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RESEARCH ARTICLE

Single Nucleotide Polymorphisms in Lactoferrin Gene are Associated with Lactoferrin Content in Milk and Somatic Cell Count in Deoni (*Bos indicus)* Cows

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Lactoferrin, a glycoprotein, is an important antimicrobial molecule present in milk which plays a vital role in antimicrobial defence of the udder in bovines. In the present investigation, polymerase chain reaction- single-strand conformation polymorphism (PCR-SSCP) studies were carried out in 122 Deoni (*Bos indicus*) cows to detect SNPs of lactoferrin gene and to analyze the association between the observed polymorphisms with milk lactoferrin content and Somatic Cell Count (SCC). The PCR-SSCP analysis revealed a total of 20 different variants in the entire coding regions of the lactoferrin gene. Analysis of exons 2, 6 and 8 of lactoferrin gene revealed 4, 3 and 5 unique SSCP patterns, respectively. The PCR-SSCP analysis of exons 3, 9, 14 and 15 revealed two unique SSCP patterns. Comparison of nucleotide sequences of lactoferrin gene of the Deoni cows with taurine cattle revealed a total of 39 point mutations, 25 of which were found to be in coding region. Conceptualized translation of nucleotide sequence revealed 15 amino acid changes. The SSCP variants of exons 2 and 6 had significant ($P<0.05$) effect on milk SCC. The SSCP variants of exon 3 and exon 15 showed significant effect on lactoferrin content. The SCC and lactoferrin content in Deoni cows was highest in 4^{th} and above parity group. Somatic Cell Count was lowest in early stage of lactation. Parity was found to have highly significant ($P<0.01$) effect on both milk SCC and lactoferrin content. The observed association between SSCP variants in lactoferrin gene with milk SCC, and milk lactoferrin content suggests the possibility of using these genetic variants in lactoferrin gene as prognostic markers for selection of animals for high lactoferrin content, low SCC and mastitis resistance.

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INTRODUCTION

In cow's milk, approximately 82% of milk protein is casein and the remaining 18% is whey protein. The whey protein family consists of approximately 50% ß-lactoglobulin, 20% α-lactalbumin, blood serum albumin, immunoglobulins, lactoferrin, transferrin, and many minor proteins and enzymes (Fox and McSweeney, 1998). Lactoferrin is a multifunctional protein present in bovine milk, which plays an important role in the innate host defence. It has a molecular weight of about 80 kDa and comprised of a single polypeptide chain containing 708 amino acids folded into two globular lobes. Concentration of lactoferrin in the cow milk varies from 80 to 500 mg/L (Drackova *et al.,* 2009). It can keep the iron in bound form even at low pH which is important at bacterial infection sites where pH is low (Valenti and Antonini, 2005) and is well known for its function as a general antibacterial, antiviral, antitumor and immunomodulatory molecule during infections of mammary gland (Gonzalez-Chavez *et al*., 2009; Hussain *et al*., 2012). The antibacterial activity of lactoferrin especially against *E. coli*, *P. aeruginosa* and *S. aureus* has been proved in various *in-vitro* as well as *in-vivo* studies (Lacasse *et al.,* 2008). Shashikumar and Puranik (2011) reported the use of lactoferrin as biopreservant for paneer to enhance its shelf life by lowering the microbial growth.

Somatic Cell Count in milk constitutes a good diagnostic tool that allows early detection of either subclinical or acute form of mastitis (De Haas *et al.*,

2004). Milk from a healthy udder contains less than 10^5 somatic cells mL^{-1}; however, the individual variation is large and reported values vary from 10^4 to 10^7 cells mL^{-1} (Hamann, 2002). According to Mrode *et al.* (1998), there is moderate to high genetic association (r_g=0.3–0.7) between SCC and clinical mastitis and it is also comparatively more heritable (h^2=0.10–0.14) than clinical cases (h^2=0.03–0.06; Shook, 2006), making it one of the most important selection tool for mastitis resistant animals. It was also reported that the polymorphism in lactoferrin gene has association with susceptibility/ resistance to mastitis (Wojdak-Maksymiec *et al.,* 2006; Zhao *et al.,* 2008). The bovine lactoferrin gene was mapped to bovine chromosome 22 (Schwerin *et al*., 1994), contains 17 exons and spreads on about 34.5 kb of a genomic DNA.

