

Alpha-Lactalbumin Gene Polymorphism: A preliminary study on two breeds of the river Buffalo (*Bubalus bubalis*)

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Abstract. An experiment was carried out to investigate Single Nucleotide Polymorphisms (SNPs) in the coding regions of α -LA gene in two Indian buffalo breeds *vis.* Murrah (the best milk yielder) and South Kanara (used in agriculture operations). SNPs in α -LA potentially alter the gene expression and may be associated with differences in milk yield and quality. High molecular weight genomic DNA was extracted from 16 peripheral blood samples. Based on the 3090 bp sequences for bovine gene for alpha-lactalbumin (NCBI GenBank X06366) four primers were designed and used for amplification. The PCR products were electrophoresed, visualized and documented. Single Stranded Conformational Polymorphisms (SSCP) technique was used to screen for SNPs using the DCode Universal Mutation Detection System with 6% acrylamide gel. The individuals (samples) showing polymorphisms and also two samples per breed for each exon were further probed by DNA sequencing. DNA was eluted from the gel using a gel elution Kit. For sequencing, PCR reactions contained 5ul of eluted DNA and 10 pmoles of primer and 4ul of reaction mix. The sequencing products were analysed with the ABI Prism 377 DNA automated sequencer. In order to detect possible polymorphisms, the buffalo α -LA gene sequences observed were compared with the sequence for the same gene in NCBI gene bank AF194373. Sequence of exon 1 of all the animals were determined by direct sequencing. All the 8 buffaloes belonging to both the breeds showed a substitution at position 864 (C \rightarrow T) and one South Kanara buffalo showed substitution at position 1264 (A \rightarrow G). The polymorphism at position 864 (C \rightarrow T) results in substitution of an amino acid P \rightarrow S while the observed polymorphism at 1264 was a silent mutation in the coding region of the gene. No SNPs were observed in other three exons. The substitution observed in all the buffaloes concerned reveals Indian buffaloes which are riverine breeds differing from the buffaloes of China.

Keywords. Single Nucleotide Polymorphisms, Alpha-Lactalbumin gene, Indian Buffaloes

INTRODUCTION

The variation in DNA sequence, as it causes the variation in the performance of animals is the basic material for improving livestock through selection. Single nucleotide polymorphisms (SNPs) represent a potential resource for analyzing DNA sequence variation in many species of animals (Stewart et al., 1987 and Soulier et al., 1989; Soumi et al., 2007). SNPs have become the markers of choice in genetic studies because of their high frequency, low mutation rates and amenability to automated analysis (Gudmundur et al., 2003).

It has been estimated that there is on an average of 0.5-1.0 heterozygous SNPs per 1000 base pairs in the human genome (Mark, 2001). In livestock, one expects a slightly lower number of SNPs because of intensive selection in their breeding programmes. But the number of SNPs still exceeds other types of genetic markers (Ramesha et al., 2002). Since SNPs are found in such large numbers, they are a superlative

marker type for characterization of economically important traits. It is well established that the quality and quantity of milk protein differ among the species, breeds as well as individuals within a breed. It is essential to identify SNPs in genes responsible for milk quality and quantity like bovine alpha lactalbumin (α -la), α S₁-casein etc., score them in a reference population and to determine their linkage map position.

The α -LA is a major whey protein of bovine milk and is essential for the biosynthesis of lactose in mammary gland (Larson, 1979). It is a constituent of lactose-synthetase, the enzyme responsible for the synthesis of lactose in the final step where glucose is linked to galactose (Larson, 1979). Dairy cows produce milk that contains approximately 1.2 mg/ml of α -LA and 5% lactose (Wagner et al., 1994).

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Table 1. Primer combinations used for PCR amplifications

Exon	Amplicon (bp)	Primer	Location	Annealing temp.(°C)
Exon1	(177bp)	f: 5' GAG CAG TGT GGT GAC CCC 3' r: 5' TGG AGG GAA AGA GTG AAG AG 3'	721-738 938-898	55
Exon2	(294bp)	f: 5' GGT CTG GGA ATA CAG GTC C 3' r: 5' GGT TAT CCC AGG AGT AGG TT 3'	1145-1163 1419-1438	55
Exon3	(239bp)	f: 5' GGC AAC AGG CAT AAG CCT C 3' r: 5' GGA CTG AGA AGA AAG AGA GG 3'	1742-1760 1962-1980	55
Exon4	(262bp)	f: 5' CCT CAG CCT TCC TGG GGA 3' r: 5' CAG GGC TCA GAG ACG AGT T 3'	2347-2364 2590-2608	55

f=forward primer; r=reverse primer

Lactose is the main osmole in milk and causes movement of water into the secretory vesicles of mammary epithelial cells. The concentration of α -LA is hypothesized to regulate milk volume through this mechanism by increasing or decreasing the amount of lactose secreted into the milk (Pervaiz and Brew, 1985). It has been shown that α -LA can be the limiting component in the lactose synthetase complex (Grimble and Mansaray, 1987). Alpha-LA is considered to be the bovine milk protein that is most lactation specific and its expression is used as a lactation specific marker in both mammary expellant and cell culture systems (Vega et al., 1988).

