DOI : 10.5958/2277-8934.2020.00029.6 *Vol 27 No 2, p 201-206*

GENETIC POLYMORPHISM AT κ-CASEIN GENE IN INDIAN CAMEL BREEDS (*Camelus dromdarius*)

S.A. Jadhav, U.D. Umrikar, M.P. Sawane, V.D. Pawar, R.S. Deshmukh, S.S. Dahiya[1] and S.C. Mehta[1]

Bombay Veterinary College, Parel, Mumbai- 400012, Maharashtra
[1]ICAR-National Research Centre on Camel, Post Box-07, Bikaner-334001, Rajasthan

ABSTRACT

The present study was carried out in 112 camels belonging to Bikaneri, Jaisalmeri, Kachchhi and Mewari breeds of Indian dromedary to detect point mutation in κ- casein encoding gene. Amplification of 488 bp fragment of κ-Casein gene spanning from -137 (5′ flanking region) to +351 bp of κ-CN gene was carried out and genotyped for the g.1029T>C SNP using the restriction enzyme *Alu*I in PCR-RFLP analysis. Three restriction patterns were resolved on 3.5% agarose gels. The pattern, comprising of 203 bp, 158 bp and 127 bp bands, was resolved successfully for the TT samples. The g.1029T>C transition created an additional restriction site for the enzyme *Alu*I leading to the digestion of 158 bp band into two fragments of 120 bp and 38 bp resulting in the 5 band pattern of 203 bp, 158 bp, 127 bp, 120 bp and 38 bp for CT genotype and 4 band pattern of 203 bp, 127 bp, 120 bp and 38 bp for CC genotype. The genotype frequency, pooled over breed, was 0.045, 0.384 and 0.571 for the CC, CT and TT genotypes, respectively. The frequency of major allele T was observed to be 0.763 and that of C was observed to be 0.237. The existence of CT genotype in sizable number documents the dynamic nature of the locus g.1029T>C SNP, in Indian dromedary breeds. Almost comparable polymorphism was observed in both the sexes. The 3 genotypes, *viz.* CC, CT, TT, were almost equally distributed among the four Indian breeds (χ^2=3.4529; P = 0.750224). The frequency of C allele was lowest in Bikaneri and highest in the Mewari breed. Though the frequency of C allele (Cytosine) in Indian dromedary is relatively low (0.237), still a rapid directional selection might be attempted in favour of the C allele, which is responsible for the creation of an extra putative site for the Hepatocyte Nuclear Factor – 1 (HNF-1) transcription factor. The HNF-1 is reported to be involved in regulation of a number of genes associated with innate immunity, lipid and glucose transport, metabolism etc.

Key words: Camel, dromedary, kappa-casein, milk, polymorphism

The protein content in dromedary milk ranges from 2.3 to 4.9% in different camel rearing countries. The casein is the major protein (1.63 to 2.76%) in camel milk and constitutes about 52 to 87% of the total protein (Konuspayeva *et al,* 2009; Nikkah, 2011a; 2011b; Singh *et al,* 2017). In camel milk κ-CN is 3.5% (El Agamy, 2006) and is encoded by CSN3 gene (Kappeler *et al,* 1998). It is reported that κ-CN plays an essential role in stabilisation of casein micelle (Alexander *et al,* 1988). It is chiefly located on micellar surface and is the specific substrate of chymosin, responsible for the hydrolysation of the κ-CN into para-κ-CN and the caseino-macropeptide (CMP) (Kappeler *et al*, 2006; Moller *et al,* 2012). Existence of 16 alleles corresponding to 13 κ-CN variants have been reported in goat (Caroli *et al,* 2006) and 19 alleles corresponding to 14 κ-CN variants have been reported in cattle (Caroli *et al,* 2009). However, the information about the DNA sequence of dromedary κ-CN is coming at a slow pace. The cDNA sequence and comparison of the 5′ flanking regions of CNS3 gene in Somali camel has been reported by Kappeler *et al* (1998) and Kappeler *et al* (2003), respectively. Recently, Pauciullo *et al* (2013) reported full length sequence of CSN3 gene along with 1045 nucleotides of 5′ flanking region of κ-CN in Sudanese dromedary. They have also reported 17 polymorphic sites in Sudanese camels. The information regarding genetic variability in dromedary populations existing elsewhere in the world is largely lacking including the Indian subcontinent. Looking at this and the increased importance of camel milk for human therapeutics, this study was planned to investigate the genetic variability in κ-Casein gene spanning from -137 (5′ flanking region) to +351 bp of κ-CN in Indian dromedary breeds.

