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Molecular Characterization of Coding Region of Partial N-lobe of Malabari Goat Lactoferrin

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ABSTRACT

Lactoferrin (*Lf*), an 80 kDa glycoprotein, is present in milk and other exocrine secretions. It is considered as a "moonlighting" protein because of its diverse functions, including antimicrobial, anti-inflammatory, immunomodulatory and anti-neoplastic activities. The present study shows the molecular characterization of partial coding region of N-lobe of lactoferrin (*Lf*) gene of Malabari goat breed of Kerala, which are reputed for their sturdiness and resistance to diseases. The RNA was isolated from the milk somatic cells of Malabari goat, followed by cDNA synthesis and subsequent amplification of partial coding region of N-lobe of *Lf* gene. The amplicons were sequenced and the sequences were analyzed using various bioinformatics tools. A 813 bp long partial coding region encoding 274 amino acids was obtained for Malabari goats. The sequences of both the breeds were 94-99% similar to *Lf* gene of other ruminant species. Six nucleotide variations were observed in Malabari of which four variations were non-synonymous leading to amino acid variations as compared to *C. hircus*. This is the first research to concentrate on of cDNA sequence and nucleotide variations of N-lobe of *Lf* gene of the Malabari goat of Kerala.

HIGHLIGHTS

- The N-lobe of Malabari goat *Lf* gene was sequenced.
- *In-silico* analysis revealed the Malabari goat *Lf* to possess unique variations in the nucleotide and amino acid sequence in comparison with the *C. hircus* gene sequence.

Keywords: Malabari goat, lactoferrin, N-lobe, amino acid variation

Lactoferrin (*Lf*), an 80 kDa glycoprotein, is considered as a "moonlighting" protein because of its diverse functions, including antimicrobial, anti-inflammatory, immunomodulatory and anti-neoplastic activities (Kell *et al.,* 2020). This red or salmon-pink coloured protein was first isolated from bovine milk by Sorensen and Sorensen (1939). It was then in 1960 that Groves identified *Lf* to be a prominent iron-binding protein in human milk. Subsequently, its presence was reported in the body secretions like tears, saliva, cervical mucus, semen and in specific granules of neutrophils.

The size of the *Lf* gene varies from 23 to 35kb among different species. The gene has 17 exons, of which 15 encode amino acids. The amino acid sequence of lactoferrin shares 70 per cent sequence homology among humans, mice, bovine and porcine. The initial 19 amino acids form the signal peptide, of which 11 amino acids are conserved among species. The N-lobe extends from the 2nd to 8th exon while the C-lobe extends from the 9th to the 17th exon (Teng, 2002). According to Legrand *et al.* (2008), the N and C lobes of Lf are divided into two domains each (N1 and N2; C1 and C2). In the bovine *Lf*, out of 689 amino acids, the 1-90 and 251-333 residues form the N-1

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domain, and 91-250 comprise the N-2 domain. Similarly, residues 345 to 431 and 593 to 676 form the C-1 domain and 432 to 592 residues form the C-2 domain. The N and C-lobes are connected by a three-turn α-helix formed by residues 334 to 344. The N terminal-derived peptides termed lactoferricins, which are basic, are reported to be more potent than the whole protein.

Malabari, an indigenous goat breed of Kerala, more common in Northern Kerala, is well known for its high productivity and ability to adapt to hot and humid climatic conditions. Reports on molecular level exploration of major and minor milk proteins of Malabari goat have been found scanty. Hence the present study was undertaken to characterize the partial coding region of N-lobe of *Lf* gene in Malabari goat breed of Kerala and to compare the sequences with *Lf* sequences available from the database.

