Ukrainian Journal of
Veterinary and Agricultural Sciences
http://ujvas.com.ua

The Stepan Gzhytskyi National University of Veterinary Medicine and Biotechnologies Lviv

Volume 6
Number 3

original article | UDC 577.112.083.3:636.2(540) | doi: 10.32718/ujvas6-3.01

Sequence Analysis of Prion Protein Gene in Bhagnari Cattle Breed from the Hottest Region of Sibi, Balochistan, Pakistan

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Article info
Received 01.09.2023
Received in revised form 20.10.2023
Accepted 21.10.2023

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Abstract

Prion diseases are a type of neurodegenerative disorder caused by the transmission of specific pathogens containing prion proteins. Due to the unique structural characteristics of Prion proteins (PrP), which differ from other types of proteins, the extended incubation period observed in the transmission of specific Prions can be attributed to these differences, at least in part. Prions are found in several other mammals and animals. Prions are unique among false protein folding abnormalities because these are infections and contain various strains of contagious agents associated with a unique in vivo phenotype. They can be acquired either by inheritance or sporadically. There are two types: classical and typical BSP. The objective of this study was to observe the Bhagnari cattle breed of Balochistan, Pakistan and compare the PrP gene sequence of the Bhagnari with other reported sequences from Pakistan and other parts of the world. This research collected 40 Bhagnari cattle blood samples from Tali, Bhag Nari, and Sibi district areas. DNA extraction of each sample was performed by inorganic method, and then DNA amplification and sequencing of PRNP Gene was performed. The results of this research work showed different polymorphic variations (SNPs) in 16 samples. In this study, while mutations causing prion diseases in cattle were detected in Italian and German breeds, none were identified in the PRNP gene of the cattle population investigated, despite its association with neurological disorders.

Keywords: Bhagnari Cattle, Prions, Polymorphic Variations, PRNP Gene, Mutations, PrP.

Citation:
Rasheed, R., Ahmad, J., Hussain, T., Wajid, A., Nudhani, S., Musthafa, M. M. M., & Marikar, F. M. M. T. (2023). Sequence Analysis of Prion Protein Gene in Bhagnari Cattle Breed from the Hottest Region of Sibi, Balochistan, Pakistan. *Ukrainian Journal of Veterinary and Agricultural Sciences*, 6(3), 3–7.

1. Introduction

Prion is also called transmissible spongiform encephalopathy (TSE). It is a rare degenerative brain illness characterized by a slight microscopic depression that gives the brain a "Porous" (Kamali-Jamil et al., 2021). Prion, written by DR. Stanley B. Prusiner in 1982, is a port animal derived from the word "Protein and infection". Prion identifies small Particles that infect proteins, which, in most cases, resist inactivation (Abrams et al., 2021). The hypothesis suggests that PrPc may play a role in copper metabolism, although its typical cellular function remains unclear (Acín et al., 2021).

Prions proteins act as an infectious agent produced from the standard components of our cells, with a high level of the Central Nervous System and fewer in the organs and system. Normal prion or PrPc-coding genes are structures that we define as "alpha" or three-dimensional structures (Hussain et al., 2017). Prion disease may also show as genetic, infections, or sporadic problems, and all of these entail modification of Prion proteins (Oamen et al., 2020).

A defining characteristic of this disease type is prolonged exposure to a contagious pathogen, which subsequently leads to clinical symptoms. Prion illness affects different types of mammals, such as humans. In animals,

Prions cause different types of diseases like scrapie, an illness of sheep and goats, Transmissible mink of encephalopathy disease of mink, Bovine spongiform encephalopathy in cattle's fine spongiform encephalopathy in cates, and exotic ungulate encephalopathy in nyala and oryx (Baiardi et al., 2019).

Prions usually have a more extended incubation period and are measured over several years, with a comparatively short clinical course and 100% mortality. Approximately customarily, it causes dementia, damage to motor control, which causes paralysis, and, finally, the demise of an organism (Scheckel & Aguzzi, 2018).

