Pakistan J. Zool., vol. 54(1), pp 443-446, 2022 DOI: https://dx.doi.org/10.17582/journal.pjz/20191201161210

Short Communication

Variations in the Prion Protein Gene (*PRNP*) Sequences of Wild Punjab Urial from Pakistan

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Article Information
Received 01 December 2019
Revised 19 May 2020
Accepted 28 July 2020
Available online 26 January 2021 (early access)
Published 17 December 2021

Authors' Contribution
NS, TH, AW and MSK conceived and designed this study. TH andAW coordinated field samples. NS, NA and MZK conducted laboratory experiment. AW and NS analyzed data. AW, FM, MEB and TH wrote and edited the manuscript.

Key words
Scrapie, Neurodegenerative disease, Prion protein gene, Polymorphisms, Wild Punjab urial

ABSTRACT

Scrapie is a fatal neurodegenerative disease caused by the accumulation of improperly folded forms of host-encoded cellular-prion protein (*PrP*^{c}) in the central nervous system (CNS) and in some peripheral tissues in sheep and goats. Scrapie resistant/susceptibility have been associated with the presence of *PRNP* gene polymorphisms. We analyzed the polymorphisms in *PRNP* gene sequences in 49 wild Punjab urial (*Ovis vignei punjabiensis*). Four novel amino acids polymorphisms (p.Q149E, p.Q155E, p.Y228L and p.L253F) were detected in *PRNP* gene. The urial was monomorphic at codon 149 and 155 and polymorphic at codon 228 and 253. These amino acid polymorphisms were combined in four alleles and six genotypes. Investigating the polymorphisms at codon 136, 154 and 171, the only genotype ARQ/ARQ was detected in Punjab urial. The *PRNP* gene sequences in Punjab urial showed low variability and is resemble to previous findings for wild animals. Prevalence-estimate, transmissibility, distribution, and host-range of prion disease in wild-animals remain undetermined.

In Pakistan, there are three subspecies of urial: the *Ovis vignei blanfordi* (Baluchistan Urial) which is present in Balochistan and Sindh provinces, *Ovis vignei vignei* (Ladakh Urial) which are located in Gilgit-Baltistan and Chitral areas and *Ovis vignei punjabiensis* (Punjab Urial) which are present in Salt and Kala Chitta Ranges in the Punjab province. The genus *Ovis* are found in western Asian countries from Northern and Western Pakistan, Western Kazakhstan to North-Eastern part of Iran and also is found in Kulun mountain range of Ladakh, Jammu, and Kashmir. Punjab Urial is endemic to Indus and Jhelum rivers, northern regions of Punjab province. Most populations of Punjab Urial are distributed at elevations of 250 to 1500m primarily reported in four areas including Kala Chitta Range, Khairimurat Range, Margalla Hills and Salt Range (Awan *et al*., 2004). According to a report of IUCC (2000), Punjab Urial is classified in endangered animals list.

Prion diseases or transmissible spongiform encephalopathies (TSEs) are rare progressive neurodegenerative diseases that can affect multiple animals as well as human beings but reveal a predominant disease in ruminants. Other TSEs are bovine spongiform encephalopathies (BSE) or mad cow disease, chronic wasting disease (CWD) in deer and elk, and Cruetzfeldt Jacob disease in human beings (Kim *et al*., 2019). Scrapie is a fatal neurodegenerative disease in domestic sheep and goats and remains undetermined in wild animals (Fantazi *et al*., 2018) is endemic to several countries of the world (Babar *et al*., 2009).The TSE is characterized by accumulation of prion protein ($PrP^{sc\ -\ scrapie}$), an abnormal isoform of the cellular protein ($PrP^{c\ -\ cellular}$) in the central nervous system and lymphoid organs (Prusiner, 1982).

