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MOLECULAR ASSESSMENT OF KAPPA CASEIN GENE BY SEQUENCING IN BIKANERI DROMEDARY CAMELS

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ABSTRACT

The aim of the present study was to investigate the genetic and phylogenetic analysis of kappa casein gene in the 70 Bikaneri breed of camels which were blood sampled for isolation of their DNA. The polymerase chain reaction (PCR) products (488bp) of kappa casein gene were sequenced using the Sanger dideoxy chain termination method. The sequence analysis of kappa casein gene revealed presence of within-species variation. Intraspecies variation within camel were observed in the form of four SNPs. Generated Sequences were submitted on NCBI GENBANK. Furthermore, a Neighborhood Joining Phylogenetic tree was constructed through MEGA7 bioinformatics software. The sequencing of the kappa casein gene in Bikaneri camel may be useful for future studies on genetic variation within and between camel populations or on associated traits due to the therapeutic importance of camel milk for human consumption.

Key words: Camel, kappa casein, milk, polymorphism, SNP

The casein fraction comprises of 52-89% in the camel milk (Ereife*et al,* 2011) and can be distributed into four fractions involving 1- CN (22%), as 2- CN (65%), b- CN (9.5%), and k-CN (3.5%) (El Agamy, 2009). These four fractions of casein are encoded by four genes such as CSN1S1, CSN1S2, CSN2 and CSN3, respectively (Kappeler *et al,* 1998). Out of all the casein protein, k casein plays a crucial role in stabilising milk micelles and keeping calcium phosphate in solution and further allowing the transfer of calcium and phosphorus from milk to consumers (Ikonen *et al,* 2008).

Substantially more research has been conducted on genetic variability of the kappa casein gene in other dairy farm animals in comparison to the camel (Dioli, 2016; Hemati *et al,* 2017). The first characterisation of the major components of camel milk casein was reported by Farah and Farah-Riesen (1985). Subsequent to this, a quantitative analysis was carried out by Kappeler *et al* (2003) on camel milk protein, which found significantly lower amounts of camel k-casein compared to the homologous cow's casein. Hinz *et al* (2012) have reported five different isoforms of k-casein in the camel milk as a result of a strong glycosylation of casein protein. Yamini *et al* (2019) carried out RFLP analysis through restriction digestion of amplified product of kappa casein gene with *Alu*I generated two genotypic patterns, CT and TT. Further, the CT pattern was identified by the presence of four separate intact fragments of 203-bp, 146-bp, 127-bp and 12-bp and the TT pattern produced three fragments 203-bp, 158-bp and 127-bp. Furthermore, Jadhav *et al* (2020) also reported polymorphism in the kappa casein gene in Indian dromedary. Further, Pauciullo *et al* (2013) characterised, for the first time, the nucleotide sequence of the whole k-casein encoding gene CSN3 plus 1045 nucleotides at the 5′ flanking region in the *Camelus dromedarius*. The length of camel k-casein gene is 13000 bp and it contains 5 exons and 4 introns.

We hypothesised that there may be genetic variation of the kappa casein gene in Indian dromedary camels. The aim of the present study was therefore, to the investigate genetic diversity in Indian dromedary camels through sequencing of the kappa casein gene along with detection of SNPs.

Materials and Methods

Sample collection

Blood samples were collected from 70 Bikaneri camels via jugular venipuncture in sterile vacutainer

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tubes containing EDTA as an anticoagulant. For this procedure, the camels were led gently into a squeeze chute facility, and blood was sampled using minimal restraint. After collection, blood samples were immediately placed on ice, and serum was separated through centrifugation at 3000 RPM for 15 min. Blood serum samples were stored at -20°C until DNA isolation.

Isolation of DNA

DNA was extracted from 200µL of blood by the spin column method following standard procedures as per the manufacture's protocol through a Blood Genomic DNA Purification Kit supplied by HIMEDIA Pvt. Ltd (Mumbai, Maharashtra). Horizontal agarose gel electrophoresis was carried out to check the quality of genomic DNA using 0.8% w/v agarose. The gel was visualised under UV trans-illuminator and documented by photography. DNA samples showing intact bands were used for further analysis.