In 1969, Pakistan initiated a program to enhance beef cattle breeds by introducing five Droughtmaster cows and one bull from Australia. These animals were reserved at the Beef Creation Study Center, Sibi, Balochistan, for crossing with resident cattle breeds. Cross-breeding experiments were initiated in 1970. The breed event arranged to prevent Droughtmaster males with Bhagnari females and next intersect Cl females (50 % Bhagnaris and 50 % Droughtrmaster) to the Bhagnari males to develop C2 (25% Droughtmaster and 75 % Bhagnari). The females from C2 were to be crossed with Droughtmaster males to perceive C3 (62.5 % Droughtmaster and 37.5 % Bhagnari). These C3 animals were crossed interse, followed by the collection manner for complex typescripts (Khan & Khan, 2001). The animals of 62.5 % Droughtmaster and 37.5 % Bhagnari inheritance were named "Narimaster".

The Bhagnari cattle, spaced out from their moral swig qualities, are careful probable beef producers. Most of this breed's animals are mainly created in the plains of Sibi, Karachi, and Nasirabad Districts of Balochistan territory in Pakistan. Some of these animals are reared on crops like sorghum and millet, irrigated essentially by the flood stream of the Nari and Indus rivers (through a smooth Feeder Canal) permanent water. Males of this breed are very tall and massive that weigh around 600 kg while females weigh about 480 kg (Waheed et al., 2003).

BSE in cattle is believed to be due to the intake of meal and bone meal contaminated with PrP^{sc}. BSE appears to cattle; however, adding on ally human public fitness due to human sickness variation CDJ. (Hlásný, 2020). Although looking at infection, Prions illness consisting of Kuru and more these days new variation CDJ has led to massive advances within the study of that illness, most people suffer from stricken sporadic CDJ (Sawas et al., 2020).

Numerous studies have characterized the genomic location of the Bovine PRNP gene polymorphism. The primary report on German BSE animals indicated a probable outcome of the PRNP Promoter variant on BSE susceptibility (Won et al., 2020). Throughout the BSE outbreak in England, an estimated one million cattle were believed to have been infected with prions. Recently, in Portugal, BSE's annual prevalence rate (quantity of bovine) was 9.08 in 2009 and 6.83 in 2010 (Lloyd-Smith et al., 2009).

2. Materials and methods

Blood collection and Ethical clearance

The research was performed at the Animal Genomics Laboratory of the Department of Molecular Biology of the Virtual University of Pakistan, Lahore. Blood samples of Bhagnari cattle were gathered from various areas of Sibi District, including villages Talli, Kurak, and Sibi Town area along with Bhagnari cattle farm at Usta Muhammad, Balochistan. By using the venipuncture procedure, 10 ml blood samples were collected from 40 cattle (male and female) and transferred into ethylenediaminetetraacetic acid (EDTA) containing tubes / Vacutainer immediately (Russell & Sambrook, 2001). The tubes were shaken to ensure the mixing of blood with EDTA to avoid coagulation. The blood samples were collected and placed in a refrigerated container, then transported to the laboratory and stored at -20 °C for future use. The consent from Ethical committe from the Virtual University of Pakistan was obtained before the experiment.

DNA Extraction

This protocol extracted DNA from the blood samples (Russell & Sambrook, 2001). The blood sample of 200 µl of Bgahnari cattle was taken in an Eppendorf tube. A Lysis buffer of 1000 µl was added to it. It was vortexed for a few seconds. It was centrifuged at 10,000 rpm for 10 minutes. Pellet formation was checked, and the supernatant was discarded. Lysis buffer of 1000 µl was added and centrifuged at 10,000 rpm for 10 minutes, and this step was repeated three times. 250 µl buffer A1, 80 µl 10 % SDS, and 20 µl proteinase K were added. It was incubated at 58 ℃ overnight for degradation of the protein. On the next day, 300 µl PCI was added to each sample. It was vortexed for a few seconds. It was centrifuged at 13,000 rpm for 15 minutes. Three layers were formed. The upper aqueous layer was carefully transferred into a separate Eppendorf tube. Isopropanol 600 µl was added and mixed gently with a pipette. It was centrifuged at 13,000 rpm for 15 minutes. The upper layer was discarded. Ethanol 1000 µl was added. It was centrifuged at 13,000 rpm for 10 minutes. The upper layer was discarded, and the pellet was dried. Injection water 150 µl was added, and the pellet was dissolved. DNA was stored at -20 °C for further use. The Inorganic method (Russell & Sambrook, 2001) was used for genomic DNA extraction. The final concentration of DNA was brought to 50 ng/uL and stored at -80 0C before further use.

