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Determination of genetic variability at α_{s1}-casein gene in Indian dromedary

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ABSTRACT

The analysis of genetic variability at α_{s1}-casein gene in 112 Indian dromedary (*Camelus dromedarius*) was carried out. The 930 bp fragment of exon 5 of α_{s1} casein gene was successfully amplified by PCR. The nucleotide substitution at g.942G>T SNP (GenBank ID: JF429140) was detected by PCR-RFLP using *Sml*I restriction enzyme and verified by sequencing. The GT genotype was not observed in Bikaneri and Kachchhi camels but was observed in Jaisalmeri and Mewari camels at the frequency of 0.143 and 0.071, respectively. Though, the frequency of GT genotype was quite low but still it was enough to document the dynamic nature of the locus g.942G>T SNP in Indian dromedary. Due to the existence of full agreement between genotyping by PCR-RFLP and Isoelectric Focusing at α_{s1}-casein, as envisaged by earlier researchers, the existence of two protein patterns corresponding to α_{s1}-casein alleles, A and C at the same frequency values in the Indian dromedary breeds may also be inferred from the present study. Hence, this DNA based test can be used for typing camel α_{s1}-casein gene variability in Indian dromedary.

Keywords: Alpha-casein, Camel, Dromedary, Genetic variability, Milk

India is the largest producer of the milk (176.3 million tons per annum) in the world; still the per capita milk availability is only 375 g per day (BAHS 2018). Bovines' milk is the main milk that is being consumed by the human population across the world but a significant proportion of the non-bovine milk is also being consumed by the human population for day to day nutritional requirement (Faye and Konuspayeva 2012). Camel remains the significant contributor to the non-bovine milk in the desert regions of the world. The family Camelidae has two main tribes: Old World *Camelini* and New World *Lamini*. The *Camelini* tribe has two genera, the genus *Camelus* having the single-humped camel (*Camelus dromedarius*) and the genus *Bactrianus* having the double-humped wild bactrian camel (*Camelus ferus*) and the bactrian camel (*Camelus bactrianus*). The *Lamini* tribe also has two genera. The genus *Lama* having llama (*Lama glama*) and guanaco (*Lama guanicoe*) and the genus *Vicugna* having alpaca (*Vicugna pacos*) and vicuna (*Vicugna vicugna*) at present time (Barazandeh *et al.* 2019). The two-humped bactrian camel and single-humped dromedary live in the extreme desert environments of Africa and Asia, and their adaptations to arid conditions include a tolerance of temperatures exceeding 40°C and water losses greater than 25% of total body weight. In contrast, their nearest relatives (*Lamini*) which have no hump live in the high altitudes of South America and do not exhibit similar adaptations to hot desert environments (Barazandeh *et al.* 2019). Camels are important as beasts of burden and also as sources of meat, milk and wool (Ghasemi *et al.* 2016). Interest in camels rearing is increasing nowadays for human food production and production of modern therapeutics (Barazandeh *et al.* 2019). As much as interest is increasing, a major concern is the potential cumulative effects of global climate change, as it is increasingly recognized to have significant impacts on biodiversity (Ghasemi *et al.* 2016). The Indian dromedary produces about 3017±148 litres of milk in a lactation spanning over 16 months in the harsh desert climate (Mehta *et al.* 2011, 2014, 2015). The population of camel in the world is increasing with the current strength of 34.8 Million heads spread over 47 countries in the year 2017. The production of camel milk has also increased until the year 2012 (3.0 million tonnes) but has recently dwindled to 2.8 million tonnes in the year 2017 (FAOSTAT 2017). In India, camels were chiefly used for their draught power but with increased mechanization, the draught utility vanished significantly and the conservation scientists and stakeholders-initiated production and marketing of camel milk and milk products. Until recent, the camel milk production figures were not available for India (Mehta *et al.* 2009). Scientists and stakeholders are also trying to exploit the therapeutic utility of camel milk for betterment of the society as well as for sustenance of the species. The camel milk proteins have been found to possess anti- bacterial and anti-viral activity (El-Agamy *et al.* 2006). Angiotensin I-Converting Enzyme

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Table 1. Genotype frequency in Indian dromedary at exon 5 of α_{s1} casein gene

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
Genotype frequency												
GG	1.0	1.0	1.0	0.83	0.86	0.86	1.0	1.0	1.0	0.92	0.93	0.93
GT	0.0	0.0	0.0	0.17	0.14	0.14	0.0	0.0	0.0	0.08	0.07	0.07
TT	0.0	0.0	0.0	0.00	0.00	0.00	0.0	0.0	0.0	0.00	0.00	0.00

M, Male; F, Female; P, Pooled sex; N, number of animals.