At present, there is no vaccine or treatment available to cure the disease. Currently, small ruminant TSEs are commonly diagnosed by post-mortem detection of PrP^{sc} in the posterior brain stem using traditional diagnostic methods. However, the recent advances in the development of *in vitro* cell-free amplification techniques such as protein misfolding cyclic amplification (PMCA) and real time quaking induced conversion (RT-QUIC) have been

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0030-9923/2022/0001-0443 $ 9.00/0

developed having high diagnostic sensitivity in a variety of biological tissues such as cerebrospinal fluid, skin, blood, saliva and nasal brushings (Favole *et al*., 2019).

PRNP polymorphism has been shown to strongly influence prion disease susceptibility and pathologies among a wide range of hosts (Kim *et al*., 2019). Variations in codons 136, 154 and 171 of the *PRNP* gene are associated with classical scrapie (Goldmann, 2008). In addition, leucine/phenylalanine mutation at codon 141 has been recognized as an increased risk factor for atypical scrapie. The most common variations observed in amino acid position 136 is A/V, at aa 154 is R/H and at aa 171 is Q/H/R of *PRNP* play an important role in classical scrapie (Vitale *et al*., 2016). The three codon system has been characterized into five haplotypes ARR, ARQ, ARH, AHQ, and VRQ that combined to form 15 genotypes, which have been constituted into five risk-group (R1 to R5): for example, the most scrapie-susceptible genotype VRQ/ARQ (R5) and the most scrapie-resistant genotype ARR/ARR (R1) (Stepanek and Horin, 2016).

The area of this study was located in the Northern regions of Punjab, the mountain ranges of Salt and Kala Chitta and distributed in Indus and Jhelum rivers from an altitude of 250 to 1500 m. Total population estimated for the Punjab urial is about 700 individuals. From the last 10 years, our group has characterized domestic and wild animals in Pakistan on the basis of microsatellite markers, mitochondrial (mtDNA) cytochrome b (*cyto b*) gene, mtDNA control region and *PRNP* gene (Hussain *et al*., 2011, 2016; Babar *et al*., 2008, 2009). The present study was conducted to determine polymorphisms of *PRNP* gene in wild Punjab urial in order to evaluate their status of resistance or susceptibility to infectious prion disease.

Materials and methods

According to a report of IUCC (2000), wild Punjab urial is currently classified as endangered species within the protected areas in the IUCN Red Data Book for mammals and also demonstrated as vulnerable by IUCN Red List of Threatened Species. The species is also protected under the Punjab Wildlife (Protection, Preservation, Conservation, and Management) Act, 1974 (Punjab Wildlife and Parks Department, 2010). This research work was approved by the Departmental Ethical Research Committee of the Virtual University of Pakistan, the samples were used only for scientific study and we didn't kill any animal for this purpose.

The present study was conducted to genetically investigate the *PRNP* gene in wild endangered Punjab urial (*Ovis vignei punjabiensis*) of Pakistan. A total of 49 samples were collected from wild Punjab Urial in this study. Skin and blood samples were collected from Chakwal, Sargodha, Attock, Mianwali and Jehlum districts, LoiBher Wildlife Park Rawalpindi, Lahore zoo and Jotana House of Lahore. The skin samples were provided by the Punjab Wildlife department and blood samples were collected from the Zoo and capture animals.

For isolation of genomic DNA, skin samples and blood samples (2ml) collected in EDTA-containing vacutainers were used for DNA extraction using DNeasy blood and tissue kit (Qiagen, USA) following the manufacturer's recommended protocol.