Materials and Methods

Experimental animals

The blood samples were collected from 112 camel belonging to Bikaneri, Jaisalmeri, Kachchhi

SEND REPRINT REQUEST TO S.C. MEHTA email: scmehta64@gmail.com

and Mewari breeds (Table 1) at the ICAR-National Research Centre on Camel, Bikaner, Rajasthan. Approximately 10 ml of venous blood was collected in EDTA and were transported to the laboratory in ice box and stored at 4°C until use.

PCR conditions

DNA was isolated using phenol-chloroform method (Sambrook *et al,* 1989) with minor modifications. The PCR primers; forward: CACAAAGATGACTCTGCTATCG and reverse GCCCTCCACATATGTCTG were utilised (Pauciullo *et al,* 2013). PCR amplifications were carried out in 12.5 µl reactions containing 50 ng DNA, 12.5 pmol each primer (Sigma-Aldrich), 1.0 U *Taq* DNA polymerase, 0.2 mM each dNTP, 1.25 µl 10X Taq DNA polymerase buffer containing 10 mM Tris – HCl (pH 9.0), 1.5 mM $MgCl_2$, 50 mM KCl and 0.01% gelatin. The PCR amplification programme, performed on Eppendorf Mastercycler Gradient, consisted of an initial denaturation temperature of 95°C for 5 min, then 34 cycles at 94°C for 30s, 56°C for 30 s and 72°C for 45s. Final extension was carried out at 72 °C for 5 min. The κ-Casein bands were visualised in 1% agarose gel containing ethidium bromide. The electrophoresis was carried out in 1XTBE at 80 volts and the results were recorded using UVP gel-documentation system.

Restriction digestion

Around 250-500 ng of amplified PCR products were digested in 10 µl reaction using 5 units of *Alu*I restriction enzyme (BioLabs) with CutSmart Buffer and incubating at 37°C for 15 minutes. The restriction bands were analysed on 3.5% Agarose gel electrophoresis with appropriate marker DNA.

Statistical analysis

The Chi-square test ($\chi 2$) was performed using IBM SPSS Statistics 20 software (2017) to test the statistical significance of the differences between observed and expected frequencies in genotypic classes.

Sequencing and sequence analysis

The PCR products were got sequenced on ABI3730 DNA Sequencer. The SNPs were visualised on chromatograms using Chromas 2.6.6 software. The sequences were analysed using BioEdit Sequence Alignment Editor (Hall, 1999). Sequence phylogeny was derived using Nucleotide BLAST programme of NCBI.

Results and Discussion

PCR amplification of κ-Casein gene promoter

Amplification of 488 bp fragment spanning from -137 of 5′ flanking region (promoter) to +351 bp of κ- Casein gene was successfully achieved by PCR in Bikaneri, Jaisalmeri, Kachchhi and Mewari Camels (Fig 1). The present results are in agreement with the findings of Pauciullo *et al* (2013) in Sudanese camel (*Camelus dromedarius*), Othman *et al* (2016) in Maghrabi camel of Egypt and Yamini *et al* (2019) in Bikaneri camel of India, where the same set of primers was utilised for the amplification of 488 bp fragment of CSN3 gene and it's 5′flanking region.

PCR-RFLP of κ-Casein gene, promoter fragment

The detection of point mutation at κ-Casein gene, promoter was attempted using PCR-RFLP. The restriction fragments were resolved in 3.5% Agarose gel. The RE digestion of PCR product (488 bp) of κ-Casein gene from the Indian camel (*Camelus dromedarius*) breeds using *Alu*I lead to 3 fragments of 203 bp, 158 bp and 127 bp for the TT genotype camels. The 158 bp long band was further restricted into 2 daughter fragments of 120 bp and 38 bp in the presence of cytosine. Thus, four bands of 203 bp, 127 bp, 120 bp and 38 bp (not resolved) were observed in animals having CC genotype. Accordingly, the

Table 1. Genotype frequency and allele frequency in Indian dromedary at κ-Casein gene, promoter.