Polymerase Chain Reaction

Genomic DNA was amplified using a prion region-specific primer pair. The sequences of prion region-specific PRNAP primers used were as follows: forward primer (5′ATCCTGGTTCTCTTTGTGGT3′) and reveres primer (5′CCCACTATGAGGAAA ATGAG3′). PCR analysis was carried out by using 25 µL of erection mixture containing 2 µL of template DNA, 2 µL of dNTPs (20 mM each), 2 µL 10X PCR buffer, 2 µL 50 mM MgCl2, 1 µL of each forward and reverse primer (10 PM), 0.5 µL Taq Polymerase2 and 14.5 µL ddH2O. Thermocycler conditions used for amplification were: initial denaturation at 94 °C for 3 min; 35 cycles of denaturation at 94 °C for 45 s, annealing at 54 °C for 45 s, extension at 72 °C for 45 s followed by final extension at 72 °C for 10 min. Amplified PCR products were electrophoresed in 1.2 % agarose gel, stained with Ethidium Bromide, and visualized under the Gel documentation system.

Sequencing and Phylogenetic Analysis

Samples requiring fragment size were selected and sent for sequencing through 1st Base, Singapore. All the sequences were aligned and edited using Codon Code aligner, and finally, 638 bp were explored for phylogenetic analysis. The essential Primary Local Alignment Search Tool was

used to confirm the identity of the sequences. The PrP sequences of various animals were obtained from NCBI (National Center for Biotechnology Information USA). The obtained sequences were analyzed first to exclude frame shifts or vague portions. The PrP-published sequences of 61 animals from different parts of the world were used to compare and analyze with the one-humped dromedary camels of Pakistan. MEGA version X was used for molecular evolution and phylogenetic analysis. The Neighbor-Joining method with bootstrap analysis of 1000 replicates was used to draw the phylogenetic tree.

3. Results and discussion

3.1. Results

Sequence Analyses of PRNP Gene

This study involved 40 DNA samples from the Bhagnari cattle breed from Balochistan, Pakistan. The DNA extraction for each sample was manually carried out using an inorganic method, followed by DNA amplification and sequencing of the PRNP gene. The results of this research work showed different polymorphic variations (SNPs) in 16 samples shown in Table 1.

Table 1

Table shows silent mutations under SNP analyzed in an exon of the PRNP gene in Bhagnari cattle from Balochistan, Pakistan

Blood Sample #	Nucleotide Change	Amino Acid change	Effect on Protein	Percentage of Change	Sequence
1	c.69C>T c.75G>A c.126A>G c.678T>C	p. 23 Leu>Leu p. 25 Lys>Lys p. 42 Pro>Pro p. 226 Ile>Ile	No No No No	2.5 % C>T 17.5 % G>A 17.5 % A>G 30 % T>C	
2	c.576C>T c.678T>C	p. 192 Asn>Asn p. 226 Ile>Ile	No No	17.5 % C>T 30 % T>C	
3	c.576C>T c.678T>C	p. 192 Asn>Asn p. 226 Ile>Ile	No No	17.5 % C>T 30 % T>C	
4	c.576C>T c.678T>C	p. 192 Asn>Asn p. 226 Ile>Ile	No No	17.5 % C>T 30 % T>C	
5	c.576C>T	p. 192 Asn>Asn	No	17.5 % C>T	
6	c. 630C>T	p. 210 Thr>Thr	No	2.5 % C>T	
7	c.576C>T	p. 192 Asn>Asn	No	17.5 % C>T	
8	c.678T>C c.576C>T c.678T>C	p. 226 Ile>Ile p. 192 Asn>Asn p. 226 Ile>Ile	No No No	30 % T>C 17.5 % C>T 30 % T>C	
9	c.576C>T	p. 192 Asn>Asn	No	17.5 % C>T	
10	c.678T>C	p. 226 Ile>Ile	No	30 % T>C	
11	c.75G>A c.126A>G c.678T>C	p. 25 Lys>Lys p. 42 Pro>Pro p. 226 Ile>Ile	No No No	17.5 % G>A 17.5 % A>G 30 % T>C	
12	c.75G>A c.126A>G c.678T>C	p. 25 Lys>Lys p. 42 Pro>Pro p. 226 Ile>Ile	No No No	17.5 % G>A 17.5 % A>G 30 % T>C	
13	c.75G>A c.126A>G	p. 25 Lys>Lys p. 42 Pro>Pro	No No	17.5 % G>A 17.5 % A>G	
14	c.75G>A c.126A>G c.678T>C	p. 25 Lys>Lys p. 42 Pro>Pro p. 226 Ile>Ile	No No No	17.5 % G>A 17.5 % A>G 30 % T>C	

