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Genomic Analysis and Comparison of Pakistani Camels (*Camelus dromedarius*) by Prion Gene

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ABSTRACT

Background: In many parts of the Old World, domesticated camels (genus - *Camelus*) are an essential resource, providing food, labor, commodities, and sport to millions of people. Of the three extant species, two have been domesticated (single-humped dromedarius, *Camelus dromedarius*, and two humped Bactrian camels *Camelus bactrianus*) and one remains wild (two-humped wild Bactrian camels *Camelus ferus*). All three species possess a variety of adaptations to harsh desert conditions, including mechanisms to tolerate extreme temperatures, dehydration, and sandy terrain. People residing in harsh climate zones of the world are being benefitted by raising camels in terms of draft, milk, meat, hides and wool from centuries. There are different breeds of dromedary camels distributed in various parts of Pakistan; however there has been scarcity of research work on camels in Pakistan. Identification of novel link between camel breeders with fatal neurodegenerative disorders is presence or not can be detected by a Prion gene and it was not carried out in Pakistan soil to date. Prion diseases which are a group of fatal neurodegenerative disorders affect both animals and humans. It is believed that the prions are infectious agents responsible for transmissible spongiform encephalopathies. In this study we report the first study on Prion protein gene in dromedary camels of Pakistan.

Material, Methods & Results: Genes are the blueprint of life and determine the functional aspects of cellular mechanisms. Genomic DNA of the enrolled blood samples was extracted using the Nucleospin® DNA extraction kit. Genomic DNA was run on Agarose gel electrophoresis, checked the Genomic DNA quality and amplified using prion region specific primer pair. Prion protein gene was amplified (770 bp) in 35 individuals of seven dromedary camel breeds from the province Balochistan and Punjab of Pakistan. Samples having required fragment size were selected and sent for sequencing through Sanger Sequencing. All the sequences were aligned through multiple sequence alignment and edited using Codon Code aligner and explored for phylogenetic analysis. A portion of 667 bp was finally selected for phylogenetic analysis of dromedary camels from Pakistan with 61 different mammalian species (drawn from GenBank) that revealed five different clades. We found 99.9% nucleotide sequence similarities among Dromedary camels (Germany), Dromedary camels (Iran), and Dromedary camels (Pakistan). We observed deletion in dromedary camels in codon region 66-69 except wild Bactrian camels that might be the causative factor for Prion protein gene resistance in camels. The Neighbor-Joining method with bootstrap analysis of 1000 replicates was used to draw phylogenetic tree.

Discussion: This study documents the presence of 14 PrP polymorphisms and shows the relationship between different camel breeds. The deletion had not previously been examined PrP allelic variation, and was found to segregate in these breeds. However, additional data are necessary to demonstrate PrP and genetic approach will be ideal for the future studies and, more investigations are necessary to demonstrate PrP genetic resistance in camels. The utility of these techniques in identifying Prion genes and selecting superior animals and culling the weak animals and making them parents of the next generation that will lead to producing more meat and milk with fewer animals are well discussed and by slaughtering of high Prion incidence will eliminate human animal fatal neurodegenerative disorders.

Keywords: dromedary camels, prion protein gene, polymorphism, phylogenetic, Pakistan.

INTRODUCTION

Camels have been grouped to class Mammalia, order Artiodactyla and family Camelidae [1,14]. *Camelus* genus contains two species domesticated, *C. bactrianus*, and *C. dromedarius*, one remains wild *C. ferus* [7,10,29]. The new world camel's classification is still a matter of debate where genus *Vicugna* and genus *Lama* [6,9,28,29]. The camels are able to survive in life-threatening drought periods, so that their reproduction and production can linger on [7,8,26]. Furthermore, camels have a significant role in transportation in rural civilizations [7,8,14,26,28].