Genotype	Bikaneri			Jaisalmeri			Kachchhi			Mewari		
	M	F	P	M	F	P	M	F	P	M	F	P
N	13	15	28	6	22	28	13	15	28	13	15	28
CC	0.00	0.00	0.00	0.00	0.04	0.03	0.00	0.13	0.07	0.08	0.07	0.07
CT	0.38	0.33	0.36	0.17	0.50	0.43	0.38	0.27	0.32	0.23	0.60	0.43
TT	0.62	0.67	0.64	0.83	0.46	0.54	0.62	0.60	0.61	0.69	0.33	0.50
Allele T	0.81	0.83	0.82	0.92	0.70	0.75	0.81	0.73	0.77	0.81	0.63	0.71
Allele C	0.19	0.17	0.18	0.08	0.30	0.25	0.19	0.30	0.23	0.19	0.37	0.29

M-Male; F-Female; P-Pooled Sex; N-Number of Animals

heterozygous animals with CT genotype showed 5 fragments of 203 bp, 158 bp, 127 bp, 120 bp and 38 bp (not resolved). The results are presented in table 1 and Fig 2.

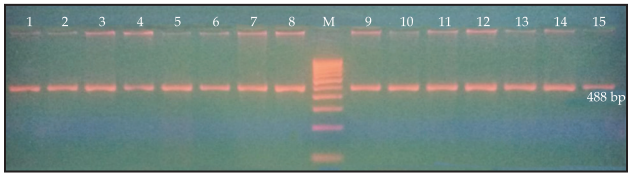

Fig 1. Amplification of 488 bp fragment of κ-Casein gene, promoter in Indian camel (Lane 1 – 15) ; M-100 bp marker.

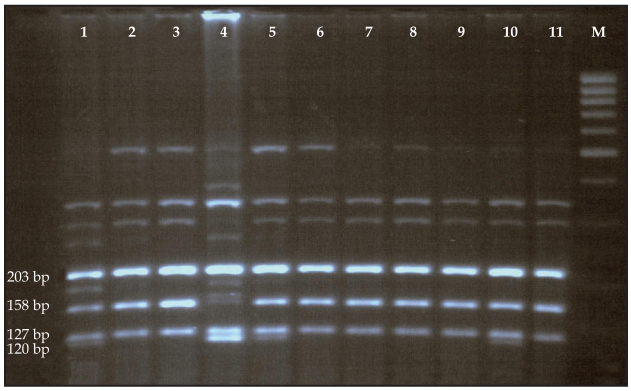

Fig 2. PCR-RFLP genotyping of camel κ- casein gene, promoter with *Alu*I. Lane 4 : CC genotype (203bp, 127 bp, 120bp and 38 bp) ; Lane 1, 5 and 10 : CT genotype (203 bp, 158 bp, 127 bp, 120 bp and 38 bp) ; Lane 2-3, 6-9 and 11 : TT genotype (203bp, 158 bp and 127 bp); M-50 bp DNA marker.

The results indicated that the TT genotype was the most numerous followed by CT, and the CC genotype was the least numerous. The nucleotide substitution at g.1029T>C SNP was observed in all the camel breeds studied but the CC genotype was not observed in Bikaneri camels analysed in the study. The frequency of CT genotype in Bikaneri, Jaisalmeri, Kachchhi and Mewari breeds were observed to be 0.357, 0.429, 0.322 and 0.429, respectively. The frequency of the CC, CT and TT genotypes, pooled over breeds, was 0.045, 0.384 and 0.571, respectively (Table 2). The existence of CT genotype in sizable number documents the dynamic nature of the locus g.1029T>C SNP, in Indian dromedary breeds. The frequency of major allele T was observed to be 0.763 and that of C was observed to be 0.237. Almost comparable polymorphism was observed in both the sexes (Table 2). The three genotypes, *viz.* CC, CT, TT, were almost equally distributed among the 4 Indian breeds (χ^2=3.4529; P = 0.750224; non-significant at 5% probability level of significance).

Table 2. Genotype and allele frequency in Indian dromedary at κ-Casein gene, promoter.