For PCR amplification of PRNP gene primer pair (forward primer 5'-1-CTTTAAGTGATTTTTACGTGG-21-3' and reverse primer5'-854-TGGCAAAGATTAAGAAGATAATG-876-3') used by Babar *et al*. (2008) were used to amplify the fragment size of 771 bp containing the complete coding region in exon 3 of the *PRNP* gene. PCR was performed using a total volume of 25µl containing genomic DNA (100 ng/µl) 1.5µl, forward and reverse primer 1 µl each, 200µM dNTPs 1.5 µl, 5 U Taq polymerase (Fermentas, USA) 0.5 µl, 2.0 mM $MgCl_2$, 10 mM Taq buffer $(NH4)_2SO_4$ 6 µl and 13.5µl UltraPure DEPC-Treated water. PCR condition was standardized of initial denaturation at 95°C for 5 min, denaturation at 95°C for 45 seconds, annealing at 60°C for 2 min, extension at 72°C for 45 seconds and final extension was optimized at 72°C for 10 min. The amplified PCR products were run on 1% agarose gel and Gene JET gel extraction kit was used for purification of amplified products as per manufacturer's instructions. The purified samples were sequenced by ABI Prism 3130xL automated sequencer (Applied Biosystems, Inc., Foster City, CA). The obtained *PRNP* gene sequences were edited, assembled and analyzed for single nucleotide polymorphisms (SNPs) through BLAST and by BioEdit v7 (Hall, 1999). To infer the evolutionary history phylogenetic tree was built using the neighbor-joining method and evolutionary distances were computed through Maximum Composite Likelihood method using the MEGA software v6 (Tamura *et al*., 2013).

Results and discussion

Sequencing analysis of the *PRNP* gene revealed overall four novel amino acid aa variations in the studied 49 samples from wild urial sheep breed at position p.Q149E, p.Q155E, p.Y228L and p.L253F (Table I). These aa variations were combined in four alleles and six genotypes detected in the studied samples. The allele and genotype type frequencies of the aa variations were calculated for Punjab urial. The maximally detected allele was $E_{149}Q_{155}Y_{228}F_{253}$ found in wild urial sheep with the frequency of 0.4497 and followed by $E_{149}Q_{155}L_{228}F_{253}$ (0.2244) and allele $E_{149}Q_{155}L_{228}L_{253}$ (0.1020) was found with lowest frequencies in the studied samples (Table II). Apparently low variability of the *PRNP* gene coding region was detected in wild Punjab urial with merely four alleles in 49 individuals

(8.16%). In Punjab urial, the maximally and minimally detected genotypes were $E_{149}Q_{155}Y_{228}Y_{253}$ /$E_{149}Q_{155}F_{228}F_{253}$ (0.5102) and $E_{149}Q_{155}L_{228}Y_{253}$/$E_{149}Q_{155}L_{228}$ L_{253} (0.0612) and $E_{149}Q_{155}L_{228}Y_{253}$/$E_{149}Q_{155}F_{228}F_{253}$ (0.0612) (Table III).

Table I. The locus, genotype, amino changes and frequencies in *PRNP* gene for Punjab wild urial.

S.N.	Locus	Genotype	Amino acid	Wild urial
1	447 [a] (149 [b])	cc	QQ	0.00
		cg	EQ	0.00
		gg	EE	100.00
2	465 (155)	aa	KK	0.00
		ac	KQ	0.00
		cc	QQ	100.00
3	684 (228)	aa	LL	28.57
		at	LY	6.13
		tt	YY	65.30
4	759 (253)	cc	LL	8.16
		ct	LF	0.00
		tt	FF	91.84

[a], Nucleotide position; [b], Amino acid position.

Table II. Allele frequency distribution of the *PRNP* gene in Punjab urial at codons 149, 155, 228 and 253.

Alleles	Punjab urial (n = 49)
$E_{149}Q_{155}L_{228}L_{253}$	0.1020
EQYL	0.1836
EQLF	0.2244
EQYF	0.4497

Table III. Genotype frequency distribution of *PRNP* gene in Punjab Urial.

Genotypes	Punjab urial (n = 49)
EQLL/EQLL	0.1020
EQLY/EQLL	0.0612
EQYY/EQFF	0.5102
EQLL/EQFF	0.1632
EQLY/EQFF	0.0612
EQYY/EQLL	0.1020

Genotyping of the wild sheep breed was performed for three significant codons 136, 154 and 171 involved with susceptibility to scrapie. $AA_{136}RR_{154}QQ_{171}$ was the only genotype (1.000) that was present in all studied samples. There are five risk groups (R1 to R5), as described by Dawson *et al*. (1998) with very low-risk group ARR/ARR to high-risk group VRQ/VRQ. The result showed that the Punjab urial at moderate risk (R-3) with genotype ARQ/ARQ (100%). In addition, the leucine into phenylalanine polymorphism at codon 141 has been recognized as an increased risk-factor for atypical scrapie. Leucine was the most prominent aa found in codon 141 in all studied samples of Punjab urial.