The above information clearly indicates the importance of lactoferrin gene as a candidate for selection of mastitis resistant cows. However, information regarding the polymorphisms within the *Bos indicus* lactoferrin gene is very scanty. In the present study, we describe the locus specific polymorphisms within all 17 exons of bovine lactoferrin gene through PCR-SSCP and DNA sequencing, and its association with lactoferrin content and SCC in milk of Deoni cows.

MATERIALS AND METHODS

Experimental animals and their management: Deoni (*Bos indicus*) are medium sized dual purpose cattle breed found in parts of Maharashtra, Karnataka and Andhra Pradesh states in India. A total of 122 Deoni cows maintained under semi-intensive system of management at Southern Campus of National Dairy Research Institute, Bangalore, Karnataka and Marathwada Agricultural Research Institute, Parbhani, Maharashtra were used in the study. Cows aged between 3 to 10 years with parity ranging from 1 to 6 were used in the study during 2013-2014. The cows were grouped into early lactation (0-90 days), mid lactation (91-180 days) and late lactation (181-300 days). Blood (8-10 ml) was collected aseptically by jugular venipuncture using vacutainers containing EDTA as anticoagulant and 50 ml of milk samples were collected from Deoni cows (n=87).

DNA isolation and PCR amplification: Genomic DNA was isolated from blood samples (n=122) by high salt method, as described by Miller *et al*. (1988) and the working solution was prepared by diluting the stock to 100 ng/µL for utilizing as DNA template in PCR. Seventeen sets of primers (Table 1) were designed based on the 35384 bp sequence for *Bos taurus* lactoferrin gene (Ensembl Ref Seq: ENSBTAG00000001292) for using primer 3 (http:/www.genome.wi.mit.edu/ cgibin/primer/ primer-3www.cgi) software and were procured from Europhins MWG operon, Bangalore, India.

The Polymerase Chain Reaction (PCR) was carried out on about 50-100 ng of genomic DNA in 25 µl per reaction volume. The PCR reaction mixture consisted of 200 µM of each dNTPs, 10X Taq Pol assay buffer, 1U Taq polymerase enzyme and 20 pM of each primer. The thermocycler conditions included an initial denaturation at 94°C for 2 min, followed by 35 cycles with denaturation at 94°C for 30 sec with varying annealing temperatures based on primer set (Table 1), extension at 72°C for 1 min followed by a final extension at 72°C for 10 min. The PCR products were electrophoresed at 100 V in 1.5% agarose gel in 1X TBE buffer containing 0.5 µg/mL ethidium bromide along with a DNA molecular size marker. The gels were visualized and documented using Gel documentation system (Gel doc 1000, Bio-Rad, USA).

Single Strand Conformation Polymorphism analysis and SNP identification: The amplified PCR products were further subjected to PAGE and visualized by silver-stained, as described by Sambrook and Russell (2001). Band patterns were characterized by the number of bands, mobility shifts and scored manually. Representative PCR products giving unique SSCP patterns were custom sequenced using automated ABI DNA Sequencer (Amnion Biosciences Pvt. Ltd., Bangalore, India) to confirm the mobility shift in each pattern. Sequence data were analyzed using Bio edit software Clustal W multiple alignments for detecting single nucleotide polymorphisms (SNPs) by comparing the observed sequence with the bovine lactoferrin gene reference sequence.

Determination of lactoferrin concentration and SCC in milk: The lactoferrin content in milk was quantitatively determined, using CUSABIO, Bovine Lactoferrin ELISA Kit (China) which is based on competitive inhibition enzyme immunoassay technique, as per manufacturer's guidelines. Calculation was done using the professional soft "Curve expert 1.3". Standard curve was created by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit and lactoferrin concentration was calculated. The SCC (cells/ mL) for each milk sample was determined using Nucleo Counter® SCC-100™ system (Chemometc, Denmark) following the protocol developed by Saleh and Faye (2011).

