MOLECULAR CHARACTERIZATION OF CODING REGION OF LACTOFERRIN GENE OF MALABARI AND ATTAPPADY BLACK GOATS OF KERALA

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Malabari goats
Attappady Black goats

ABSTRACT

Lactoferrin (Lf), an iron binding glycoprotein mainly found in the secretions of the body like milk, tears, saliva, pancreatic juice, bile, genital fluids etc is well noted for its role in innate immunity as well as biological functions like antibacterial, antifungal, anti-tumorigenic, antiviral and other metal binding properties. The present study unveils the molecular characterization of coding region of lactoferrin (Lf) gene of Malabari and Attappady Black goat breeds of Kerala, which are reputed for their sturdiness and resistance to diseases. Thirty minutes post milking milk samples from early lactating goats of both the breeds were collected for RNA isolation followed by cDNA synthesis and subsequent amplification of partial coding region of Lf gene. The amplicons were sequenced and the sequences were analyzed using various bioinformatics tools. A 1914 bp long partial coding region encoding 638 amino acids was obtained for Malabari goats while that of Attappady Black goat yielded 1975 bp encoding 657 amino acids. The sequences of both the breeds were 94-99% similar to Lf gene of other ruminant species. Eight nucleotide variations were observed in Malabari whereas ten variations were seen in the nucleotide sequences of Attappady Black when compared with Lf gene of Capra hircus (Gen Bank Acc. No. NM_001285548). The five non-synonymous amino acid variations observed in both the breeds as compared to C. hircus were p.Arg88Leu, p.Lys124Gln, p.Pro154Phe, p.Leu357Val and p.Gly414Asp. This is the first report of cDNA sequence and nucleotide variations of Lf gene of the indigenous goat breeds of Kerala.

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1 Introduction

Lactoferrin (Lf), a multifunctional single polypeptide glycoprotein, is composed of around 690 amino acids, having a molecular mass of 80kDa (Baker & Baker, 2005). It is secreted by epithelial cells and is found in almost all the secretions of the body (Alexander et al., 2012). Lf belongs to the transferrin family and is capable of binding to and transporting Fe³⁺ ions (Metz-Boutique et al., 1984). It exhibits a range of biological activities including antioxidant, antibacterial, antiviral, antifungal as well as metal binding properties (Adlerova et al., 2008). It has been found to modulate innate immune mechanism by reducing pro-inflammatory cytokines (Valenti et al., 2011). As it is present in specific granules of neutrophils (Bennett & Kokocinski, 1978), it is having an essential role in cell mediated immunity.

Lactoferrin (Lf) gene is composed of 17 exons and 16 introns (Kim et al., 1998; Seyfert et al., 1994; Kang et al., 2011) and has a size of 34.5 Kb. In cattle and goats, it is located on chromosome 22 (Schwerin et al., 1994). The intron-exon distribution pattern of Lf has been observed to be very similar in cattle, sheep and goat, where all these species were having same exon length but varied intron length (Kang et al., 2011).

Kerala, the southern state of India, has two native goat breeds viz, Malabari and Attappady Black. Malabari goats, more common in Northern Kerala, are well adapted to the hot humid climate of this region and are popular for their high prolificacy. Attappady Black goats are found in the hilly region of Attappady in Palakkad district and are reared mainly by tribal people for meat purpose. These indigenous goat breeds are lean built, eat tree leaves which are pungent and bitter, consume less water and do lot of physical activities. These breeds are well known for their disease resistance and their adaptability to extreme agro-climatic conditions. Reports on molecular level exploration of major and minor milk proteins of these breeds have been found scanty. Hence the present study was undertaken to characterize the coding region of Lf gene in these indigenous goat breeds of Kerala and to compare their sequences with Lf sequences available from the database.

2 Materials and Methods

2.1 Isolation of milk somatic cells

A volume of 50 mL milk samples (thirty minutes post milking) were collected from Malabari and Attappady Black goats (in early lactation period) maintained at University Goat and Sheep Farm, College of Veterinary and Animal Sciences, Mannuthy, Kerala and transported in ice. The fresh milk samples were processed immediately as per the protocol of Boutinaud & Jammes (2002) with slight modifications. The milk samples were centrifuged at 2000 x g for 15 min at 4°C to remove the fat layer and the supernatant was discarded. The pellet containing milk somatic cells (MSC) was washed twice with ice-cold PBS (Phosphate buffered saline)(pH 7.2) supplemented with 0.5mM EDTA (Ethylenediaminetetraacetic acid) and 0.1% DEPC (Diethyl pyrocarbonate) and finally resuspended in 200 µL of PBS-EDTA.

