. The general recurrence of the most sheep scrapie-safe polymorphism (Q171R) was ascertained to be 0.107. The most scrapie- susceptible polymorphism (A136V) was not identified in any of the concentrated on sheep. The general recurrence of scrapie-related polymorphism (H143R) in goats was observed to be 0.152. Alongside already known amino polymorphisms, two novel polymorphisms were additionally recognized for each of sheep (Q171N and T191I) and goats (G22C and P63L).

There is a settled relationship between sheep prion protein (PrP) genotype and the danger of death from scrapie. Certain genotypes are plainly connected with vulnerability to the infection and others to resistance. In the established structure, scrapie susceptibility is very identified with changes in particular amino acids that prompt a modified type of the prion protein (PrPsc). Polymorphisms in the host-encoded prion quality (PRNP) are real determinants of susceptibility to exemplary scrapie, with varieties at codons 136, 154, and 171 passing on variable degrees of resistance. There are more than 15 polymorphisms reported in PrPn Of these, just three codons (codon 136, 154 and 171) have been accounted for to influence susceptibility to the illness. Susceptibility to ovine scrapie is likewise controlled by the infective scrapie strain. Two strains of scrapie have been characterized. Sort A produces the ailment in sheep that are either homozygous or heterozygous for a valine at codon 136 while sort C causes illness in sheep that are homozygous for a glutamine at codon 171.

The codon 136, valine (V) is connected with high scrapie vulnerability while alanine (An) is connected with low susceptibility, despite the fact that this may rely on upon the strain of scrapie operators (Goldmann 1994). At codon 154, arginine (R) is connected with susceptibility while histidine (H) is connected with halfway resistance. At codon 171, glutamine (Q) and histidine (H) are connected with vulnerability while arginine (R) is connected with resistance (Baylis et al. 2004). Codon variations at positions other than 136, 154, and 171 are additionally connected with scrapie resistance. A M112T variation on the ARQ haplotype has been connected with scrapie resistance in orally-immunized Suffolk sheep in the U.S. (Laegreid et al. 2008). M137T and N176K variations on the ARQ haplotype have been connected with scrapie resistance in intercranially-inoculated, orally-inoculated, and naturally-infected Italian Sarda breed sheep. Varieties at codon 141 can be identified with the atypical scrapie structure Nor98.

Zečević et al. conducted a similar study on the Pramenka sheep breed, Vlasic (Dubski) strain, of Bosnia and Herzegovina. Their research showed that locus

(codon) 171, which is responsible for the synthesis of four amino acids (arginine, glutamine, histidine, and lysine), was present in their study with only two variants: glutamine and arginine. Arginine synthesis at codon 171 forms non-risk haplotype ARR, while the glutamine variant increases the risk of TSE. In the studied Bosnian population, arginine is represented with a frequency of 83.33%, while glutamine was with a frequency of 16.67%. A combination of the alanine variant from codon 136 with the arginine variant of codon 171 makes the scrapie risk-free ARR haplotype. Besides investigations on polymorphisms of those three well-known scrapie susceptible codons (loci), our examination was extended to investigations on polymorphisms of four other loci (codons 145, 185, 231, and 237) with unknown effects on the development of scrapie in sheep (unknown and undefined levels of risk). The absence of any polymorphisms was found at codons 145 and 185 in the Bosnian population under investigation, while silent mutations were recorded on codons 231 (AGG → CGG) and 237 (CTC→ CTG). In both cases, there was no amino acid changes found. The silent mutation that was found at locus (codon) 231, which is responsible for the synthesis of amino acid arginine, had a frequency of variant AGG 85.71%, while a variant CGG was present with a frequency of 14.29%. A silent mutation that was found at codon 237 (responsible for the synthesis of amino acid leucine) was present with 88.10% frequency of variant CTC and 11.90% frequency of variant CT.

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