Statistical analysis: The association study of SSCP variants and effects of non-genetic factors like parity and stage of lactation on milk SCC and lactoferrin content was done by fixed model analysis using Least Squares Maximum Likelihood (LSML) (Harvey, 1990). Duncan's Multiple Range Test (DMRT) as modified by Kramer (1957) was used for testing differences among least squares means. Somatic Cell Count and lactoferrin content were transformed to a logarithmic (log10) scale in order to balance the distribution.

RESULTS

Fourteen nucleotide sequences (partial) of Deoni (*Bos indicus)* cow's lactoferrin gene were submitted to NCBI gene bank and are available at Accession Numbers KM213016-KM213029. BLAST analysis of entire coding sequence revealed 99, 98, 97, 95, 94 and 78% homology with *Bos taurus*, *Bubalus bubalis*, *Bubalus arnee bubalis*, *Capra hircus*, *Ovis aries* and *Homo sapiens,* respectively.

Single Strand Conformation Polymorphism variants and SNP identification: The PCR-SSCP analysis of amplicons of exons 2, 3, 6, 8, 9, 14 and 15 showed polymorphism while the remaining exons were monomorphic.

Table 1: Details of primer sequences (5'to 3' sequences) used for amplification

Exon	Sequence	Location (bp)	Amplicon length (bp)	Annealing Temperature (°C)
1	F- AACTTGAGAGGTGGGTGTGG R- ACCCAGACGCCTACACTGAG	601 – 782	443	59
2	F- CCTCCATCAGAGGAGAGTGG R- CACTTGGATCCCTTCCCTTT	5304 – 5467	353	60
3	F- TCTGGGCTCATACCACCTCT R- TCTGTGCCTTGTCCACTCTG	8444 - 8552	354	60
4	F- ATTCAGGGAGGGCTGTTTCT R- TGGCTTGTGAAAGTGACACC	8848 - 9030	324	60
5	F- CTTCTGAGCTCCTGGCTTTG R- CATGGTCCCCACTAGCTCAC	9647 - 9794	372	59
6	F- GTCAGGAGCCCACAGAAGAC R- TGATAATCGTGTGCCAGGAA	10738 - 10793	285	59
7	F- TGGCTCATGGCTACTGACTG R- TTCCAGAGTGGCAGGAAGTT	16141 - 16319	336	59
8	F- TCTCAGAATCATCCCGGAGT R- ACCATGGTGAAGACCTCCAG	16747 - 16921	445	59
9	F- TCAGCTGTGGCTCTTCCTCT R- GCTCTGTCTGTCCCCACTCT	17447 - 17601	408	59
10	F- TCAATCCTGCTGCTTCACTG R- AGTGGGGTTACTGCTCTCCA	19558 - 19648	277	57
11	F- ACAAAGCCATGCCAATATGC R- GTGCTCAGGACACAGTGAGG	21875 - 21922	365	57
12	F- CAGGCTTGAGCTACCAGGAG R- GACCGCTTACACCAAACGAG	22545 - 22700	472	57
13	F- CACCTGTTAGGCAGAGACCA R-AAATGTGATGTGCCAAGATCC	24611 - 24752	289	59
14	F- CATTCGTGCCATGTTATTGG R- CTGGGACTGCCACAACAGTA	29510 - 29577	362	58
15	F- CTCCATCGTGAGCACTGGTA R- AGCTCTGTGGTTGCTCACCT	31498 - 31682	431	58
16	F- TGCCTCCCAAGTCCATAATC R- CCCACATCACCCCTAAAATG	32813 - 33002	346	59
17	F- AGGGCAGAGGGAAGTCTGTT R- TGGGCAGATATTTGGTCAGA	34559 - 34784	399	57