The phylogenetic tree included *PRNP* gene sequences from domestic and wild sheep buffalo, cattle, yak, horse, deer, camel, and human being sequences. Phylogenetic analysis based on the *PRNP* gene confirmed the evolutionary relationships among the animals and consistent with previous studies using other gene sequences (Hussain *et al*., 2016). The phylogenetic tree grouped the related *PRNP* gene sequences into three main clades of Caprinae, Bovinae and Cervidae and camel, horse and human being sequences as an outgroup (Fig. 1).

This is the preliminary study, to investigate the genetic structure of *PRNP* gene in wild Punjab urial sheep population in any Asian country. The analysis included 49 animals sampled from different geographical regions in Pakistan.

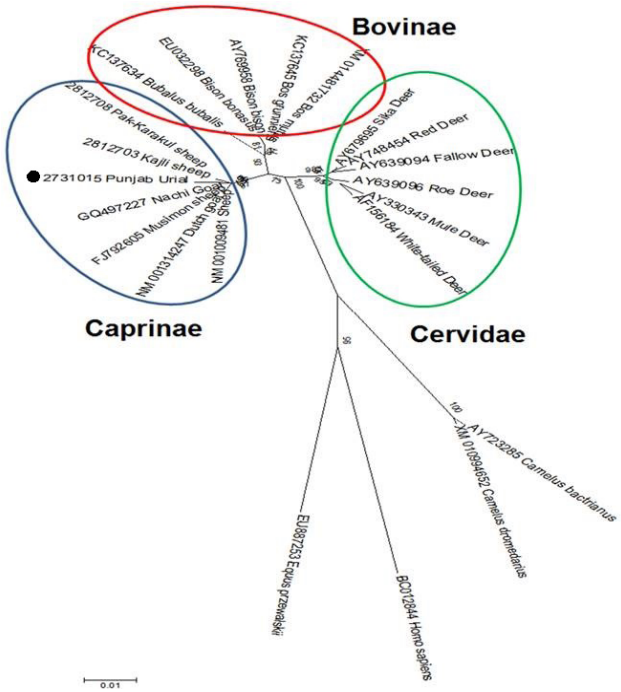

Fig. 1. Neighbor-joining phylogenetic analysis performed using prion gene sequences of wild Punjab urial with other mammalian species.

Sequencing of the *PRNP* gene of 49 Punjab urial showed three alleles of which allele EQYF was maximally found with an overall frequency of 0.4497 followed by second most frequent allele EQLF (0.2244), and minimally found allele was EQLL with allele frequency of 0.1020. The maximally detected genotype was EQYY/EQFF (0.5102) and minimally detected genotype was EQLY/

EQLL (0.0612) and EQLY/EQFF (0.0612).

The present study suggests the studied wild Punjab urial is vulnerable to scrapie if expose to typical scrapie agents. Genotyping of *PRNP* gene of 49 Punjab urials at major codons 136, 154 and 171 showed a frequency of 100% for AA, RR and QQ genotypes. The five risk groups in term of haplotypes characterized by Dawson *et al*. (1998). The *PRNP* gene carrying polymorphism Q171R confers the highest resistance to sheep scrapie. Thus, all the individuals of Punjab urial were observed with ARQ/ARQ genotype (100%) classified into the moderate risk group. The most scrapie-susceptible variant (A136V) was not detected, conversely, the most scrapie-resistant variant (Q171R) was also not detected in this study. Babar *et al*. (2008) showed the allele ARQ was most frequently observed in other Pakistani domestic sheep breeds with the total frequency of 0.75% followed by ARH (0.16%), AHQ (0.03%) and ARR (0.06) (Babar *et al*., 2008). ARR has been considered the most resistant allele to classical scrapie was detected with very low frequency in other studied Pakistani sheep breeds i.e. Damani and Hashtngri (average = 0.107) (Hussain *et al*., 2011), Kajli (0.07), Lohi (0.07), Thali (0.03), Hissardale (0.20) and Awassi (0.05) (Babar *et al*., 2008), hence, lower value raising the scrapie risk in the studied animals. The present data along with previous studies suggest that the native breeds of Pakistan are vulnerable to infection by typical scrapie agents, so strict regulations need to be implemented on the importation of sheep from infected countries.