PCR Conditions

PCR amplification of 488bp fragments were carried out using specific primers (Forward 5'- CAC AAA GAT GAC TCT GCT ATC G -3' and reverse 5'- GCC CTC CAC ATA TGT CTG -3') designed by Othman *et al* (2016). The PCR reaction was carried out using a final volume of 50 µl, containing GeneTaq PCR Master Mix 25 µl, Forward primer 0.5 µl, Reverse primer 0.5 µl, Template DNA 4 µl, and Nuclease free water 20µl. Thermal cycler conditions for PCR were initial denaturation at 95°C for 5-min, followed by 36 cycles of 94°C for 45s, 57.2°C for 1-min, 72°C for 1-min, a final extension at 72°C for 10-min, and then holding at 4°C for 5-min. PCR amplified DNA products were analysed by analytical agarose gel electrophoresis as per the procedure described by Sambrook *et al* (2001).

Restricted fragment length polymorphism

The PCR products for each tested gene were digested with *Alu*I restriction enzyme. A total 15µl PCR products were digested with1.5 µl *Alu*I overnight at the optimum temperature for maximum activity of each restricted enzyme. Molecular size of the digested fragments was measured by analyzing gel images with Gel Analyser software with 100 bp DNA ladder as the DNA size marker.

Sequence analysis and SNP's detection

The six representative samples of amplified products of each electrophoresed RFLP pattern were purified through the Exosap method and sequencing in both directions was carried out in a private laboratory (X Celris Genomics Pvt. Ltd, Ahmadabad, India) using an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA) through the Sanger dideoxy chain termination method. Forward and reverse sequences of each gene fragment was assembled against the most closely related reference sequences of respective genes and similarity was checked into the non-redundant database of GenBank with BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST).The raw sequences were analysed by Codon Code Aligner for detection of sequence anomaly, if any, on the basis of the chromatogram generated. Multiple sequence alignment for each sequenced region of kappa casein gene was carried out by Clustal W software to detect the exact location of nucleotide where polymorphism was present. An total of 6 sequences were generated for kappa casein genes of different animals and were submitted to the National Centre for Biotechnology Information (NCBI) GenBank database, an international bank of gene and genomic sequences, through online gateway.

Phylogenetic analysis

Phylogenetic analysis was conducted using the Neighbor-Joining method (Tahmoorespur *et al,* 2016) with a bootstrap test of phylogeny in MEGA7 software to evaluate the evolutionary relationship within the studied indigenous camel breeds.

Results and Discussion

PCR Amplification and sequencing

The present study focused on the identification of genetic divergence and ev*Alu*ation of SNPs within the kappa casein gene of *Camelus dromedarius*. The genomic DNA of Bikaneri camel was successfully extracted from all of the blood samples and 488 bp specific fragments of exon4 of kappa casein (CSN3) gene were successfully amplified. Further, the nucleotide sequence information generated through Sanger sequencing was checked for accuracy through Codon Code Aligner software. The sequences were assembled with the help of reference sequence already available in the NCBI database (GenBank Accession No. KU055605). The assembled sequence of 488-bp, generated for camel were submitted to the NCBI GenBank database through the online Banklt gateway after proper annotation and accession numbers were obtained (Table 1).

The detection of sequence variation is the core of all genetic analysis for identifying areas across