(ACE) Inhibitory peptides have also been found in fermented camel milk (Singh *et al.* 2017). The camel milk has also been considered as a new source of protein to the children allergic to bovine milk (El-Agamy *et al.* 2006). Apart from these, several other therapeutic utilities have been tagged to camel milk (Singh *et al.* 2017). The production and marketing of camel milk and its products and production of designer milk is another way to add value to the camel milk and ensuring increased returns to the camel owners. The protein content in Indian dromedary has been estimated to be 2.3%, however it ranges from 2.3 to 4.9% in different camel rearing countries (Singh *et al.* 2017). The total protein content is divided into caseins and whey proteins (Kappeler *et al.* 2003). The casein is the major protein (1.63 to 2.76%) in camel milk and constitutes about 52 to 89%. The casein fraction is distributed into four fractions: α_{s1}-, α_{s2}-, β -, and κ-CN (El-Agamy *et al.* 2006, Ochirkhuyag *et al.* 1997), encoded by four genes, *CSN1S1*, *CSN1S2*, *CSN2* and *CSN3,* respectively (Kappeler *et al.* 1998). In camel milk α_{s1}-CN (22%) is the second main fraction after β-CN (65%) and before α_{s2}-CN (9.5%) and κ-CN (3.5%) (El-Agamy *et al.* 2006). This study was therefore conducted to document the genetic variability at α_{s1} -casein gene in the Indian dromedary breeds for the possible production of different types of milk with reference to α_{s1}-casein composition.

MATERIALS AND METHODS

Experimental animals: Indian dromedary herd maintained at the ICAR-National Research Center on Camel, Bikaner, Rajasthan was utilized for the study. Twenty-eight adult (≥4 years) unrelated camels of each breed were selected for the study. The breed and sex-wise distribution of the animals used in the study is presented in Table 1. Thus, in all 112 blood samples (5 ml each) were collected from jugular vein using vacutainer tubes containing EDTA as anti-coagulant. The samples were 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 TGAACCAG-ACAGCATAGAG and reverse CTAAACTGAATGGGT-GAAAC were utilized (Shuiep *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 program, performed on Eppendorf Mastercycler Gradient (Germany), consisted of an initial denaturation temperature of 95°C for 5 min, then 34 cycles at 94°C for 30s, 58°C for 30 s and 72°C for 1 min. Final extension was carried out at 72°C for 5 min. The α_{s1} -casein bands were visualized on 1% agarose gel containing ethidium bromide. The electrophoresis was carried out in 1X TBE at 80 volts and the results were recorded using UVP gel-documentation system (UVP–GDS 7600, UVP International, UK).

Restriction digestion: Around 250–500 ng of amplified PCR products were digested in 10 µl reaction using 5 units of *Sml*I restriction enzyme (New England BioLabs Inc, USA) with recommended CutSmart Buffer and incubating at 55°C for 120 minutes. The restriction bands were analyzed on 2% Agarose gel electrophoresis (Scie-Plas Ltd., UK), using Phage Lambda DNA *Eco*RI / *Hind* III digest (Promega Corporation, USA) DNA marker.

Sequencing and sequence analysis: The PCR products were got sequenced on ABI 3730 DNA Analyser (Applied Biosystems, USA). The SNPs were visualized on chromatograms using Chromas 2.6.6 software (Technelysium DNA Sequencing Software, Australia). The sequences were analysed using multi-align editor of GeneTool Lite 1.0 software (BioTools Incorporated, US). Sequence phylogeny were derived using Nucleotide BLAST programme of NCBI (Altschul *et al.* 1990).

Statistical analysis: To test the distribution of genotypes in the studied dromedary populations for Hardy-Weinberg equilibrium, Chi-Square (χ^2) goodness of fit test was performed (Snedecor and Cochran, 1989).

RESULTS AND DISCUSSION

PCR amplification of α_{s1} casein gene locus: The PCR amplification of 930 bp fragment of exon 5 of α_{s1} casein gene was successfully achieved in Bikaneri, Jaisalmeri, Kachchhi and Mewari Camels (Fig. 1). The present results are in agreement with the findings of Shuiep *et al.* (2013) where PCR amplification of 930 bp fragment of exon 5 of α_{s1} casein gene in Sudanese camel (*Camelus dromedarius*) was achieved using the same primer pair.