Genotype	Male	Female	Pooled	Allele	Frequency
CC	0.02	0.06	0.045	C	0.237
CT	0.31	0.43	0.384	T	0.763
TT	0.67	0.51	0.571		

Othman *et al* (2016) studied the genetic polymorphism of κ-casein gene in Maghrabi camel reared in Egypt. The amplified fragments at 488-bp of κ-CN gene were digested with *Alu*I endonuclease. The results showed the presence of 3 genotypes; CC (12%), TT (48%) CT (40%). The finding of Othman *et al* (2016) are in agreement with the present findings in Indian camel breeds except the reporting of higher frequency of CC genotype in Maghrabi camel. Comparable results have also been reported by Pauciullo *et al* (2013) in Sudanese camel breeds where they also observed little higher frequency of CC genotype (0.18) as against (0.045) in present investigation indicating relatively higher replacement rate at the locus g.1029T>C SNP. Accordingly, the frequency of C allele in the sample of 188 Sudanese camels was 0.38, with a variation among the breeds ranging from 0.30 to 0.46 and that of T allele was 0.62 with the variation among the breeds ranging from 0.54 to 0.70. However, Yamini *et al* (2019) reported 3 fragments of 203 bp, 158 bp and 127 bp upon restriction digestion of 488 bp κ-Casein gene fragment with *Alu*I restriction enzyme in TT genotyped camels of Bikaneri breed, which is in agreement with the present findings but restriction digestion of 158 bp fragment with *Alu*I enzyme leading to 2 daughter bands of 146 bp and 12 bp was not observed in the present investigation involving Bikaneri, Jaisalmeri, Kachchhi and Mewari breeds of Indian dromedary; however, these were observed by Pauciullo *et al* (2013) in Shanbali, Kahli, Arabi and Lahaoi breeds of Sudanese camel; and by Othman *et al* (2016) in Maghrabi camel reared in Egypt. Thus, the CT genotype reported by Yamini *et al* (2019) was different from the CT genotype referred in above 3 studies. Though, the frequency of C allele (Cytosine) in Indian dromedary is relatively low (0.237), still a rapid directional selection might be attempted in favour of the C allele, which is responsible for the creation of an extra putative site for the Hepatocyte Nuclear Factor – 1 (HNF-1) transcription factor. The HNF-1 is reported to be involved in regulation of a number of genes associated with innate immunity, lipid and glucose transport, metabolism etc. Here it will be worth mentioning that the camelids are considered as evolutionary innovation because of their only heavy chain antibodies (Hamers-Casterman *et al,* 1993). Although, the three genotypes, *viz.* CC,

CT, TT, were almost equally distributed among the 4 Indian breeds (χ^2=3.4529; P = 0.750224), it was observed that the replacement to thymine with cytosine at g.1029 locus was lowest in Bikaneri breeds followed by Kachchhi and Jaisalmeri, and highest in Mewari breed of camel (Table 1). The Bikaneri, Jaisalmeri, Kachchhi and Mewari breeds of Indian dromedary are adapted in different geo-climatic