2.2 Isolation of total RNA from MSC and cDNA synthesis

The total RNA from MSC was isolated by TRI-reagent (Sigma Aldrich) as per manufacturer’s instructions and then treated with DNase 1 (Sigma Aldrich; amplification grade) 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 Revert Aid First strand 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.

2.3 PCR amplification of Lf gene

The oligonucleotides FgLf (5’ TGCCGGAGTGGTCACAAAATGTA3’) and RgLf (5’GCCTTCCTTTCAGGCTTACCT3’) were designed based on the C. hircus sequence (Gen Bank Acc. No. NM_001285548) retrieved from database. The custom synthesized primer pair was used in a 25 µL PCR reaction containing 10 picomoles of each primer; 200µmol L⁻¹ each of dATP, dCTP, dGTP and dTTP; 1.5 mmol L⁻¹MgCl₂ and 0.5 U Jumpstart AccuTaq LA DNA polymerase (Sigma Aldrich) for the amplification of Lf gene. The thermal cycling profile consisted of denaturation at 95°C for 10sec, annealing at 60°C for 30sec and extension at 68°C for 90 sec for 35 cycles followed by a final extension at 68°C for 10 min. The PCR products were electrophoresed in 1% agarose gel for 40 min.

2.4 Sequence analysis

Using FgLf and RgLf 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) provided by the National Centre for Biotechnological Information (NCBI). Using GeneTool Lite software, the pair wise identity matrix was derived with Lf sequences of different species obtained from GenBank. Multiple sequence alignment of the Lf gene sequences of Malabari (MgLf) and Attappady Black (AgLf) with C. hircus sequence (Gen Bank Acc. No. NM_001285548) 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 \textit{MgLf} and \textit{AgLf}. The amino acid sequences thus obtained were compared with different Lf protein sequences of mammalian origin present in the database using BLASTp tool of NCBI to find out the similarity between species. Multiple sequence alignment of Malabari and Attappady Black lactoferrin protein sequences with that of the database \textit{C. hircus} Lf amino acid sequence was done using Clustal Omega program. The secondary structure 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). The phylogenetic relationship of Lf gene with that of different species was analyzed using MEGA version 6.0 software.

3 Results

3.1 Amplification and sequencing of Lf cDNA

The coding region of Lf gene of Malabari and Attappady Black goat breeds were successfully amplified using custom-synthesized primer pair. On electrophoresis, the amplicons showed a single band of about 2Kb size (Figure 1). The gel purified amplicons from both the goat breeds were sequenced. The sequences obtained were of size 1917 bp for Malabari Lf gene (\textit{MgLf}) (GenBank Acc. No: MG980401) and 1975 bp for Attappady Black Lf gene (\textit{AgLf}) (GenBank Acc. No: MG980402).

3.2 Sequence analysis

3.2.1 Nucleotide similarity analysis

The obtained nucleotide sequences, \textit{MgLf} and \textit{AgLf}, were subjected to BLASTn analysis to ascertain their identity as Lf gene. The pair wise identity matrix of \textit{MgLf} and \textit{AgLf} with Lf sequences of different mammalian species retrieved from the NCBI database showed 99% homology with the GenBank \textit{C. hircus} sequence (NM_001285548.1) and 94-99% similarity with that of other ruminants.

3.2.2 Multiple sequence alignment

Using Clustal Omega program, the multiple sequence alignment of \textit{MgLf}, \textit{AgLf} and \textit{C. hircus} sequence (NM_001285548) was obtained. On comparing with the GenBank sequence, \textit{MgLf} sequence showed 8 nucleotide variations while \textit{AgLf} showed a total of 10 variations.

3.2.3 Prediction of amino acid sequences

The sequences of the proteins encoded by \textit{MgLf} and \textit{AgLf} obtained by \textit{in silico} translation were found to be of size 638 amino acids for Malabari Lf and 657 amino acid residues for Attappady Black Lf.