PCR-RFLP of α_{s1} casein gene fragment: In the present investigation, the detection of point mutation at α_{s1} -casein gene locus was attempted using PCR-RFLP. The restriction

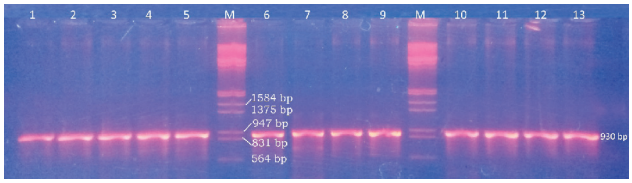

Fig. 1. Amplification of 930 bp fragment of exon 5 of α_{s1} casein gene in Indian camel (Lane 1–13). M- Phage Lambda DNA *Eco*RI/ *Hind* III digest.

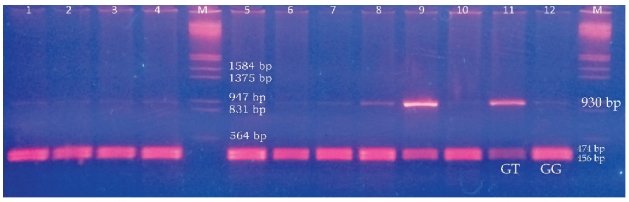

Fig. 2. PCR-RFLP genotyping of camel CSN1S1 with *Sml*I. Lane 1–8,10 and 12 are CSN1S1- GG genotype (474 bp and 456 bp) and Lane 9 and 11 are CSN1S1- GT genotype (930 bp, 474 bp and 456 bp); M- Phage Lambda DNA *Eco*RI / *Hind* III digest.

fragments were visualized on 2% agarose gel. The restriction digestion of the PCR product (930 bp) of α_{s1} - casein gene from the Indian domestic camel (*Camelus dromedarius*) using *Sml*I lead to two digested bands of 456 bp and 474 bp when guanine base was occurring. The G>T nucleotide substitution destroys the restriction site of *Sml*I; therefore, PCR-products of such animals were not digested (Fig. 2).

The breed-wise observation of data indicated that the GT genotype was absent in Bikaneri and Kachchhi camels showing that the nucleotide substitution at g.942G>T SNP (GenBank ID: JF429140) has not occurred in the randomly selected animals of these two breeds. However, the frequency of GT genotype in Jaisalmeri and Mewari was observed to be 0.143 and 0.071 respectively. The frequency of the GT genotype pooled over breeds was 0.054. Though, the frequency of GT genotype was quite low but still it was enough to document the dynamic nature of the locus g.942G>T SNP (GenBank ID: JF429140) in Indian dromedary. Almost comparable polymorphism was observed in both the sexes. The distribution of 3 genotypes in the two sexes and pooled over sexes was tested for Hardy-Weinberg equilibrium. The value of χ^2 in male, female and pooled over sexes was estimated to be 0.025, 0.063 and 0.08 indicating non-significant (P<0.05) deviation from Hardy-Weinberg equilibrium frequencies (Table 2). The frequency of major allele G was 0.973 and that of T was observed to be 0.027 (Table 2). Here it may be pertinent to write that the Bikaneri, Kachchhi and Mewari are considered better for milk production then Jaisalmeri which is recognized more for its race potential and capacity to travel long distances. Thus, the existence of this single nucleotide polymorphism at g.942G>T SNP (GenBank ID: JF429140) in Jaisalmeri and Mewari and absence in Bikaneri and Kachchhi appears independent of the ancestral selection (preferential mating) of the Indian camel breeds for various purposes especially the milk production.

Almost comparable results have been reported in Sudanese camel breeds. There were no camels of the TT genotype and the frequency of GG genotype was 0.723 and 0.643 in Lahaoi and Shanbali populations respectively. Accordingly, the major allele "G" had very high frequency (0.8615 in Lahaoi and 0.8214 in Shanbali) in the Sudanese camel populations studied (Shuiep *et al.* 2013). The only difference that was noticed in the Sudanese and Indian dromedary populations was the little higher replacement rate at the locus g.942G>T SNP (GenBank ID: JF429140). Shuiep *et al.* (2013) also did Isoelectric focusing of all 93 camel milk samples and reported dominance of α_{s1}-CN A in the two ecotypes in Lahaoi and Shanbali populations. The genotype AA was therefore predominant, whereas genotype CC was not detected, probably due to the small sample size. Furthermore, CSN1S1*C shows a single G > T nucleotide substitution in exon 5, leading to a non-synonymous amino acid exchange (p.Glu30>Asp30;

Table 2. Test of Hardy-Weinberg equilibrium, genotype frequency and allele frequency in Indian dromedary at exon 5 of α_{s1} casein gene

Genotype	Male			Female			Pooled		
	f	N	χ^2	*f*	N	χ^2	*f*	N	χ^2
GG	0.96	43	0.025	0.94	63	0.063	0.95	106	0.08
GT	0.04	2		0.06	4		0.05	6	
TT	0.00	0		0.00	0		0.00	0	
Allele frequency	G=0.978; T=0.022			G=0.97; T=0.03			G=0.973; T=0.027		

f, Frequency; N, Observed number of animals.