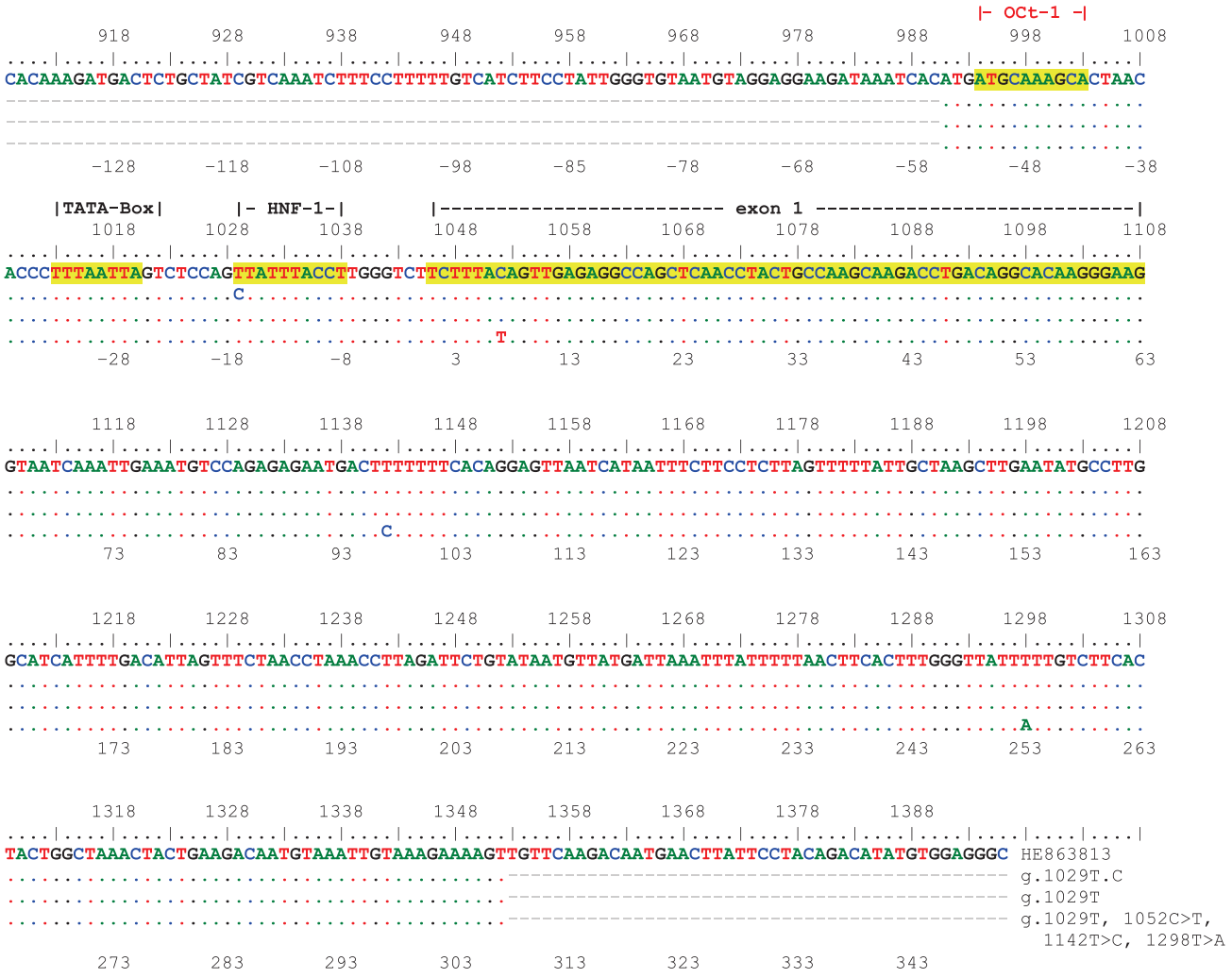

Fig 3. Alignment of nucleotide sequences of the promoter region, exon-1 and intron-1 of *C. dromedarius* CSN3 gene variants observed in the present study with the published sequence of the camel (NCBI GenBank ID HE863813). The first line numbering is as per the reference sequence HE863813 and the lower numbering is relative to the first nucleotide of first exon (+1).

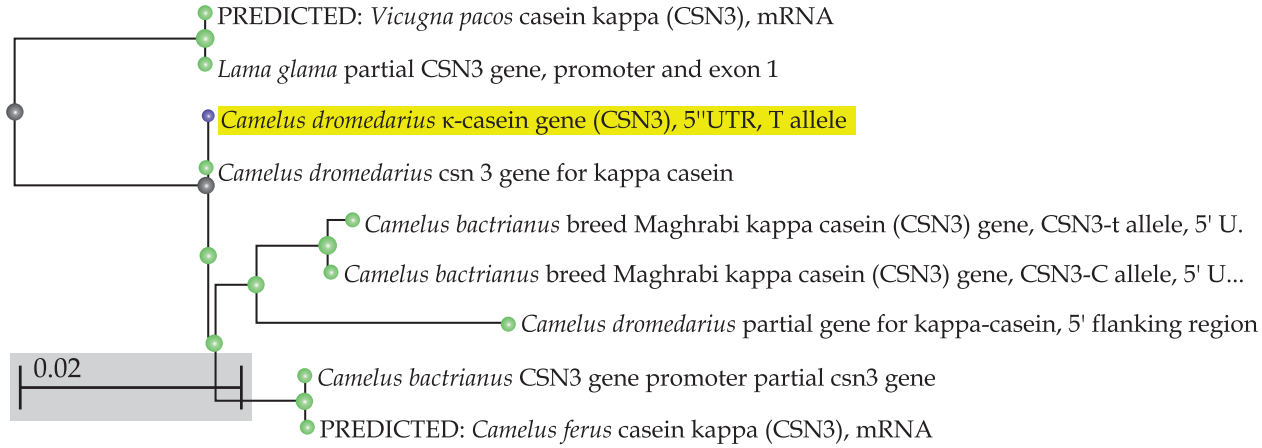

Fig 4. Phylogenetic analysis of sequence containing g.1029 T allele in κ-casein gene, promoter.