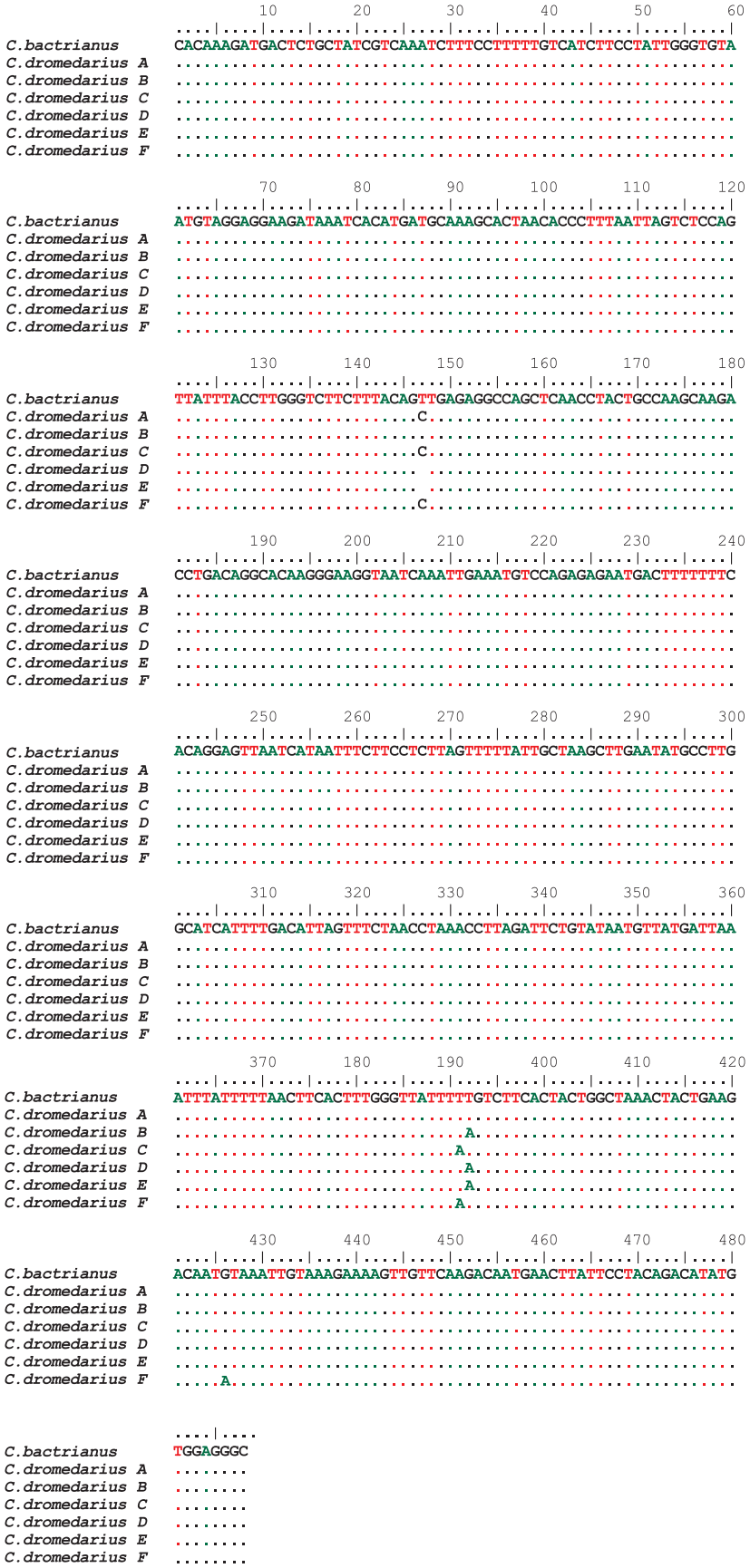

Fig 1. Multiple sequence analysis of the kappa casein gene in dromedary camel.

the candidate gene that may affect the trait of interest (Nickerson *et al*, 1997). Single nucleotide base changes including deletion, insertion and substitution, may play an important role in gene transcription and amino acid sequences of mature proteins. The RFLP banding patterns revealing different nucleotide sequences for different animals were sequenced to identify the position and nature of possible SNPs responsible for sequence variation. The multiple sequence alignment (MSA) of generated sequences (query sequences) was carried out with the help of Codon Code Aligner software to detect the position of SNPs in amplified fragments through comparison with the reference sequence for camel (Fig 1). Results reveal that polymorphism contained in different RFLP patterns of 488-bp fragments of the kappa casein gene in camel were the result of substitution of four bases at four different positions in the nucleotide sequences (Table 2). The four different SNPs were observed to be located at the 147th, 391st, 392nd and 426th position in the amplified fragment of 488-bp of the CSN3 gene. Two out of 4 detected SNPs were transition transitory in nature and the remaining two were transversion mutations (Table 2).

The nucleotide substitutions detected at the 391st position were found to be non-synonymous in nature, with the predicted protein sequence detecting the presence of tyrosine instead of leucine. The present study agrees with the study conducted by Othman *et al* (2016), which reported one SNP (C>T) at position 121 in the 488 bp amplified fragments which are responsible for destruction of site (AG/CT) in Maghrabi camels. Similarly, Pauciullo *et al* (2013) identified 17 SNPs in the kappa casein gene in *Camelus dromedarius* and thereof