Fig. 3. Sequence of GT and GG genotype at exon 5 of α_{s1} casein gene in Indian dromedary.

GenBank ID: JF429138), Though, in the present study, the polymorphism in the camel milk proteins has not been studied but due to the existence of full agreement between genotyping by PCR-RFLP and isoelectric focusing at CSN1S1 (Shuiep *et al.* 2013), the existence of two protein patterns corresponding to α_{s1}-casein alleles, A and C at the same frequency values in the Indian dromedary breeds may also be inferred from the present results. Therefore, PCR-RFLP technique can be used for typing camel CSN1S1 variability independent of age, sex and stage of lactation of animals, which can be useful for broader analysis of camel milk protein variability. This information can be utilized to produce diversified camel milk as well as to increase the economic value of camel milk production and to regulate the supply of the nomads. Erhardt *et al.* (2016) also studied casein variability in camel milk by isoelectric focusing in 193 samples of Sudanese camel. Three protein patterns named α_{s1}-casein A, C, and D were identified. The major allele A revealed frequencies of 0.79 (Lahaoi), 0.75 (Shanbali), 0.90 (Arabi Khali), and 0.88 (Arabi Gharbawi) in the different ecotypes. CSN1S1*C shows a single G> T nucleotide substitution in the exon 5, leading to a non-synonymous amino acid exchange (p.Glu30 >Asp30) in comparison to CSN1S1*A and D. The findings are quite comparable with the present results except the reporting of an additional protein pattern α_{s1}-casein D. However, Othman *et al.* (2016) studied the genetic polymorphism of α_{s1}-casein gene in Maghrabi camel reared in Egypt using the primers reported by Shuiep *et al.* (2013). The results of *Sml*I digestion did not showed any restriction site whereas the digestion with *Alu*I endonuclease revealed the presence of two restriction sites AG^CT at positions 68^69 and 631^632 in amplified fragments in Maghrabi camels of Egypt. This variation can be due to the existence of genetic variation in the different populations studied.

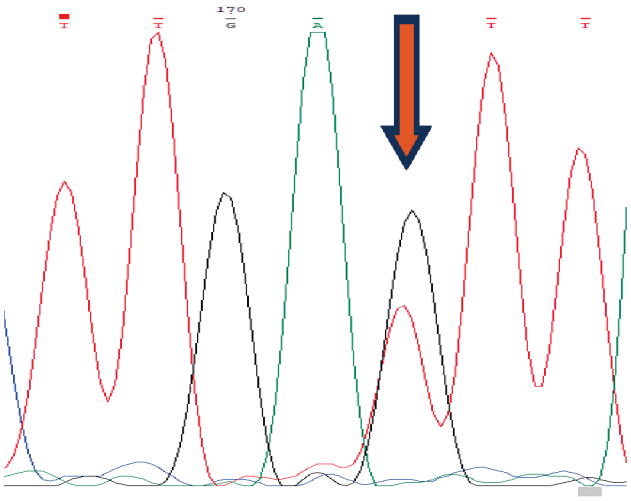

Fig. 4. A four-colour chromatogram showing GT genotype at exon 5 of α_{s1} casein gene in Indian dromedary.

Nucleotide-substitution: SNP verification by sequencing: The samples identified as representing GT and GG genotype with respect to the single G > T nucleotide substitution in exon 5 by RFLP were sequenced. The phylogenetic analysis of sequence(s) confirms the specificity of the sequences as well as the SNP genotype identified (Figs 3, 4). The sequence containing G>T substitution formed a separate node placing it between mRNA and partial sequences of α_{s1} casein gene (Fig. 5). However, the sequence containing GG genotype was closely placed with α_{s1} casein gene sequences of single-humped camel (*Camelus dromedarius*), double-humped camel (*Camelus bactrianus*) and lama (*Lama glama*) (Fig. 6). The results suggest that the G>T nucleotide substitution is of recent origin in the dromedary.