Prions are infectious agent different from the rest of others due to their mode of replication, nature and disease [12,20]. Genes encoding prion proteins seem to be existed in higher species of animals, including mammals and avian [12,13,17,18,21,30]. Prions proteins cause serious neurodegenerative disorder in animals and humans due to transformation of cellular prion protein (PrP^c) into pathological isoform (PrP^{sc}) central nervous system causes neurodegenerative disease [11,12,22,23,27].

Prion PrP is similar to the susceptibility and regulate species barrier in prion disorder [15,25]. Prions in mammals are linked to the onset of transmissible spongiform encephalopathy [2]. Upon commencement of transmission, conformational changes occur in animal prion protein [16,24]. The incident of transmission of disease mainly depends on genotype, mammalian breed, exposure and succetibility to the infectious agent [3]. The main objective of this study is to identify the genetic analysis and relationship of Pakistani Camels (*Camelus dromedarius*) interrogated by PrP Gene.

MATERIALS AND METHODS

Animal selection

Two humped camels are small in numbers and typically found in northwest part of the country. One humped *Camelius dromedarius* species of Pakistani camels was selected for the current study. All the enrolled subjects were healthy and belonged to different geographical regions of Pakistan.

Blood Sample Collection

Blood samples of all the enrolled subjects were collected from different geographical regions of Pakistan. The Venous blood samples were collected in 50 mL falcon tube having anticoagulant (EDTA) and brought to the laboratory for further analysis. The blood samples were stored in -20°C maintained freezer until required.

Genomic DNA Isolation and PCR

Genomic DNA of the enrolled blood samples was extracted using the Nucleospin® DNA extraction kit¹ according to the manufacturer protocol. Genomic DNA was amplified using prion region specific primer pair. The Sequences of prion region specific primers used were as follow: forward primer (5'ATCCTGGTTCTCTTTGTG-GT3') 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 Gel documentation system³.

Sequencing and Phylogenetic Analysis

Samples having required 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. Basic 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 US). The obtained sequences were analyzed first to exclude frame shift or vague portion. 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 6 was used for molecular evolution and phylogenetic analysis. The Neighbor-Joining method with bootstrap analysis of 1000 replicates was used to draw phylogenetic tree.

RESULTS

Fourteen polymorphisms were observed in six types of camels PrP, named as Bacterian camel (China), Dromedary camel (Germany), Dromedary camel (Iran), Dromedary camel (Pakistan), Dromedary camel (UK) and Wild Bacterian camel. The amino acid substitution in codons 49, 50, 53, 60, 65, 66, 67, 68, 69, 70, 71, 103, 104 and 219 and their configurations are shown in Figure 1. Dotted areas are conserved regions of the gene and highlighted with the relevant polymorphisms.

[illegible]

Figure 1. Alignment of the amino acid sequences of the prion protein gene of six types of camels including Pakistan. Sequence dissimilarity is indicated and the conserved regions are denoted by a dot.

Therefore, genetic analysis of the PrP nucleotide sequence from camels will trace the evolution process and especially camel breeds of Pakistan might have a high potential when compared with other breeds from all over the world. In the present study, PrP nucleotide sequence of 61 different mammalian species from all over the world were aligned with single humped camels of Pakistan and phylogenetic tree was constructed using MEGA6 software comprises of 5 Clads, A, B, C, D and E starting from the base of tree, which are further divided into different subclads and each subclad also has different nodes and animals at their tips (Figure 2). Phylogenetic tree presented in Figure 2 suggested that, among all 61 species, which were given in the tree the highest similarity was observed with the hippopotamus and the lowest similarity with harbour seal. Amino acids percentages of six camels were given in Table 1, and it reflects that Iran and Pakistan camels' amino acids percentage varies dramatically. Further each node has a good value of support.