continuation of table 1

15	c.75G>A	p. 25 Lys>Lys	No	17.5 % G>A	
	c.126A>G	p. 42 Pro>Pro	No	17.5 % A>G	
	c.678T>C	p. 226 Ile>Ile	No	30 % T>C	
16	c.75G>A	p. 25 Lys>Lys	No	17.5 % G>A	
	c.126A>G	p. 42 Pro>Pro	No	17.5 % A>G	

Therefore, genetic analysis of the PrP nucleotide sequence from Bhagnari cattle will trace the evolutionary process. The Bhagnari cattle of Pakistan might have a high potential when compared with other breeds worldwide. In the present study, the PrP nucleotide sequences of 8 different mammalian species worldwide were aligned with the Bhagnari breed from Pakistan. The phylogenetic tree was constructed using MEGA X software, comprising 2 Clads, A and B, starting from the tree's base, which are further divided into different subclades. Each subclade has different nodes and animals at their tips (Figure 1).

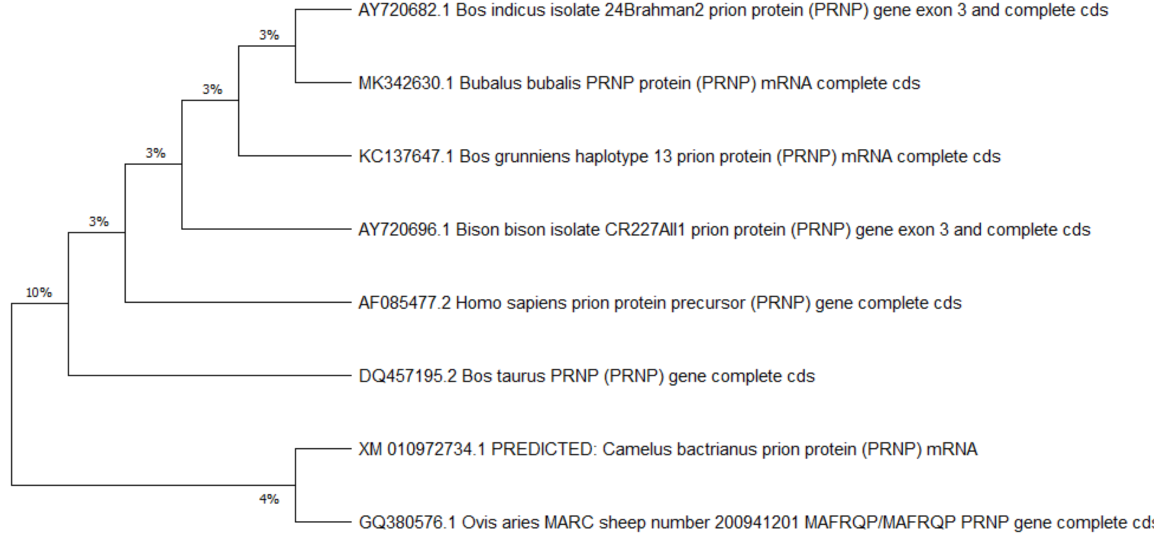

Fig. 1. Phylogenetic tree shows evolutionary relationships between cattle breeds and other mammalians created on MAGA X software, and sequences are taken from NCBI.