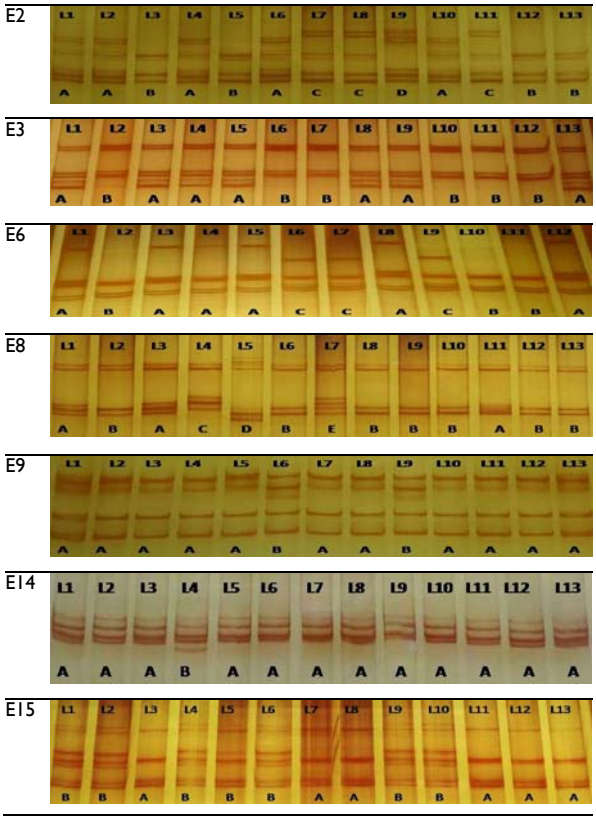

Fig. 1: PCR-SSCP patterns of different exons of lactoferrin gene in Deoni cows. E2=Exon2, E3=Exon3, E6=Exon6, E8=Exon8, E9=Exon9, E14=Exon14, E15=Exon15.

The analysis of exons of lactoferrin gene revealed five, four and three unique SSCP patterns for exon 8, exon 2 and exon 6 (Fig. 1), respectively. Two unique SSCP patterns were observed for the exons 3, 9, 14 and 15. The frequencies of SSCP variants for each exon in 122 Deoni cows genotyped in the present study are summarized in Table 2. Twenty five SNPs have been observed as compared to *Bos taurus* reference sequence. Out of the 25 SNPs observed, only 15 SNPs showed change in amino acid in the transformed products, whereas remaining 10 SNPs were silent mutations. In the intronic region a total of 14 SNPs were detected (Table 3). Clustal W and Chromatograph analysis of exon 2 as representative sample is shown in Fig. 2 and Fig. 3, respectively.

Association of Genetic variants with SCC and lactoferrin content: Milk SCC (million cells/mL) ranged from 0.01 to 0.75 with mean 0.15±0.02 and milk lactoferrin content (mg/L) ranged from 9.09-297.29 with mean 99.95±7.48 in Deoni cows. The association study revealed significant ($P<0.05$) effect of SSCP variants of exon 2 and 6 on milk SCC. The SSCP variants of exon 3 had significant ($P<0.05$) effect and that of exon 15 had highly significant ($P<0.01$) effect on lactoferrin content in milk. Parity and stage of lactation showed highly significant ($P<0.01$) effect on SCC and lactoferrin content.

The least squares mean for SSCP patterns in different exons, different parity and different stages of lactation on SCC and lactoferrin content are presented in Table 4. Among the four SSCP variants of exon 2, cows with

Table 2: Frequencies of SSCP variants in exon2, 3, 6, 8, 9, 14 and 15 of lactoferrin gene in Deoni cows

Exon	Pattern	No of observations	Frequency of SSCP variants
8	A	31	0.2541
	B	55	0.4508
	C	11	0.0902
	D	14	0.1147
	E	11	0.0902
2	A	51	0.4180
	B	32	0.2623
	C	27	0.2213
	D	12	0.0984
6	A	67	0.5492
	B	30	0.2459
	C	25	0.2049
9	A	102	0.8361
	B	20	0.1639
14	A	106	0.8689
	B	16	0.1311
3	A	67	0.5492
	B	55	0.4508
15	A	58	0.4754
	B	64	0.5246

Table 3: Summary of Single nucleotide polymorphisms observed in lactoferrin gene in Deoni cows