The clad A is further sub divided into 5 subclads: A1, A2, A3, A4 and A5. The subclad A1 comprises of 9 animals, Skunk bear at the bottom and the American black bear at the top, which shows that Skunk bear is the oldest breed of this subclad. Also this skunk bear is at the bottom of the phylogenetic tree, representing the ancestor of all animals. The second subclad A2 has three animals: harbour seal at the bottom and walnut at the top, denoting that harbour seal is the oldest of these three animals. Subclad A3 consists of small Asian Mongoos which are equally related to all animals of subclad A4 having 6 animals in this clad: clouded leopard at the bottom and Canada lynx at the top. The subclad A5 has two very closely related animals: Red fox and Grey wolf. The clad B is divided into two subclads B1 and B2. B1 has 6 animals: wild horse is the oldest and Grays Zebra is the successor. Animals in B2 are equally related with B1 animals. Clad C has three subclads C1, C2 and C3. C1 has Pig only while 5 animals are included in C2 subclad: Dromedary camel

of Pakistan at the bottom and Bactrian camel of china at the top the subclad C3 has 3 animals in which Alpaca is equally related to two very close animals (Llama and wild Bactrian camel). The three animals of C2 sub clad Dromedary camel of Pakistan, Iran and Germany are identical in genotypes. The clad D again comprises of two subclads D1 and D2, D1 has and the single animal Hippopotamus while D2 has 6 animals: bottlenose dolphin, the ancestor and Minke whale, the successor of this subclad. The last Clad E is also divided into four subclads: E1, E2, E3 and E4 and each subclad comprises of 3, 7, 4 and 8

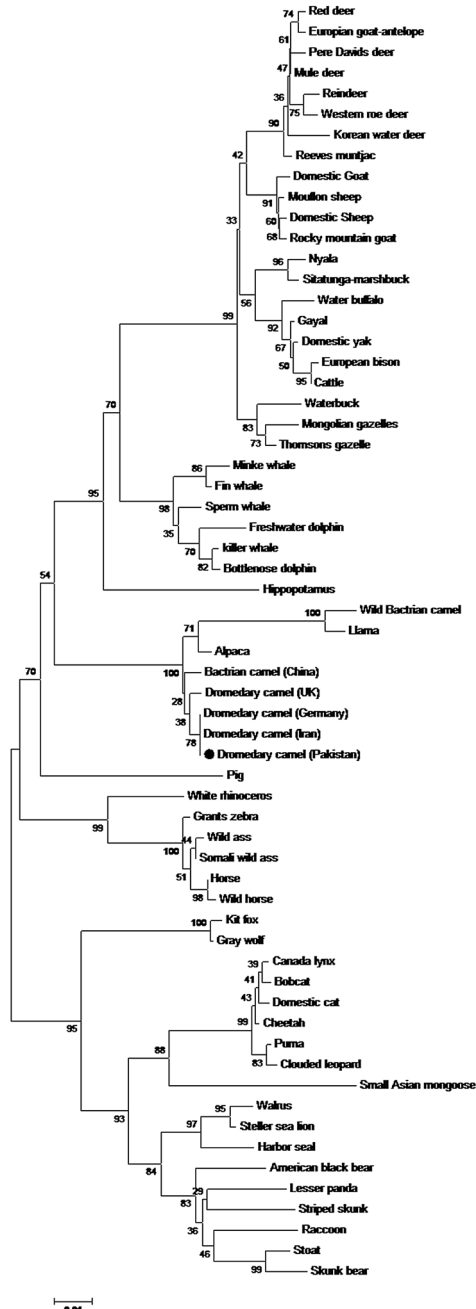


Figure 2. Molecular Phylogenetic analysis by Maximum Likelihood Method of animals of different origin

animals respectively. Thomson Gazelle is the ancestor while Red deer is the latest animal of the clad E, and it is also the successor of all animals included in this phylogenetic tree and Skunk bear is the ancestor of all animals presented.

After alignment we found that at certain positions for example at 102 there is deletion of an amino acid glycine in all camels. At 109 there is amino acid alanine in camels while there is serine or threonine in other species. At position 111 there is Glycine in all camels and some other species but serine is also present in some species. At position 122 all camels have serine while other species have asparagines. At position 245 all camels have tyrosine while all the other species have serine at that position. At position 250 all camels have serine while other species have tyrosine at that position. At position 252 all camels have glycine amino acid while other species have glutamine at that position. These are the main polymorphisms which can be seen between camels and other species and should be responsible for resistant against prion diseases in camels (Data not shown).

