

Molecular cloning and characterization of alpha lactalbumin gene of Vechur cattle

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ABSTRACT

A 1756 bp fragment of the alpha-lactalbumin (α -LA) gene of Vechur cattle covering the entire transcriptional unit was PCR-amplified from genomic DNA and was sequenced by the dideoxynucleotide method after cloning in a T-vector. The Vechur α -LA gene has an open reading frame of 426 nucleotides encoding a signal peptide of 19 amino acid residues and a mature protein of 123 amino acid residues with NH₂ terminal glutamic acid and COOH- terminal leucine (Accession # EU 200932). Sequence analysis showed that 122 amino acid residues are identical with that of *Bos taurus* α -LA and 73% identity was observed with human α -LA protein. The nucleotide sequence of Vechur cattle α -LA gene was having 99% homology with that of *Bos taurus*, 98% with that of yak and 95% with that of sheep α -LA gene. The exon-intron boundaries are conserved in Vechur and *Bos taurus* as well as in human. This strict homology in the sites of insertion of introns suggests that the exon-intron organization of these genes was established before the divergence of these species. The positions of the exon-intron boundaries are also conserved as evidenced from similar sizes of the exons. The degree of structural or functional similarity between Vechur and human α -LA proteins was almost similar to the similarity between *Bos taurus* and human α -LA proteins.

Key words: Alpha-lactalbumin, Cattle, DNA sequencing, Molecular cloning, Protein structure, Vechur cattle

The milk of Vechur cattle is considered to have medicinal value and was extensively used as an ingredient in Ayurvedic system of medicine (Pillai 1940). These cattle became the first among the native cattle of Kerala to get the approval as a distinct breed (Iype and Venkatachalapathy 1997). The populations and genetic base of several valuable indigenous cattle including Vechur began to shrink rapidly due to the extensive crossbreeding policy of the state. The Vechur cattle have now gained international recognition and attention among the scientific community due to their smaller size and the believed medicinal value of their milk. The smaller size of the fat globules in the milk makes it easily digestible and hence it is widely recommended for children and convalescents (Venkatachalapathy and Iype 1997). However, scientific studies on Vechur milk components and their beneficial effects are scarce.

Alpha lactalbumin (α -LA) is a multifunctional whey protein found in milk of all species studied so far. The α -LA gene occurs in cattle on the BTA 5q21 chromosome (Vilote *et al.* 1987). The α -LA locus can be used as a genetic marker for milk production and the marker may be directly

responsible for increased milk production in cattle (Bleck and Bremel 1993). Alpha-lactalbumin participates in lactogenesis and constitutes a subunit of the enzymatic complex that catalyzes the final phase of lactose biosynthesis in milk (Voelker *et al.* 1997). This protein enhances the synthesis of 1 α interleukin, which stimulates immunological response at many levels (Wong *et al.* 1997), exhibits the ability to inhibit the development of breast cancer cells in women by inducing apoptosis of neoplastic cells (Svensson *et al.* 1999), has bactericidal properties (Hakkanson *et al.* 2000), protects the stomach mucous membrane against harmful factors, thus counteracting the ulcer disease (Matsumoto *et al.* 2001) and has anxiolytic-like and rewarding effects (Orosco *et al.* 2004). This protein has the essential amino acid requirements of newborn infants and so important in infant nutrition (Lonnerdal and Lien 2003). Therefore, information on the degree of homology of Vechur α -LA with human α -LA would help to determine its proportion of incorporation in infant formulae.

The objectives of the study were to deduce the main features of the Vechur α -LA gene such as its DNA sequence, nature and patterns of exon-intron length distributions and its protein structure and to compare its features with that of other species so as to have an idea about the evolutionary status of the breed.

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MATERIALS AND METHODS

The study was carried out on Vechur cattle (*Bos indicus*) maintained at the Vechur cattle conservation unit of Kerala Agricultural University.