Region	Transversion	Transition	Loci (SNPs)	Amino acid change
Exon-2		T/C	T5359C	No Change
		T/C	T5366C	Phe → Leu
		G/A	G5376A	Arg → His
		C/T	C5442T	Ala → Val
		G/A	G5465A	Ala → Thr
Exon-3		G/A	G8455A	No Change
		G/A	G8494A	No Change
	G/T		G8499T	Arg → Leu
Exon-6		T/C	T10774C	Leu → Ser
		A/G	A10777G	Lys →Arg
Exon-8		A/G	A16752G	Ile→Val
		T/C	T16755C	Trp→Arg
	C/G		C16771G	Ser→ Trp
		G/A	G16818A	Val→ Ile
		G/A	G16872A	Ala→ Thr
		A/G	A16900G	His→ Arg
Exon-9		T/C	T17502C	No Change
		T/C	T17577C	No Change
		C/T	C17583T	No Change
Exon-14		C/T	C29525T	No Change
		C/T	C29552T	No Change
		G/A	G29573A	No Change
Exon-15	A/T		A31566T	Thr→Ser
	A/C		A31649C	No Change
		A/G	A31659G	Lys→Glu
TOTAL	4	21	25	15
Intron-2		G/A	G5481A	
Intron-5		T/C	T10698C	
		C/T	C10711T	
Intron-8		G/A	G16932A	
	G/T		G16941T	
		G/A	G16957A	
		T/C	T16965C	
		G/A	G16966A	
		A/G	A16981G	
	G/T		G16986T	
Intron-9		C/T	C17630T	
		G/A	G17635A	
		T/C	T17686C	
		G/A	G17720A	
TOTAL	2	12	14	

pattern B had highest SCC. In case of SSCP variants of exon 6 the highest SCC was observed in pattern B. The SCC was highest in 4th and above parity. Somatic Cell Count was lowest during 0-90 days of lactation. The SSCP analysis revealed two distinct patterns in exon 3 and 15. The lactoferrin content was higher in cows with pattern B in both exon 3 and exon 15. Lactoferrin content in Deoni cows was highest in 4th and above parity group and lowest in 1st parity. An increasing trend of lactoferrin content was observed in successive lactation stages (Table 4).

Table 4: Least squares mean of Somatic Cell Count (million cells/mL) and lactoferrin content (mg/L) in Deoni cows

Effects	No. observations	Least squares mean±SE of somatic cell count	Least squares mean±SE of lactoferrin content
Overall Mean	87	4.86±0.05	1.83±0.02
Parity 1**	21	4.72±0.09^{a}	1.44±0.04^{a}
Parity 2**	22	4.72±0.09^{a}	1.92±0.04^{b}
Parity 3**	19	4.75±0.09^{a}	1.96±0.04^{b}
Parity 4**	25	5.25±0.08^{b}	2.01±0.04^{b}
Stage of Lactation 1**	26	4.48±0.13^{a}	1.58±0.04^{a}
Stage of Lactation 2**	30	5.00±0.13^{b}	1.86±0.03^{b}
Stage of Lactation 3**	31	5.09±0.28^{b}	2.06±0.04^{c}
Exon 2 Pattern A*	37	4.65±0.14^{ab}	-
Exon 2 Pattern B*	30	5.58±0.26^{c}	-
Exon 2 Pattern C*	12	4.72±0.16^{b}	-
Exon 2 Pattern D*	8	4.50±0.15^{a}	-
Exon 3 Pattern A*	45	-	1.75±0.03^{a}
Exon 3 Pattern B*	42	-	1.89±0.04^{b}
Exon 6 Pattern A*	45	4.84±0.07^{b}	-
Exon 6 Pattern B*	26	5.26±0.13^{c}	-
Exon 6 Pattern C*	16	4.64±0.13^{a}	-
Exon 15 Pattern A**	48	-	1.75±0.03^{a}
Exon 15 Pattern B**	39	-	1.91±0.04^{b}

Figures bearing different superscripts in a column differ significantly (**P<0.01; *P<0.05).