Further, for the confirmation of the relationship among the Bhagnari cattle breed, we separated the tree only with six types of cattle breeds and drew the phylogenetic tree. Figure 1 shows that the Bhagnari cattle and *Ovis aries* MARC Sheep belong to the same clade, while others are part of a separate clade.

3.2. Discussion

The PRNP gene codes the prion protein and is active in the brain and several other tissues. The proposed roles of the prion protein are the transport of copper into cells and the protection of brain cells (neurite outgrowth and neuronal survival) (Nguyen et al., 2019) and the formation of synapses (synaptic function) (Kawahara et al., 2021). During conversion into PrPSc, the prion protein cellular (PrPc) undergoes substantial structural rearrangement by post-translational modification mechanism). However, the molecular structure of prion protein scrapie needs to be better understood (Salzano et al., 2019; Uddin et al., 2021). The published prion protein sequences (amino acid sequences) were obtained from NCBI six different species during the present study.

Furthermore, to date, Prion has not been reported exclusively in the Bhagnari cattle breed; due to some reasons, such as the PrP sequence as compared to mouflon (*Ovis aries*) and cattle (*Bos taurus*) in which there are more reports of scrape and Mad Cow Disease. These sequences were aligned using CLUSTAL W software with the different camel breeds to find out the variability between these. As the first step of this study, it was revealed that 16 different PrP polymorphisms were present. This study unveiled that the codon region 66–69 deletion was observed in all six sheep species during evolution, and it remained conserved exclusively in wild sheep. This might be a good indicator for the determination of the nucleotide sequence of the PrP camels from all parts of the world. To find out the degree of diversity in the genetics of the PrP gene, we performed the multiple sequence alignment and did a phylogenetic tree (Figure 1); 99.9 % similarities were found in nucleotide sequence comparison based upon alignment among Bhagnari cattle from Pakistan.

Multiple sequence alignment reflects the main polymorphisms seen between cattle and other species and should be responsible for resistance against prion diseases in cattle. Previous studies on transgenic mice have demonstrated that the species barrier is linked to the level of sequence homology, similarity in amino acid sequences, and the three-dimensional structure of PrP among various species (Marín-Moreno et al., 2020). TSE outbreak is neither reported in the Bhagnari cattle breed in Pakistan. There is limited available information regarding prion gene variability in the Bhagnari cattle (Zeineldin et al., 2021). A polymorphism in the PrP gene primarily affects the prion protein expression in the host, e.g., sheep, deer, and humans.

4. Conclusions

Prion protein is a neurodegenerative disorder of humans and animals. Mutations in the Prion Protein gene are located on chromosome 31, causing BSE, also known as prion syndrome, which is always lethal in cattle. We analyzed40 DNA samples of the Bhagnari cattle breed from Tali, Bhagnari, and Sibi district and Bhagnari cattle farm, Usta Muhammad in Balochistan, Pakistan under this study. DNA extraction of each sample was performed manually by inorganic method, then DNA amplification and sequencing of PRNP Gene was performed. The results of this research work show different polymorphic variations (SNPs) in only 16 samples. Mutation in this gene shows neurological disorders in cattle, but we cannot find any mutation in this study. Our results show that heterogenous variations (SNPs) in the prion protein gene have been observed in Bhagnari cattle, but these variations do not affect the standard. Proteinaceous structure, such variations are found in different cattle in Pakistan. To date, no case of Bovine spongiform encephalopathy (BSE) disease has been found. Therefore, it is concluded that all Pakistani cattle, including Bhagnari cattle of Balochistan, are accessible from this neurological disease.

Acknowledgments

We would like to thank Livestock & Dairy Development Department of Balochistan and Bhagnari Cattle Farm, Usta Muhammad, Balochistan for their kind cooperation, We would also like to express gratitude to Mr. Khalid Hussain from B&R Sibi, Pakistan, Mr. Sana Ullah from the Department of IT, APSACS Sibi, Pakistan, and Dr. Mukhtyar Khan Khajjak from SAU Tandojam, Sindh Pakistan, for their support. We also acknowledge the HEC-NRPU 4485 research project for the provision of facilities and consumables for this study.

Conflict of interest

The author claims no conflict of interest.

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