Genomic DNA isolation and design of PCR primers

Genomic DNA was extracted from whole blood using the standard phenol-chloroform extraction procedure (Sambrook *et al.* 1989). The primers for amplification of α -LA gene were designed based on the α -LA gene sequence of *Bos taurus* cattle available in the database. The forward and reverse primers were designed online using the software Primer3 at the site <http://frodo.wi.mit.edu> from a 3090 bp sequence (GenBank Accession X06366; Vilotte *et al.* 1987). The primers designed for this study amplified a 1756 bp product from position 754 to 2509. The nucleotide sequences of the designed primers were as follows:

Forward primer: 5'-GGG GTC ACC AAA ATG ATG TC-3'

Reverse primer: 5'-GCA AAG ACA GCA GGT GTT CA-3'

The primers were custom synthesized commercially. They were reconstituted to make a stock solution of 200 pM/ μ l, which were incubated at room temperature for 1 h and then stored at -20°C .

Amplification of α -LA gene by PCR: The PCR assay was performed in 200 μ l reaction tubes with 50 μ l, containing 100 ng of DNA template, 1 X PCR buffer, 1.5 mM MgCl_2 , 30 pM of each primer, 200 μ M of each dNTP and one unit *Taq* DNA polymerase. The PCR cycling conditions included an initial denaturation at 94°C for 3 min and 35 cycles each of denaturation at 94°C for 1 min, annealing at 57.4°C for 1 min and extension at 72°C for 1.5 min followed by final extension at 72°C for 10 min. The PCR products were checked in 1% agarose gel in 1 X TAE buffer in a horizontal submarine electrophoresis unit.

Gel purification and cloning PCR product in T- vector: The PCR product was purified from the gel slice using the DNA purification kit. The purified PCR product was cloned using the cloning kit. The ligation reaction was carried out in 10 ml volume and contained 3 ml of PCR product, 5 ml of 2X instant ligation buffer, 1 ml T- Vector and 1 ml instant T4 DNA ligase. The ligated product was examined in 1% agarose gel in 1 X TAE buffer in a horizontal submarine electrophoresis unit and kept at -20°C till transformation.

Transformation and isolation of recombinant plasmids: Transformation was induced by treating host *E. coli* cells with ice-cold calcium chloride followed by a brief exposure to high temperature using the transformation kit as per the manufacturer's instructions. The recombinant plasmid carrying the α -LA gene insert was isolated from the white clones by a modified SDS-alkaline lysis method using the plasmid miniprep kit.

DNA sequencing: Sequencing was carried out commercially by the dideoxynucleotide sequencing method

in an automated DNA sequencer. The initial sequencing was performed from each end using T7 and SP6 primers, respectively, and sequencing was completed using 3 additional primers designed for primer walking.

Sequence analysis and structure prediction: The 1756 bp sequence obtained through sequencing was analysed at the National Centre for Biotechnology Information (NCBI) site (<http://www.ncbi.nlm.nih.gov/BLAST>) using BLASTn programme. The raw sequence was annotated based on the published bovine sequence (Accession #X06366; Vilotte *et al.* 1987). The intron, exon and other structural and functional regions of the sequence were established. For interspecies nucleotide sequence homology between exonic regions of the α -LA gene in Vechur with human and other livestock species, individual exons were compared by BLAST2 programme.

The exon sequences were compiled together and using the programme ExPASy Translate tool (<http://www.expasy.ch/tools/dna.html>), the amino acid sequence of the signal peptide and the 123 amino acid residues of the polypeptide chain, was determined. The protein sequence was analysed by using the BLASTp programme at the NCBI site. A multiple sequence alignment of the predicted protein sequence with other mammalian species was also carried out by ClustalW programme to highlight the areas of homology and divergence and the results were presented as a phylogram. The secondary structure of the Vechur α -LA was predicted using the PAPIA system (<http://mbs.cbrc.jp/papia/papia.html>) of Computational Biology Research Center, Japan. The 3 dimensional structure of the sequenced protein was predicted using the automatic comparative modeling servers: Swiss Model Server (<http://swissmodel.expasy.org/>) and ESyPred3D (<http://www.fundp.ac.be/urbm/bioinfo/esypred/>). The predicted structure was viewed with the molecular visualization software RasMol.

RESULTS AND DISCUSSION

The nucleotide sequence of the α -LA gene obtained from the cloned PCR product revealed 99% homology with α -LA gene of *Bos taurus*, 98 and 95% homology with that of yak and sheep, respectively (Table 1). The annotation of the Vechur α -LA gene sequence based on bovine sequence revealed the presence of 4 exons of 133, 159, 76 and 58 bp separated by 3 introns of 321, 473 and 504 bp. The lengths of exon 1, exon 2 and exon 3 were 133, 159 and 76 nucleotides in all species compared including human (Table 2).