Further, for the confirmation of the relationship among the camels, we separated the tree only with 6 types of camels and drew the phylogenetic tree. According to Figure 3, it is evident that Dromedary camel (Germany), Dromedary camel (Iran) and Dromedary camel are (Pakistan) in one Clads, Dromedary camel (UK) in another Clad and finally wild Bactrian camel and Bactrian camel (China) are in a different Clads.

The number of amino acid substitutions per site from between sequences is shown in the Table 2. Analyses were conducted using the Poisson correction model which estimated of evolutionary Divergence between sequences [1]. The analysis involved 6 amino acid sequences. All positions containing gaps and missing data were eliminated for this study. There were a total of 187 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [2]. It further confirms that our hypothesis is correct, which Dromedary camel (Germany), Dromedary camel (Iran), and Dromedary camel (Pakistan) evolved in somewhere Arab region or Pakistan. According to the phylogenetic tree the Figure 3 and Table 2 confirms that wild Bactrian camel conserved region is the highest percentage and it will be the oldest camel of evolution and the rest might have evolved by breeding.

Table 1. Different Amino Acids percentages found in Prion gene in camels.

	Ala	Cys	Asp	Glu	Phe	Gly	His	Ile	Lys	Leu	Met	Asn	Pro	Gln	Arg	Ser	Thr	Val	Trp	Tyr
Bactrian camel (China).seq	7.34	2.29	2.29	4.59	1.83	10.55	7.80	0.46	0.46	8.72	0.46	0.92	11.47	8.26	15.14	7.34	1.38	3.67	4.59	0.46
Dromedary camel(Germany).seq	7.39	2.46	2.46	4.43	0.99	10.34	6.90	0.49	0.49	9.36	0.00	0.99	11.33	8.87	15.76	8.37	1.48	3.94	3.45	0.49
Dromedary camel (Iran).seq	8.00	1.50	1.50	5.00	1.00	11.50	7.50	0.50	0.50	7.50	0.50	1.00	10.00	8.50	17.00	8.00	1.50	4.00	4.50	0.50
Dromedary camel (Pakistan).seq	7.34	2.29	2.29	4.59	1.83	10.55	7.80	0.46	0.46	8.72	0.46	0.92	11.01	8.26	15.60	7.80	1.38	3.67	4.13	0.46
Dromedary camel (UK).seq	6.88	2.29	2.29	4.59	1.83	10.55	7.80	0.46	0.46	8.72	0.46	0.92	11.01	8.26	14.68	7.80	1.83	3.67	5.05	0.46
Wild Bactrian camel.seq	7.58	2.37	2.37	3.79	1.90	10.43	7.58	0.47	0.47	9.00	0.00	0.95	12.32	8.53	16.59	7.11	1.42	3.79	2.84	0.47
Average	7.41	2.21	2.21	4.50	1.58	10.65	7.57	0.47	0.47	8.68	0.32	0.95	11.20	8.44	15.77	7.73	1.50	3.79	4.10	0.47

All frequencies are given in percent in domain data.

Table 2. Estimates of Evolutionary Divergence between sequences.

	Bactrian camel (China).seq	Dromedary camel (Germany).seq	Dromedary camel (Iran).seq	Dromedary camel (Pakistan).seq	Wild Bactrian camel.seq
Bactrian camel (China).seq					
Dromedary camel (Germany).seq	0.011				
Dromedary camel (Iran).seq	0.011	0.000			
Dromedary camel (Pakistan).seq	0.011	0.000	0.000		
Dromedary camel (UK).seq	0.027	0.016	0.016	0.016	
Wild Bactrian camel. seq	0.022	0.033	0.033	0.033	0.049