3.2.4 Protein similarity search

The predicted amino acid sequences of both the goat breeds were analyzed for similarity with the database Lf sequences belonging to different species using the BLASTp tool of NCBI. Both Malabari and Attappady Black Lf showed 99% similarity to \textit{C. hircus} Lf protein in the database. The multiple sequence alignment of the amino acid sequences of Malabari and Attappady Black goats with that of the GenBank sequence was done using Clustal Omega program. Five non-synonymous variations were seen in both Malabari and Attappady Black goat breeds when compared with the \textit{C. hircus} protein sequence (Table 1).

3.2.5 Protein structure prediction

The secondary structure prediction by SOPMA indicated that Lf protein of both the goat breeds consisted of α helices, extended strands, β turns and random coils. Comparison of the secondary structures of \textit{MgLf} and \textit{AgLf} with that of \textit{C. hircus} is shown in Table 2.
The fully automatic procedure on the SWISS-MODEL server was used to construct a 3D structural model of Lf protein of both the goat breeds (Figure 2). The predicted 3D structure will provide the basis for further structure–function studies of Lf.

### 3.2.6 Phylogenetic analysis

Phylogenetic analysis of the coding sequences of Lf gene of different species was done using MEGA version 6.0 software and a phylogenetic tree was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Figure 3) which revealed that Malabari and Attappady Black breeds belonged to the same clade as that of goat and were very closely related to sheep and antelope. The goats, sheep and antelope were found to be related to the clade formed by cattle, yak and buffalo. This clarified that all the members of the family Bovidae; cattle, buffalo, goat, sheep, antelope and yak shared a recent common ancestor. All these members of Bovidae shared a distant lineage with pig, horse and camel. Humans formed a separate clade.

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Table 1 Nucleotide and amino acid variations of Lf gene of Malabari (MG980401) and Attappady Black (MG980402) goats on comparison with C. hircus (NM_001285548)

<table>
<thead>
<tr>
<th>Nucleotide variation</th>
<th>Codon change</th>
<th>Amino acid variation</th>
<th>Nucleotide variation</th>
<th>Codon change</th>
<th>Amino acid variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>G337T</td>
<td>CGG→CTG</td>
<td>Arg88Leu</td>
<td>G337T</td>
<td>CGG→CTG</td>
<td>Arg88Leu</td>
</tr>
<tr>
<td>A444C</td>
<td>AAG→CAG</td>
<td>Lys124Gln</td>
<td>A444C</td>
<td>AAG→CAG</td>
<td>Lys124Gln</td>
</tr>
<tr>
<td>T464C</td>
<td>GTT→GGC</td>
<td>Syn(Gly)</td>
<td>T464C</td>
<td>GTT→GGC</td>
<td>Syn(Gly)</td>
</tr>
<tr>
<td>C534T/C535T</td>
<td>CCC→TTC</td>
<td>Pro154Phe</td>
<td>C534T/C535T</td>
<td>CCC→TTC</td>
<td>Pro154Phe</td>
</tr>
<tr>
<td>T1143G</td>
<td>TTG→GTG</td>
<td>Leu357Val</td>
<td>T1143G</td>
<td>TTG→GTG</td>
<td>Leu357Val</td>
</tr>
<tr>
<td>G1102A</td>
<td>GGT→GAT</td>
<td>Gly414Asp</td>
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<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>A1787G</td>
<td>ACA→ACG</td>
<td>Syn(Thr)</td>
</tr>
<tr>
<td>T1985C</td>
<td>TTT→TTC</td>
<td>Syn(Phe)</td>
<td>T1985C</td>
<td>TTT→TTC</td>
<td>Syn(Phe)</td>
</tr>
</tbody>
</table>

Table 2 Features of secondary structure of Lf protein of Malabari and Attappady Black goats and C. hircus (NM_001285548)

<table>
<thead>
<tr>
<th>Protein parameters</th>
<th>Malabari</th>
<th>Attappady Black</th>
<th>Capra hircus</th>
</tr>
</thead>
<tbody>
<tr>
<td>α helices</td>
<td>197</td>
<td>200</td>
<td>231</td>
</tr>
<tr>
<td>β turns</td>
<td>138</td>
<td>141</td>
<td>147</td>
</tr>
<tr>
<td>Extended strands</td>
<td>73</td>
<td>75</td>
<td>78</td>
</tr>
<tr>
<td>Random coils</td>
<td>230</td>
<td>241</td>
<td>252</td>
</tr>
</tbody>
</table>
4 Discussion