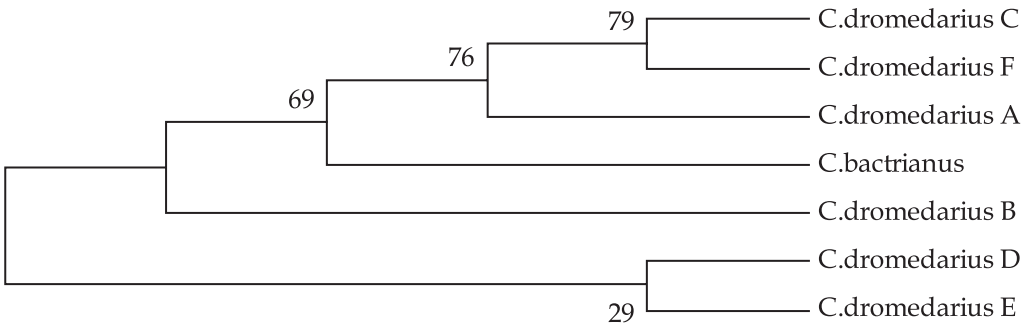

Fig 2. Phylogenetic analysis of the kappa casein gene by Neighbor-Joining method.

Table 1. List of accession numbers obtained for different RFLP patterns of the kappa casein gene.

RFLP Pattern	Product size	Accession number
kCN Variant A	488-bp	MG586889
kCN Variant B	488-bp	MG586890
kCN Variant C	488-bp	MG586891
kCN Variant D	488-bp	MG586892
kCN Variant E	488-bp	MG586893
kCN Variant F	488-bp	MG586894

Table 2. List of position and nature of SNPs observed in the kappa casein gene.

147	T>C	Transition	Synonymous	No change
391	T>A	Transversion	Non-Synonymous	(Leucine to Tyrosine)
392	T>A	Transversion	Synonymous	No change
426	G>A	Transition	Synonymous	No change

these SNPs were found in exon 4. Tahmoorespur *et al* (2016) using in SNP screening of the CSN3 gene in Iranian dromedarius and Bactrian camels, indicated that there were 3 and 1 single mutation found in 10 samples of dromedary and 5 samples of Bacterian camel, respectively. Consistent with our results, studies with other species such as buffalo, sheep, goat, and cattle, revealed many nucleotide changes in exon 4 of the kappa casein gene. Mukesh *et al* (2005) reported seven nucleotide changes: 14 (Asp–Glu), 19 (Asp/Ser–Asn), 96 (Ala–Thr), 126 (Ala–Val), 128 (Ala/Gly–Val), 156 (Ala/Pro–Val) and 168 (Ala/Glu–Val) and they were limited to exon 4 only. Rachagani *et al* (2008) revealed two allelic variants in exon 4 of bovine and these variants were differentiated by PCR-RFLP in indigenous Tharparkar and Sahiwal breeds. Our study concluded that increasing the number of camel populations in future studies may identify more SNPs in the CSN3 gene, which may be useful for determining effective milk camel breeding strategies.

Phylogenetic Analysis

The FASTA converted nucleotide sequences of the kappa casein gene of camel were tested for their evolutionary relationship with the variants using MEGA7 software. A dendrogram was considered an effective way to represent genetic distances through construction of tree-based diagrams. A phylogenetic tree was constructed through the algorithm-based Neighbor-Joining (NJ) method of Saitou and Nei (1987) to observe the evolutionary between the studied variants of the kappa casein gene (Fig 2). The Tamura 3-parameter model (Tamura, 1992) was selected from 24 different maximum likelihood substitution models for construction of the dendrogram as it had the lowest minimum BIC value (1481.512). Bootstrapping was carried out to obtain confidence intervals for the grouping of variants and to test the validity of the clusters obtained. A bootstrap consensus tree, inferred from 1000 replicates (Felsenstein, 1985), was taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% of the bootstrap replicates were collapsed. The NJ tree showed bootstrap values ranging from 29 to 79. The *Camelus dromedarius* C is identical to *Camelus dromedarius* F and they are forming one cluster and *Camelus dromedarius* A and *Camelus bactrianus* are separated and they are little bit distant and other forming two lineages with *C. dromedarius* D and E are more distant. While *Camelus dromedarius* D and E are in same cluster and they are identical.

In conclusion, the CSN3 gene of Bikaneri dromedary was investigated in order to detect molecular variation through sequencing methods and determine the divergent evolution of Bikaneri camel breed. The presence of within variation in Bikaneri camel suggests that it is non-conserved in nature and this may be due to dilution of the breed. Intra-specific variation suggests that increasing the number of camel populations included in future

studies could identify more mutations in the kappa casein gene which may be useful in determining camel breeding strategies.

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