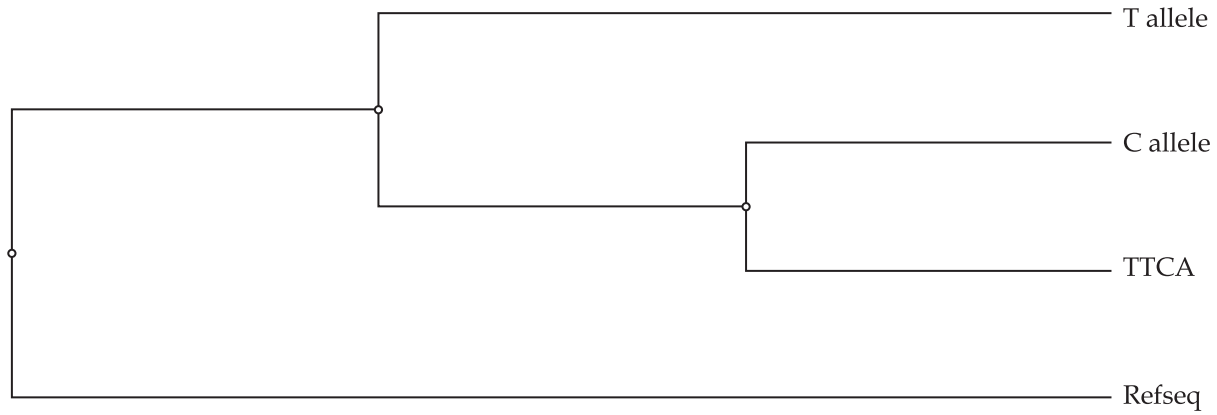

Fig 5. Phylogenetic analysis of sequence containing T allele (g.1029 T), C allele (g.1029T>C), TTCA alleles (g.1029T, 1052C>T, 1142T>C, 1298T>A) in κ-casein gene, promoter in Indian camel in relation to the published sequence (NCBI GenBank ID HE863813).

conditions and have recently been evaluated and/or selected for milk production (Mehta *et al,* 2011; 2014; 2015). Nevertheless, it will be too early to correlate this SNP with above classification and selection of Indian camel breeds because of relatively small sample size (28 animals per breed) to establish this relationship.

Nucleotide-substitution: SNP verification by sequencing

The samples identified as representing TT, CT and CC genotype with respect to the single g.1029T >C nucleotide substitution in CSN3 gene were sequenced. Fig 3 presents the alignment of nucleotide sequences of the promoter region, exon-1 and intron-1 of *C. dromedarius* CSN3 gene variants observed in the present study with the published sequence of the camel (NCBI GenBank ID HE863813). The analysis of sequences confirms the specificity of the sequences for the identified SNP genotypes. The TT homozygous animal was also observed to be heterozygous for g.1052C>T, g.1142 T>C and g.1298 T>A (Fig 3). The phylogenetic analysis reveals that the sequence containing g.1029T allele was placed at a distance of 0.017 from evolutionary point of reference in the node containing CSN3 gene and 5'UTR sequences of *Camelus dromedarius*, *Camelus bactrianus* and *Camelus ferus*. The other node of the phylogenetic tree had CSN3 gene and 5'UTR sequences of *Lama glama* and *Vicugna pacos* (Fig 4). The phylogenetic relationship between sequences containing g.1029T allele; g.1029T allele along with g.1052C>T transition; g.1142T>C transition and g.1298T>A trans-version; and g.1029T>C transition was studied in relation to the published sequence (NCBI GenBank ID HE863813) and presented in the Fig 5. The analysis suggested that the sequences containing C allele and other transition and transversion are of subsequent origin (Fig 4-5), which is also substantiated by the paucity of CC genotype animals and lower frequency of C allele in all the 4 breeds covered in the present investigation. The SNPs g.1052C>T transition; g.1142T>C transition and g.1298T>A trans-version needs to be further investigated by increasing the sample size and working out possible correlation with the traits of economic importance.

The study documents existence of genetic polymorphism in Indian dromedary breeds in the CSN3 gene promoter just upstream of the exon 1 creating extra putative site for the transcription factor HNF-1. The influence of HNF-1 allelic variants on CSN3 needs to be further substantiated. Nevertheless, the presence of C allele at g.1029 locus in the 5'UTR of CSN3 gene with the frequency 0.24 gives the opportunity for the rapid directional selection in favour of such allele. This DNA based PCR-RFLP test can be used for typing camel CSN3 variability independent of age, sex and stage of lactation of animals for selecting them in breeding and production programmes.