Acknowledgment

We thank Punjab Wildlife Department and National Zoos providing the samples for this study.

Statement of conflict of interest

The authors have declared no conflict of interest.

References

Awan, G.A., Ahmad, T. and Festa Bianchet, M., 2004. *J. Sci.,* **14**: 1-14.

Babar, M., Farid, A., Benkel, B.F., Ahmad, J., Sajid, I.A., Imran, M., Hussain, T. and Nadeem, A., 2008. *J. Genet.,* **87**: 187-190. https://doi.org/10.1007/s12041-008-0029-z

Babar, M.E., Farid, A., Benkel, B.F., Ahmad, J., Nadeem, A. and Imran, M., 2009. *Mol. Biol. Rep.,* **36**: 561–565. https://doi.org/10.1007/s11033-008-9214-7

Dawson, M., Hoinville, L.J., Hosie, B.D. and Hunter, N., 1998. *Vet. Rec.,* **23**: 623-625.

Fantazi, K., Migliore, S., Kdidi, S., Racinaro, L., Tefiel, H., Boukhari, R., Federico, G., Marco, VD., Presti, L., Gaouar, SBS. and Vitale, M., 2018. *Ital. J. Anim. Sci.,* **17**: 578–585. https://doi.org/10.1080/1828051X.2017.1420430

Favole, A., Mazza, M., Costassa, E.V., D'Angelo, A., Lombardi, G., Marconi, P., Crociara, P., Berrone, E., Gallo, M., Palmitessa, C., Orrù, C.D., Caughey, B., Acutis, P.L., Caramelli, M., Casalone, C. and Corona, C., 2019. *Sci. Rep*., **9**: 6173. https://doi.org/10.1038/s41598-019-42449-7

Goldmann, W., 2008. *Vet. Res.,* **39**: 30. https://doi.org/10.1051/vetres:2008010

Hall, T.A., 1999. *Nucl. Acids Symp. Ser.,* **41**: 95-98. https://doi.org/10.1099/00222615-48-1-95

Hussain, A., Babar, M.E., Imran, M., Haq, I.U. and Javed, M.M., 2011. *Virol. J.,* **8**: 246. https://doi.org/10.1186/1743-422X-8-246

Hussain, T., Babar, M.E. and Wajid, A., 2016. *J. Anim. Pl. Sci.,* **26**: 1890-1893.

IUCN 2020. *The IUCN red list of threatened species.* Version 2020-2. Available at www.iucnredlist.org.

Kim, S.K., Kim, Y.C., Won, S.Y. and Jeong, B.H., 2019. *Sci. Rep*., **9**: 15293. https://doi.org/10.1038/s41598-019-51621-y

Prusiner, S.B., 1982. *Science,* **216**: 136–144. https://doi.org/10.1126/science.6801762

Punjab Wildlife and Parks Department (2010). *Protection, preservation, conservation and management. Amendment, Act 2007*. Amendment in rules up to 31st December 2010. pp. 55.

Stepanek, O. and Horin, P., 2016. *J. appl. Genet.*, **58**:111-121.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S., 2013. *Mol. Biol. Evol.*, **30**: 2725–2729. https://doi.org/10.1093/molbev/mst197

Vitale, M., Migliore, S., Giglia, M.L., Alberti, P., Presti, V.D.M.L. and Langeveld, J.P.M., 2016. *BMC Vet. Res*., **12**: 141. https://doi.org/10.1186/s12917-016-0766-9