DNA sequence analysis: The BLAST2 analysis revealed a high degree of exon sequence homology of Vechur with human and livestock species, which ranged from 77 to 100% (Fig.1). Evidence for some homology was also seen in intron 2. In contrast, intron 1 has diverged considerably both in length and in sequence and intron 3 in sequence. The intron 1 is much larger in the human gene as a consequence

Table 1. Results of BLASTn of the Vechur α -LA gene nucleotide sequence showing the % of identity with other species

Accession	Description	Maximum score	Total score	Query coverage	E value	Maximum identity
X06366.1	<i>Bos taurus</i> gene for α -LA	3166	3166	100%	0.0	99%
AF194372.1	Yak α -LA gene, complete CDS	3107	3107	100%	0.0	98%
AF194373.1	Buffalo α -LA gene, complete CDS	2968	2968	100%	0.0	97%
AB052168.1	Sheep gene for α -LA, complete CDS	2843	2843	100%	0.0	95%
M63868.1	Goat α -LA gene, exons 1-4	2772	2772	100%	0.0	95%
AY122023.1	<i>Bos taurus</i> α -LA gene, intron 2 and partial CDS	883	883	27%	0.0	100%
EF419240.1	Buffalo α -LA gene, intron 3	843	843	28%	0.0	96%
X05153.1	Human α -LA gene	542	542	83%	2e-150	74%

Table 2. Comparison of the length of exons and introns of the α -LA gene in different species

Species	No. of nucleotide residues						
	Exon 1	Intron 1	Exon 2	Intron 2	Exon 3	Intron 3	Exon 4
Vechur	133	321	159	473	76	504	330
<i>Bos taurus</i>	133	321	159	473	76	504	330
Buffalo	133	326	159	469	76	505	61
Yak	133	321	159	471	76	503	61
Goat	133	327	159	474	76	503	58
Human	133	648	159	489	76	499	333
Rat	133	341	159	429	76	1016	328

of the insertion of *Alu* family repeat sequence as reported earlier (Deininger *et al.* 1981, Hall *et al.* 1987). The di- and tri-nucleotide repeats of (TG), (TCC) and (TAT) present in intron 1 and 3 of Vechur α -LA gene are not present in the human gene. Alignment of the Vechur and human α -LA genes, allowing gaps for best fit, showed homology between exons 1, 2, 3 and 4 to be 77, 86, 90, and 90% s, respectively.

Comparison of the human and Vechur α -LA genes showed identical structural organization and identifies extensive homology within the transcription unit. The exon-intron boundaries in the human α -LA gene occur at the codons of the amino acid residues, Leu-26 (intron 1), Lys-79 (intron 2) and Trp-104 (intron 3). The latter 2 residues are conserved in Vechur and *Bos taurus*, whereas Leu-26 is replaced by

Trp-26 in the Vechur and *Bos taurus* proteins. This strict homology in the sites of insertion of introns suggests that the exon-intron organization of these genes was established before the divergence of bovine and human species. The positions of the exon-intron boundaries are also conserved as evidenced from similar sizes of the exons.

The nucleotide sequence data of Vechur α -LA gene was submitted to the GenBank DNA database and obtained the unique Accession Number: EU 200932. The sequence data of 1756 bp obtained from sequencing of the cloned PCR product provide the complete structural organization of the transcription unit of the Vechur α -LA gene. The transcription unit with a size of 1724 bp begins at position 13 as evidenced by the presence of the start codon 'ATG'. It is composed of 4 exons and 3 introns and sequences that respond to hormones and regulatory elements. The hexanucleotide sequence TGT(C/T)CT at positions 17 and 1698 might be possible sites for glucocorticoids as suggested by Scheidereit *et al.* (1986). The 3 intervening sequences begin and end with the consensus sequences (GTGAGT...) and (...CAG), respectively, and these sequences involve in the splicing process in which the sequences corresponding to the introns are removed from the primary transcript of the gene as reported by Breathnach and Chambon (1981). The sequences upstream from the intron 32 end such as the consensus sequence (CTGATCTCTCT) identified in the human globin gene (Rutmann and Breathnach 1985) seem to be necessary for the splicing process to occur (Ruskin and Green 1985). Sequences similar to the above mentioned occur at positions