The study documents existence of genetic polymorphism in Indian dromedary breeds at the locus. The PCR-RFLP technique can be used for typing camel CSN1S1 variability independent of age, sex and stage of lactation of animals for selecting them in breeding and production programs.

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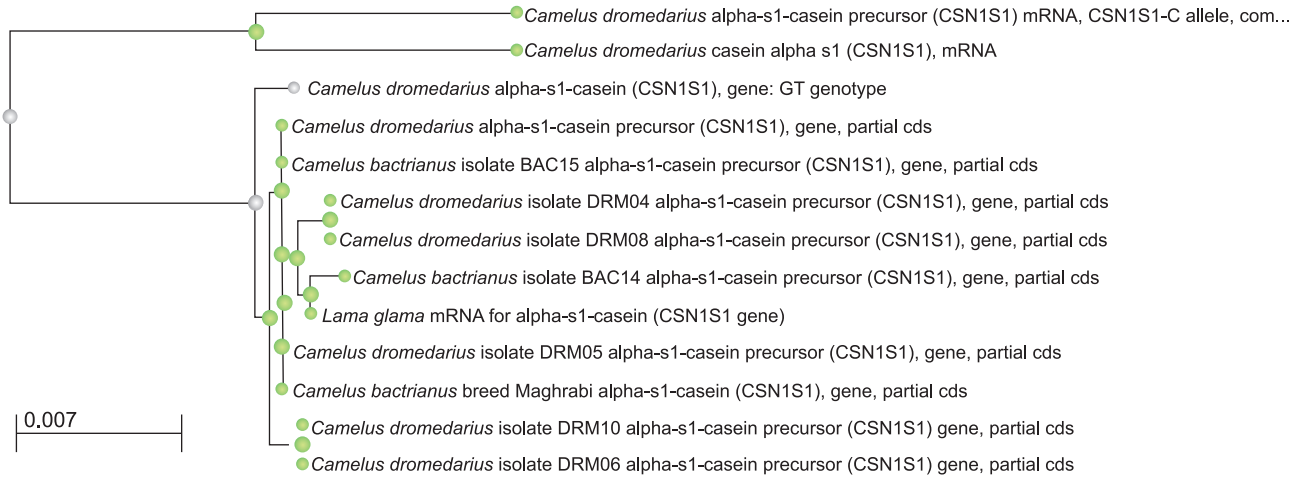

Fig. 5. Sequence phylogeny of GT genotype at exon 5 of α_{s1} casein gene in Indian dromedary.

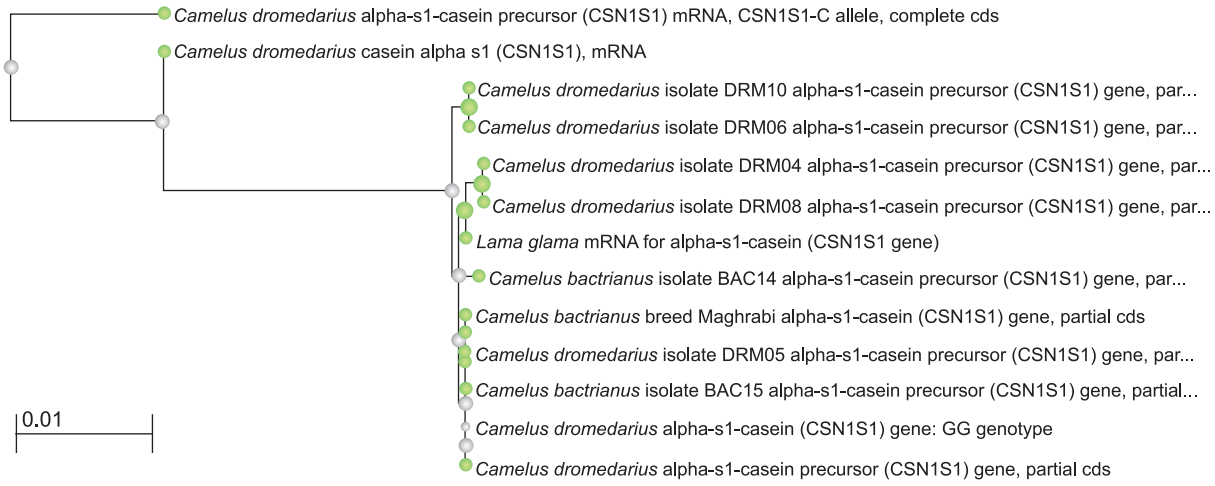

Fig. 6. Sequence phylogeny of GG genotype at exon 5 of α_{s1} casein gene in Indian dromedary.

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