DISCUSSION

Lactoferrin is an important component of the antimicrobial defences of the mammary gland and is poorly understood compared with other milk proteins. It is involved particularly in the mechanism of alimentary immunity (Seyfert *et al.,* 1997). The present investigation is aimed to characterize lactoferrin gene in *Bos indicus* cows and to elucidate the genetic variation in the entire coding region of bovine lactoferrin gene. Single Nucleotide Polymorphisms give a better chance to explain a part of the genetic variance of different traits through understanding of the biochemical or physiological mechanisms in which a candidate SNP might be involved. Perusal of Table 3 indicates high degree of mutations in lactoferrin gene in Deoni cows.

In this investigation, milk SCC exhibited an ascending trend with the progression of lactation. Increasing trend of milk lactoferrin content was observed with the advancement of stage of lactation. The immunity due to lactoferrin results from the fact that possible infection factors have a limited availability of iron (as well as other growth agents, such as phosphorus and zinc), since its concentration in an organisms fluids is reduced (Persson *et al.,* 1992). Identification of genetic markers associated with SCC might be helpful in improving cows' health by implementing appropriate breeding programs.

The results observed in the present study support the findings of Wojdak-Maksymiec *et al*. (2006), who observed association between genetic variants and SCC. Lei *et al.* (2006) reported association between SSCP markers with clinical mastitis residuals and SCC in milk

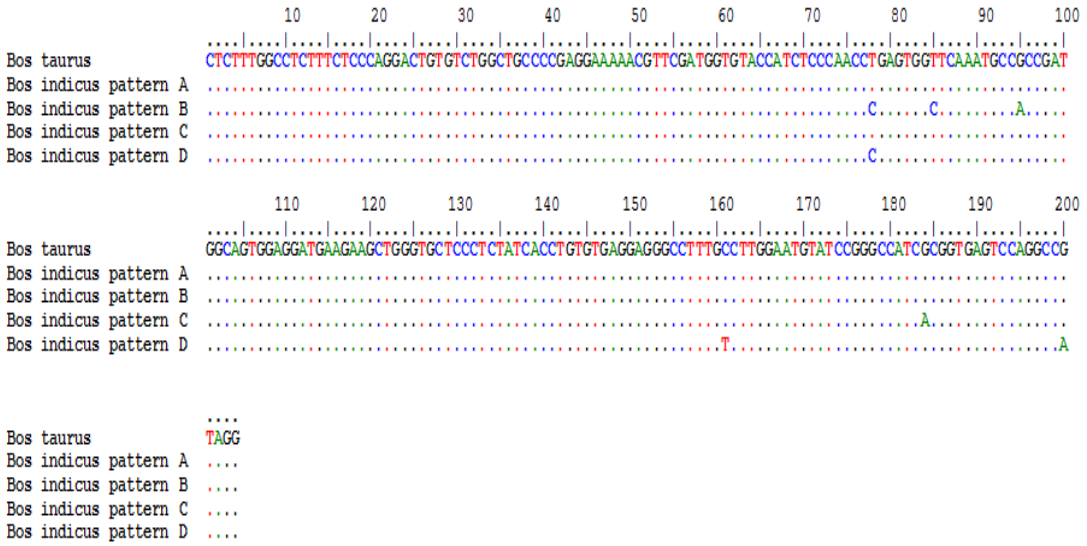

Fig. 2: Clustal W multiple alignment sequence of exon 2 of lactoferrin gene in Deoni cows