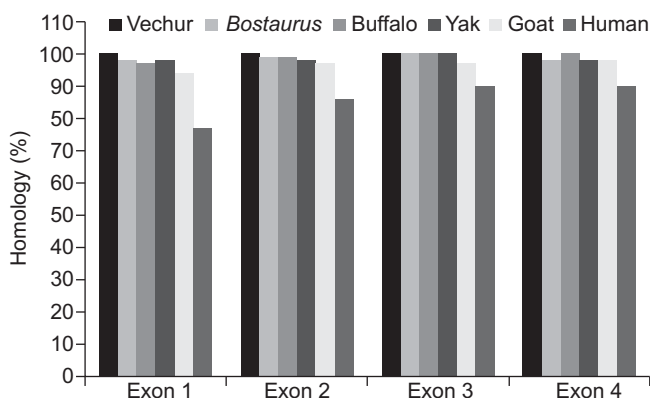


Fig 1. Interspecies nucleotide sequence homology of the exonic regions of α -LA gene based on Vechur sequence as a reference.

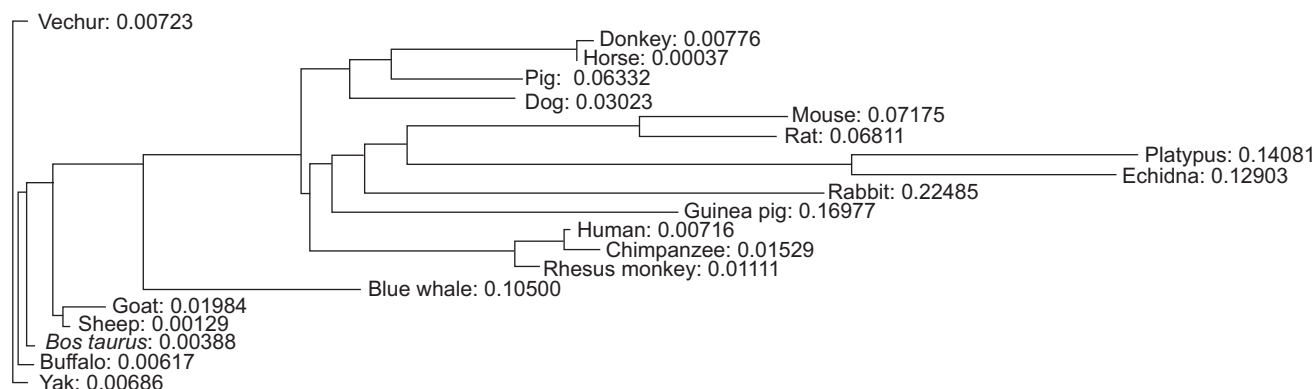


Fig 2. Phylogram constructed by ClustalW depicting the evolutionary relationships between species.

-29 (73% homology), -39 (82%) and -43 (73%) of the 32 end of introns 1, 2 and 3, respectively.

Protein sequence analysis: The derived amino acid sequence revealed the presence of 19 amino acid residues of the signal peptide and 123 amino acid residues of the mature polypeptide starting with the NH₂-terminal glutamic acid and ending with the COOH-terminal leucine. The Vechur α -LA comprises only a single residue of methionine and 2 residues each of arginine and proline. The 19 amino acid signal peptide was 100% homologous with the *Bos taurus* counterpart while 7 variants were found with the human counterpart. The signal peptide was also identical to that reported for bovine and ovine α -LA (Gaye *et al.* 1987).

The protein sequence analysis by BLASTp also revealed 99% identity with the corresponding protein of *Bos taurus* and 98 and 73% identity with that of yak and human, respectively. The ClustalW results of amino acid sequences also produced a high alignment score with *Bos taurus*, buffalo, goat and human with scores of 99, 98, 94 and 72, respectively, and the constructed distance phylogram of mammals placed Vechur along with bovine species while human and other primate species were in distant group (Fig. 2).

The predicted secondary structure of Vechur α -LA showed that the larger α -helical lobe is formed by the amino- and carboxyl-terminal sections of the polypeptide chain (residues 1–16 and 86–123) while the smaller lobe, which encompasses a small 3 stranded antiparallel beta-sheet, is formed by the central section of the polypeptide chain (residues 17–85).