MATERIALS AND METHODS

Isolation of total RNA from milk somatic cells and cDNA synthesis

Somatic cells were isolated from colostrum of newly kidded apparently healthy Malabari goats maintained at University Goat and Sheep Farm, Mannuthy, Kerala as per the protocol of Anjusekar *et al.* (2018). The total RNA from milk somatic cells was isolated by TRI-reagent (Sigma Aldrich) as per the manufacturer's instructions and then treated with *DNase* 1 (Sigma Aldrich) to remove DNA contamination if any. RNA samples were quantified by Nano Drop spectrophotometer (Thermo Scientific, USA) and checked for the integrity on 1% agarose gel. Reverse transcription was performed to synthesize cDNA from the isolated RNA using verso cDNA synthesis kit (Thermo Scientific) and oligo dT primers with 0.1 µg of RNA in a reaction volume of 20 µL and were stored at −80 °C until use.

PCR amplification of partial N lobe coding region of Malabari goat *Lf*

The oligonucleotides FcLf (5'CATGCCATGGTGCCGGAGTGGTCCAAATGC3') and RcLf (5'CTCAGGAAGGCACAGGAGAAGCTCGAGCGG3') were designed based on the *Capra hircus* sequence (GenBank Acc. No. NM_001285548.1) retrieved from database to amplify the partial N lobe coding region of Malabari *Lf* (*MgLf-N*). The custom synthesized primer pair was used in a 20 µL PCR reaction containing 10 picomoles of each primer, 10mM dNTPs and 0.5 U Phusion polymerase (Thermo Fischer Scientific) for the amplification of partial N-lobe of *Lf* gene. The thermal cycling profile consisted of denaturation at 98ºC for 10sec, annealing at 65ºC for 30sec and extension at 72ºC for 40 sec. for 35 cycles followed by a final extension at 72ºC for 5 min. The PCR products were electrophoresed in 1% agarose gel for 40 min.

Sequence analysis

Using the above mentioned primer set, the amplicons were sequenced at the DNA sequencing facility at AgriGenom Pvt. Ltd, Kochi, Kerala. The sequence similarity search was performed using Basic Local Alignment Search Tool (BLASTn) (www.ncbi.hlm.nih.gov/BLASTn). Multiple sequence alignment of the sequences corresponding to *MgLf-N* with that of *C. hircus* (Gen Bank Ac. No. NM_001285548.1) was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo) program. The 'Translate' tool of the online portal ExPASy was used to predict the amino acid sequences encoded by *MgLf*. The amino acid sequences thus predicted were compared with different mammalian Lf sequences in the database using BLASTp tool of NCBI to find out the similarity between species. Multiple sequence alignment of the predicted aminoacid sequences with that of the database *C. hircus* Lf amino acid sequence was done using Clustal Omega software. The secondary structure of the partial protein was predicted by SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) (Geourjon & Deleage, 1995). The modeling of the tertiary structure of the protein was done by using SWISSMODEL server (https://swissmodel.expasy.org) (Biasini *et al.,* 2014).

RESULTS AND DISCUSSION

Lactoferrin, a member of the transferrin family, is a glycoprotein with a molecular weight of about 80 kDa. It is considered as a "moonlighting" protein because of its diverse functions, including antimicrobial, anti-inflammatory, immunomodulatory and anti-neoplastic activities. Lactoferrin and its derivative peptides have numerous applications in cancer therapy. The Malabari

goat breed accounts for majority of the goat population in Kerala, and are disease resistant and adaptable to extreme agro-climatic conditions; hence their gene pool provides a valuable platform to explore the potentials of different bioactive peptides including *Lf*.

The concentration of *Lf* is significantly higher in colostrum than in milk (Legrand *et al.,* 2008), so the total RNA was isolated from the somatic cells of Malabari goat colostrum. The isolated RNA was converted into cDNA followed by amplification of *MgLf-N* using custom- synthesized primer pair. On electrophoresis, the amplicons showed a single band of about 813bp size (Fig. 1). The gel purified amplicons from both the goat breeds were sequenced and the sequences submitted to NCBI Genbank (Acc. No: OP494313). The obtained nucleotide sequences were analysed using BLASTn to ascertain them as *MgLf-N*. The pair wise identity matrix of *MgLf-N* with *Lf* sequences of different mammalian species in database showed 99.24% homology with *C. hircus* (NM_001285548.1), 98.99% similarity with sheep (NM_001024862.1), 93.85% with buffalo (NM_001290860.1) and 93.81% with cattle (NM_180998.2).