Lactoferrin is an iron binding glycoprotein with an array of molecular functions. Any significant variation in the nucleotide sequence of Lf gene could alter its biological properties. The native goat breeds of Kerala, Malabari and Attappady Black 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 total RNA extracted from MSC of Malabari and Attappady Black goat breeds was utilized for cDNA synthesis followed by amplification of Lf gene (about 2Kb size) and sequencing of the amplicons. The sequences of MgfL and AgfL which were of size 1914 bp and 1975 bp respectively were compared with C. hircus Lf nucleotide sequence retrieved from GenBank. The sequences of coding region of Lf gene of both Malabari and Attappady Black goat breeds revealed more than 99% identity with goat lactoferrin mRNA sequences in the database retrieved by BLASTn. In MgfL there were 8 nucleotide variations, out of which 3 were transversions and 5 were transitions while AgfL sequence showed a total of 10 variations comprising of 3 transversions and 7 transitions. Conceptualized translation of nucleotide sequence revealed 5 non-synonymous amino acid changes (p.Arg88Leu, p.Lys124Gln, p.Pro154Phe, p.Leu357Val, p.Gly414Asp) in both the breeds. At position 88 of the protein, arginine, a basic amino acid was replaced by leucine, a hydrophobic non-polar amino acid. At position 124 of the protein, lysine, a polar positively charged amino acid residue was replaced by glutamine, a polar amino acid with no charge in its side chain. The 154th residue proline, a non-polar amino acid was found to be replaced by phenylalanine, another non-polar amino acid. At position 357, leucine, a non-polar amino acid was found to be replaced by valine, another non-polar amino acid. The 414th residue, glycine, a polar amino acid with no charge on its side chain was found to be replaced by aspartic acid, a polar amino acid with negative charge.

Le Provost et al. (1994) reported the characterization of caprine Lf coding region of 2411bp size. Chen et al. (2007) expressed and purified goat Lf in Pichia pastoris and their amplified product was 2235 bp in size. In the present study the PCR amplified products were sequenced by primer walking technique and the end regions of the products could not be sequenced properly, that led to sequences shorter than the expected product size of 2048 bp. Pauciullo et al. (2010) sequenced the full reading frame (2127 bp) of Lf cDNA of Italian Nicastrese goat breed which was famous for its disease resistance and compared with Saanen goat breed Lf sequences. They found 11 nucleotide variations responsible for 5 amino acid changes which were the same as observed in the present study. Kang et al. (2008) noted 6 novel amino acid variations while analyzing the sequences of goat Lf gene.

The evolutionary relationship of Lf nucleotide sequences with 11 other species was generated. Analysis of the phylogenetic tree of Lf gene confirmed the presence of a common ancestor for the members of Bovidae family i.e., cattle, buffalo, goat, sheep, yak and antelope. Horse, camel and pig were distantly related to Bovidae while human beings formed a separate clade. Similar results regarding the phylogenetic relationship of Lf gene in cattle, goat, sheep, horse and camel were reported by many researchers (Yakubu et al., 2014; Akumbugu & Olusegun, 2017) by conducting in silico analysis of the sequences retrieved from the database.

Salient changes in the primary structure of proteins can alter their 3D structure and consequently change their functional properties. Kang et al. (2008) considered Lf amino acid variations within species to be related to antibacterial property or other biological activities. The non-synonymous amino acid variations revealed by the present study, at 88th and 414th positions of Lf seems to be highly relevant regarding to its biological functions as they are contributed by amino acids with totally different physical and chemical properties. These changes could be significant to the disease resistance exhibited by these autochthonous goat breeds. Moreover the synonymous amino acid variations though do not lead to altered protein structure, could alter the substrate specificity to mRNA binding and thereby down-regulate the translation process resulting in modified conformation of protein (Kimchi-Sarfaty et al., 2007). Lf is considered as one of the house keeping genes modulating iron homeostasis and immune
responses; hence special emphasis has to be given to the two unique synonymous variations of Attappady Black goats to rule out their contributions with respect to biological functions. Detailed population level studies on these nucleotide variations could throw light on the relevance of LF in the herd immunity and disease resistance of these goats.

Conclusion

LF is attributed with multiple biological functions besides taking part in iron homeostasis in the intestine. The present study reveals that the LF of native goat breeds of Kerala, though very similar in genetic makeup to other members of the Bovidae family, possesses unique variations in its amino acid sequences which could be relevant for its antimicrobial and other biological properties.

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Conflict of Interest

Authors would hereby like to confirm that there is no conflict of interests that could possibly arise.

References