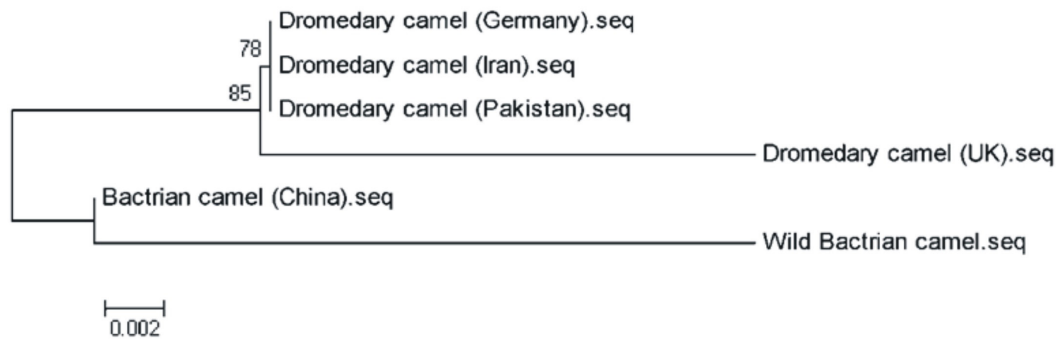


Figure 3. Molecular Phylogenetic analysis by Maximum Likelihood Method of Camel breeds.

DISCUSSION

The prion protein is coded by PRNP gene, which is active in brain and several other tissues. The proposed roles of prion protein are the transport of copper into cells and protection of brain cells (neurite outgrowth and neuronal survival) [3] also formation of synapses (synaptic function) [4]. The prion protein cellular (PrP_C) during conversion into PrP^{Sc} undergoes substantial structural rearrangement by post translational modification mechanism). However, molecular structure of prion protein scrapie is poorly understood [5,19]. During the present study the published prion protein sequences (amino acid sequences) were obtained from NCBI five different species and Pakistan was sequenced in which prion disease, which was more common in camels. Furthermore, till to date Prion has never been reported in camel, due to some reasons such as PrP sequence in camel 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, this was revealed that 14 different PrP polymorphisms were present. In this study it was revealed that all six camels codon region 66-69 deletion was observed with the evolution, and it was conserved only in Wild Bactrian camel. This might be a good indicator for 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 in PrP gene, we performed the multiple sequence alignment (Figure 1); 99.9% similarities were found in nucleotide sequence comparison based upon alignment among Dromedary camel (Germany), Dromedary camel (Iran), and Dromedary camel (Pakistan). It

is not clear from where Dromedary camel (Germany) migrated with the record of history. A small difference was observed six camels evolutionary in this samples which highlight relationship suggesting a simple evolutionary history (Table 2 & Figure 3).

Multiple sequence alignment reflects the main polymorphisms that can be seen between camels and other species, and should be responsible for resistant against prion diseases in camels. Species barrier was associated with the degree of sequence homology, homology in amino acid sequence and its three dimensional structure of PrP among different species as demonstrated by previous studies on transgenic mice [3,30]. TSE outbreak is neither reported both in two humped and in one humped camel. In camels there is limited information available regarding prion gene variability [4]. The expression of prion protein in host e.g sheep, deer, and human is affected largely by polymorphism in PrP gene. The susceptibility and phenotypic expression of the disease are both attributed to polymorphism in codon 129 which results in either valine or methionine [19].

CONCLUSION

In summary, this study documents the presence of 14 PrP polymorphisms and shows the relationship between different camel breeds. The deletion had not previously been examined PrP allelic variation, and was found to segregate in these breeds. However, additional data are necessary to demonstrate PrP and genetic approach will be ideal for the future studies.

MANUFACTURERS

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Ethical approval. According to the Guidelines for the Care and Use of Animals of Virtual University of Pakistan, this study was carried out, and animal experiments were approved by Care and Use of Animals Center, Virtual University of Pakistan.

Declaration of interest. The authors declare that they have no conflict of interest. The authors alone are responsible for the content and writing of paper.

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