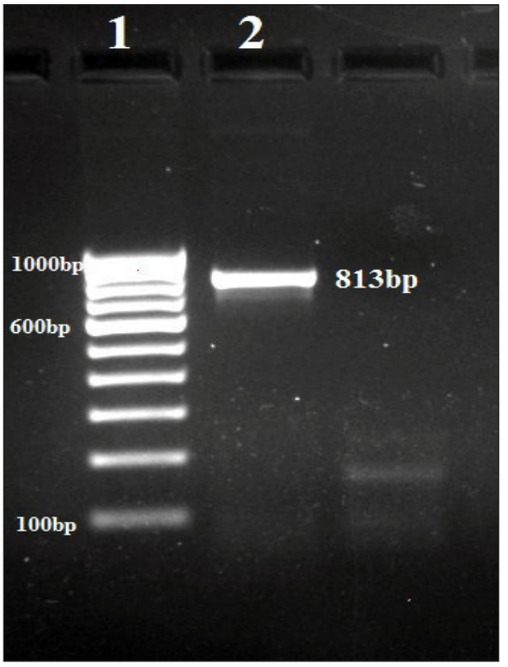

Fig. 1: PCR amplified partial N-lobe coding region of Malabari goat *lactoferrin* Lane 1: Molecular Marker (1kb) Lane 2: *MgLf-N* amplicon

Using Clustal Omega software, the multiple sequence alignment of *MgLf-N* and *C. hircus* sequence (NM_001285548.1) was obtained. On sequence comparison with *C. hircus* the Malabari *Lf* N-lobe (*MgLf* –N) showed 6 nucleotide variations, of which 4 were transitions and 2 were transversions (Table 1). The sequences of the protein encoded by *MgLf-N* (N-lobe) as predicted by *in silico* translation were formed of 274 amino acids with a molecular weight of 30kDa. The predicted amino acid sequences were analysed for similarity with the database *Lf* sequences belonging to *C. hircus* sequence (NM_001285548.1) by BLASTp. The MgLf-N showed 97.94% similarity to *C. hircus* Lf protein (NP_001272477.1). Multiple sequence alignment of the predicted amino acid sequences with that of the *C. hircus* Lf (NP_001272477.1) revealed 1 synonymous and 4 non-synonymous variations.

Table 1: Nucleotide and amino acid variations of partial N-lobe of Malabari (OP494313) goat lactoferrin on comparison with that of *C. hircus* (NP_001272477.1)

Nucleotide variation	Codon change	Amino acid variation
G170T	CGG→CTG	Arg57Leu
A276C	AAG→CAG	Lys93Gln
T297C	GGT→GGC	Syn(Gly)
T314C	ATG→ACG	Met105Thr
C367T	CCC→TTC	Pro123Phe
C368T		

At position 57 of the predicted protein, Arginine, a polar basic amino acid was replaced by a hydrophobic non-polar amino acid Leucine. At position 93, the positively charged basic amino acid Lysine was replaced by a polar amino acid with no charge on side chain Glutamine. At position 105, the non-polar sulphur-containing amino acid Methionine was replaced by a hydroxyl group containing amino acid Threonine which is polar with no charge on the side chain. A non-polar amino acid Proline was replaced by another non-polar amino acid Phenylalanine at 123rd position. Most of the variations observed by the study were consistent with Anjusekar *et al.* (2018) who sequenced a 1914 bp long partial coding region encoding 638 amino acids of Malabari goats and reported 8 nucleotide variations of which 5 were non-synonymous leading to variation in amino acids. However, in the present study, a novel variation in nucleotide sequence of Malabari goat *Lf*

was observed, T314C, which corresponds to replacement of Methionine with Threonine in the amino acid sequence of the protein. This change from a sulphur containing amino acid to a polar hydroxyl group containing amino acid could result in significant changes in the protein structure, which could in turn bring about changes in the biological functions of the protein. Pauciullo *et al.* (2010) reported 11 nucleotide variations responsible for 5 amino acid changes on comparison of *Lf* cDNA sequence of Italian Nicastrese goat breed with Saanen goat breed *Lf* sequences. Kang *et al.* (2008) reported 6 novel amino acid variations while analyzing the sequences of goat *Lf* gene.