The bovine α -LA consists of four α -helices, five 3_{10} helices, 2 α -structures, and an appreciable amount of random coil, representing 30, 20, 6 and 44%, respectively (Acharya *et al.* 1989). The predicted tertiary structure of Vechur α -LA also showed high homology with the bovine and human α -LA structures (Fig. 3).

Comparison with *Bos taurus* and human α -LA: A comparison of the amino acid sequence of the Vechur α -LA with the amino acid sequence of *Bos taurus* and human α -LA is presented in Fig. 4. The amino acid composition of the Vechur α -LA was in good agreement with the corresponding sequences of *Bos taurus* (Vilotte *et al.* 1987) with a single variant amino acid of Arginine (AGG) at position 122 in Vechur against Lysine (AAG) in *Bos taurus*. The alignment of the Vechur and human α -LA showed a high degree of homology (74%) with 31 variant amino acid residues in human protein. A relatively higher percentage of valine in the protein of Vechur and *Bos taurus* was found in comparison to human alpha lactalbumin. The percentage of tryptophan was also higher in the α -LA of bovine species than in the human counterpart. The percentages of essential amino acids in the α -LA of human and Vechur milk were found to be 52 and 51, respectively.

Overall, the structures of Vechur α -LA was found to be very similar to that of *Bos taurus* and moderately close to human reflecting their high degree of amino acid sequence identity (74–99%). Similar results were also reported by Brew and Grobler (1992) with respect to human and *Bos taurus* proteins. The degree of structural or functional



Fig. 3. The 3 dimensional structure of alpha lactalbumin. (A). Vechur alpha lactalbumin predicted using Swiss Model (B).Vechur alpha lactalbumin predicted using ESyPred3D. (C). Human alpha lactalbumin (PDB code 1A4V).

	10	20	30	40
Vechur	MMSFVSLLLV	GILFHATQAE	QLTKCEVFRE	LKDLKGYGGV
<i>Bos taurus</i>	MMSFVSLLLV	GILFHATQAE	QLTKCEVFRE	LKDLKGYGGV
Human	MRFFVPLFLV	GILFPAILAK	QFTKCELSQL	LKIDIDGYGGI
	50	60	70	80
Vechur	SLPEWVCTTF	HTSGYDTQAI	VQNNDSTEYG	LFQINNKIWC
<i>Bos taurus</i>	SLPEWVCTTF	HTSGYDTQAI	VQNNDSTEYG	LFQINNKIWC
Human	ALPELICTMF	HTSGYDTQAI	VENNESTEYG	LFQISNKLWC
	90	100	110	120
Vechur	KDDQNPSSN	ICNISCDKFL	DDDLTDDIMC	VKKILDKVGI
<i>Bos taurus</i>	KDDQNPSSN	ICNISCDKFL	DDDLTDDIMC	VKKILDKVGI
Human	KSSQVPQSRN	ICDISCDKFL	DDDIITDDIMC	AKKILDIKGI
	130	140		
Vechur	NYWLAHKALC	SEKLDQWLCE	RL999	
<i>Bos taurus</i>	NYWLAHKALC	SEKLDQWLCE	KL	
Human	DYWLAHKALC	TEKLEQWLCE	KL	

Fig. 4. The deduced amino acid sequence of Vechur α -LA and the amino acid sequence of *Bos taurus* and human α -LA. The variants amino acids are shown in red bold letters.

similarity between Vechur and human α -LA proteins was almost similar to the similarity between *Bos taurus* and human α -LA proteins. The higher percentage of fat and total solids and lower fat globule size than other breeds of cattle is considered to be some reasons for the superiority of Vechur milk. The superiority of human milk and its high suitability to infants could be due to the higher content of α -LA and might not be attributed to any structural variations of the protein. This view is supported by the fact that the ratios of whey proteins to casein in human and bovine milks are 60 : 40 and 20 : 80, respectively, as reported by Lien (2003). Since the higher content of α -LA in human milk could be due to the high expression of this gene, further studies may be carried out to find out sequence variations, if any, occur in the regulatory sequences upstream of the gene. Another possibility could be the presence of *enhancer* sequences or variation in the regulation of expression of this protein in human. Hence, it is worth to carry out a comparative expression study of this gene in human and Vechur cattle.

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