Acknowledgement

This work was carried in the Institutional Project of ICAR-NRC on Camel, Bikaner. The authors thank the Indian Council of Agricultural Research, New Delhi for providing the financial support and other facilities for the research.

References

Alexander LJ, Stewart AF, Mackinlay AG, Kapelinskaya TV, Tkach TM and Gorodetsky SI (1988). Isolation and characterisation of bovine kappa-casein gene. European Journal of Biochemistry 178:395-401.

Caroli A, Chiatti F, Chessa S, Rignanese D, Bolla P and Pagnacco G (2006). Focusing on the goat casein complex. Journal of Dairy Science 89:3178–87.

Caroli AM, Chessa S and Erhardt GJ (2009). Milk protein polymorphisms in cattle: effect on animal breeding and human nutrition. Journal of Dairy Science 92: 5335–52.

El Agamy EI (2006). Camel milk. In: Handbook of Non-Bovine Mammals, eds., Park Y W and Haenlein F W, Blackwell Publisher Professional, Iowa, NJ, USA. pp 297-344.

Hall TA (1999). BioEdit : a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41:95-8.

Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hammers C, Bajyana Songa E, Bendahman N and Hammers R (1993). Naturally occurring antibodies devoid of light chains. Nature 363:446-8.

IBM Corp. (2017). IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.

Kappeler S, Farah Z and Puhan Z (1998). Sequence analysis of *Camelus dromedarius* milk caseins. Journal of Dairy Research 65:209-22.

Kappeler S, Farah Z and Puhan Z (2003). 5′ Flanking regions of camel milk genes are highly similar to homologue regions of other species and can be divided into two distinct groups. Journal of Dairy Science 86:498-508.

Kappeler SR, van den Brink HJ, Rahbek-Nielsen H, Farah Z, Puhan Z, Hansen EB and Johansen E (2006). Characterisation of recombinant camel chymosin reveals superior properties for the coagulation of bovine and camel milk. Biochemical and Biophysical Research Communications 342(2):647-54.

Konuspayeva G, Faye B and Loiseau G (2009). The composition of camel milk: a meta-analysis of the literature data. Journal of Food Composition and Analysis 22:95-101.

Mehta SC, Bissa UK, Patil NV and Pathak KML (2011). Importance of camel milk and production potential of dromedary breeds. Indian Journal of Animal Science 81(11):1173-7.

Mehta SC, Yadav SBS, Singh S and Bissa UK (2014). Sire evaluation and selection of Indian dromedary for milk production: issues and strategies. Journal of Camel Practice and Research 21(1):93-8.

Mehta SC, Sharma AK, Bissa UK and Singh S (2015). Lactation persistency, yield and prediction models in Indian dromedary. Indian Journal of Animal Science 85(8): 875-82.

Møller KK, Rattray FP, Sørensen JC and Ardö Y (2012). Comparison of the hydrolysis of bovine κ-casein by camel and bovine chymosin: a kinetic and specificity study. Journal of Agricultural and Food Chemistry 60(21):5454-60.

Nikkah A (2011a). Equidae, camel, and yak milks as functional foods: a review. Journal of Nutrition and Food Sciences 1(5):100-116.

Nikkah, A (2011b). Science of camel and yak milks: human nutrition and health perspectives. Food and Nutrition Sciences 2:667-73.

Othman EO, Nowier Am and El-Denary ME (2016). Genetic variations in two casein genes among Maghrabi camels reared in Egypt. Biosciences Biotechnology Research Asia 13(1):473-80.

Pauciullo A, Shuiep ES, Cosenza G, Ramunno L and Erhardt G (2013). Molecular characterisation and genetic variability at k-casein gene (CSN3) in camels. Gene 513:22-30.

Sambrook J, Fritsh E F and Manities T (1989). Molecular cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbour Laboratory Press, New York.

Singh R, Mal G, Kumar D, Patil NV and Pathak KML (2017). Camel Milk: An Important Natural Adjuvant. Agricultural Research 6(4):327-40.

Yamini, Gahlot GC, Pannu U, Ashraf M and Choudhary S (2019). Assessment of genetic variability in kappa Casein gene in Indian dromedary. Journal of Camel Practice and Research 26(3):255-8.