The protein's secondary structure was predicted by SOPMA, which indicated the protein was composed of 31.02 per cent α helix, 17.15 per cent extended strand, 9.12 per cent β turn and 42.70 per cent random coil. The amino acid composition of the predicted *MgLf* (N-lobe) is depicted in Table 2. The SWISS-MODEL server was used to construct a 3D structural model of partial N-lobe of Malabari goat (Fig. 2).

Table 2: Predicted amino acid composition of partial N-lobe of Malabari goat lactoferrin

Amino acid	No.	Percentage (%)
Ala (A)	28	10.2%
Arg (R)	14	5.1%
Asn (N)	9	3.3%
Asp (D)	11	4.0%
Cys (C)	13	4.7%
Gln (Q)	14	5.1%
Glu (E)	18	6.6%
Gly (G)	20	7.3%
His (H)	10	3.6%
Ile (I)	7	2.6%
Leu (L)	23	8.4%
Lys (K)	18	6.6%
Met (M)	3	1.1%
Phe (F)	10	3.6%
Pro (P)	14	5.1%
Ser (S)	17	6.2%
Thr (T)	10	3.6%
Trp (W)	5	1.8%
Tyr (Y)	9	3.3%
Val (V)	21	7.7%

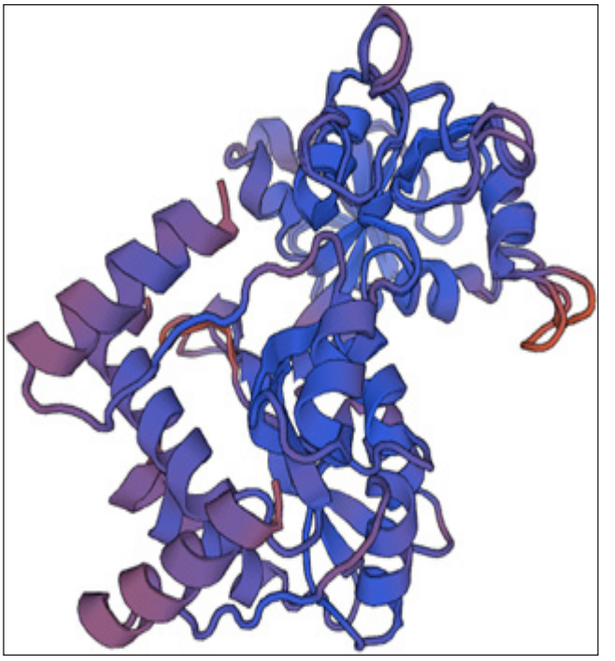

Fig. 2. Three dimensional structural model of predicted partial N-lobe of Malabari goat lactoferrin

The changes in the primary structure contributes to the variations in the three dimensional structure, further leading to the changes in biological activity. According to Kang *et al.* (2008) variations in amino acid sequence within species is related to antibacterial property or other biological activities. The non-synonymous amino acid variations found in the present study might contribute to the disease and stress resistance exhibited by this indigenous goat breed.

CONCLUSION

Lf possesses antibacterial, antiviral, antifungal and immunomodulatory activities besides taking part in iron homeostasis. Many studies have emphasized the potent antimicrobial activity of lactoferricin, the peptide derived from N-lobe of *Lf* upon action by proteases. The present study demonstrated the N-lobe of *Lf* of Malabari goat to possess unique variations in the amino acid sequence, compared to *C. hircus* sequences in the database which might be responsible for its antimicrobial, anticancer and other biological properties.

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