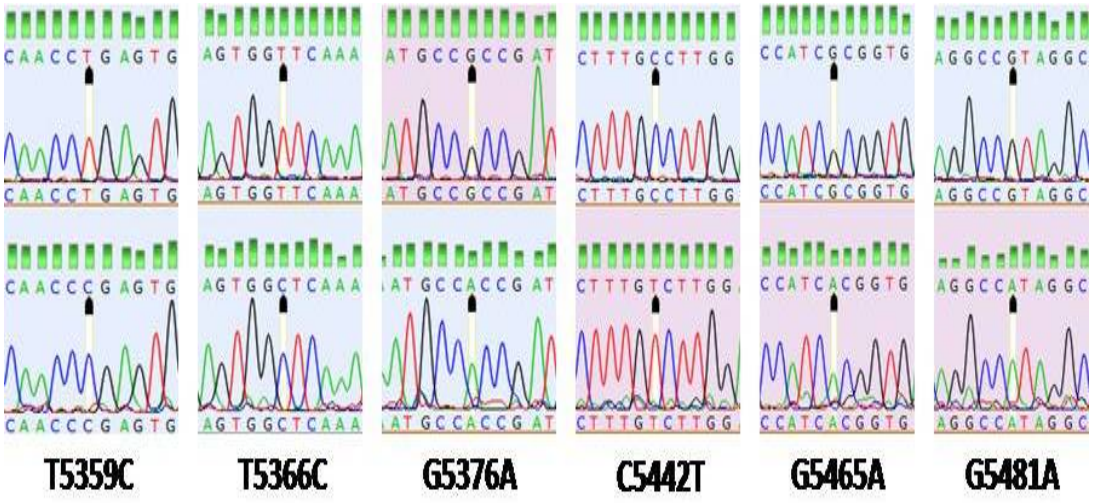

Fig. 3: Chromatograph analysis of observed SNPs in exon 2 of lactoferrin gene in Deoni cows

in bovines. The observed association between genetic variants and milk SCC is in agreement with the earlier report of Rahmani *et al.* (2012) in crossbred cattle.

The SCC in Deoni cows was highest in 4th and above parity group which was significantly different from other parities. Mitev *et al*. (2012) also observed higher risk for occurrence of mastitis consequently to the higher chance of infection due to damaged teat sphincter with advancing of parity number, the share of teats with higher milk SCC, respectively could be seen, because of the more prolonged effect of milking (more lactation) and occurring teat end changes, predisposing to teat bacterial infection. Somatic Cell Count was lowest in first stage of lactation and was significantly different from that of other stages which is in agreement with the earlier report of Wojdak- Maksymiec *et al*. (2006). Similarly, our results are in accordance with Krol *et al.* (2010) and Litwinczuk *et al.* (2011), who observed a significant effect of stage of lactation on lactoferrin content in bovine milk, which could be due to high correlation coefficient (r=0.557) between the lactoferrin content and stage of lactation Cheng *et al*. (2008). Krol *et al.* (2010) reported significant differences in lactoferrin levels in the successive lactations, the primiparous cows were observed to produce significantly less lactoferrin compared to the multiparous cows. According to Lindmark-Mansson *et al*. (2000, 2006), a higher SCC leads to increase of lactoferrin content in milk, and a close relationship between this milk component and the status of udder health has been reported by very high correlation coefficients (r = 0.962 and r=0.918 at P<0.001) between milk lactoferrin concentration and SCC.

The lactoferrin has important relation with the innate immunity, thus this protein gene is supposed to be a promising candidate gene for the mastitis-resistance. Our findings in the present study indicate that there is high variability in lactoferrin gene and genetic variants are associated with SCC and lactoferrin content in Deoni cows. Further studies on the association between SNPs in lactoferrin gene with lactoferrin content and SCC using large population could result in identification of markers for udder health and milk lactoferrin content. Present

study could be a step towards identification of genetic markers for selecting cows for udder health and immunity.

Conclusion: The present study revealed high degree of genetic variation as indicated by different SSCP patterns which resulted into presence of 25 SNPs in lactoferrin gene in Deoni cows. The observed SSCP patterns in exon 2 and 6 were found to have significant effect on milk SCC and exons 3 and 15 were found to have effect on milk lactoferrin content in Deoni cows. The present study indicated the possibilities of using SSCP patterns as marker for milk SCC and lactoferrin content. The cows with SSCP patterns with low SCC could be selected for producing next generations to increase mastitis resistance. However, further studies using large number of animals need to be carried out to validate the marker data before using them in the Marker Assisted Selection (MAS).

Authors' contribution: APS executed the experiment, analyzed the data, conceived and designed the review. KPR, SI and DND designed and supervised the work. PD, AR, MB and UM helped in sample collection. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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