Many reports suggest that SNPs in α -LA potentially alter the gene expression and may be associated with differences in milk yield and quality (Bleck and Bermal, 1993; Moe, 1994 and Ramesha et al., 2002). Variation in SNF and contributed proteins may be attributed to polymorphism in sequence of causative genes.

There are many buffalo breeds in India which are kept for diverged purposes. Among them, Murrah are well known to be the best milch and meat breed of buffaloes with increasing popularity among Indian farmers. While the South Kanara buffaloes are used in agricultural operations and Kambala and they are considered for high content of butter fat and tier ability to convert coarse feed into butter fat (buffalo bulls racing in muddy field). The current study was formulated intended to verify possible SNPs in the coding regions of α -LA gene in Murrah and South Kanara breeds of buffaloes.

MATERIALS AND METHODS

Experimental Animals. Sixteen randomly chosen buffaloes (8 from Murrah and 8 from South Kanara breeds) were used for blood collection. The animals were taken from a parental stock maintained at NDRI, Southern Campus, Adugodi, Bangalore, India. High molecular weight genomic DNA was extracted from peripheral blood as described by Miller et al. (1988). Samples were adjusted to a concentration of 20

μ g/ml before PCR amplification.

PCR amplifications. Based on the 3090 bp sequences for bovine gene for alpha-lactalbumin (GenBank Acc:X06366) primers designed and obtained from Bangalore Genei (P) Ltd., Bangalore, India, were used for amplification. Polymerase chain reaction primers, their location in X06366, their annealing temperature and the expected product sizes are summarized in Table 1.

The amplifications were carried out in 0.2 ml PCR reaction tubes using a programmable thermal cycler (Perkin Elmer, Weiterstadt, Germany). The 25 μ l reaction mix comprised of 20 ng template DNA, 20 pM each primer, 100 μ M each of the four d-NTPs, P³² label 2.5 units, 1 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore), 1.5 mM MgCl₂ and 1X PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl and 0.1% TritonX-100). A PCR programme with an initial denaturation at 94 °C for 5 min., second denaturation for 1 min at 94 °C, annealing at 55 °C for 60sec and extension duration of 1 min at 72 °C was used. Temperature cycling included 35 cycles of 60 sec at 94 °C, 60 sec at 55 °C, 60 sec at 72 °C and final extension at 72 °C for 10 min. The PCR products were electrophoresed at 100 V in 1% agarose gel in 1X TAE buffer containing 0.5 μ g/ml ethidium bromide along with a DNA molecular size marker and were visualized and documented using Gel documentation system (Gel doc 1000, Bio-Rad, USA).

Single Stranded Conformational Polymorphisms (SSCP).

SSCP technique was used to screen for SNPs using the DCode Universal Mutation Detection System (Biorad, USA) with 6% acrylamide gel according to the manufacturer's protocol. About 300ng of amplified DNA of each sample along with 2x SSCP gel loading dye was loaded onto the SSCP gels after denaturing the samples at 95 °C for 10 minutes and snap chilling using ice. The gel was run at 40 W constant power for 5 hours in 1 x TBE. Gels were exposed to a storage phosphor-imaging screen after drying the gel for 60 min at 60 °C. Imaging screen were scanned on a storage phosphor imaging system. The images were screened for SNPs in any

Table 2. Summary of single nucleotide polymorphisms observed in the study

Gene	Segment	Position ^a	Variation ^b	Amino acid exchange ^c	Polymorphic allele frequency
α -LA gene of buffalo	Exon1	864	GTTT <u>GC</u> /TCTGAA	P 52 S	1.00
	Exon2	1264	AAGCC <u>A</u> /GTAGTA	-	0.06

^a based on the sequences from the GenBank Acc:AF194373 for buffaloes.

^b polymorphic residues underlined (the common nucleotide followed by the variant).

^c based on transeq predict database of EMBL-EBI

individual and the individuals showing polymorphisms were identified based on number of bands detected. The polymorphic samples and also two samples per breed for each exon were further probed by DNA sequencing.

PCR was carried out using the same primers and temperature and other conditions as above. The PCR products were electrophoresed at 100 V in 1% agarose gel in 1X TAE buffer containing 0.5 μ g/ml ethidium bromide along with a DNA molecular size marker. DNA was eluted from the gel using gel elution Kit (Invitrogen, USA). For sequencing PCR reactions contained 5ul of eluted DNA and 10 pmoles of primer and 4ul of reaction mix. The sequencing products were analysed with the ABI Prism 377 DNA automated sequencer (Perkin Elmer, Germany). Unique sequences (SNPs) and ambiguous sequences were confirmed by repeating the PCR and sequencing in both directions. Sequence of exon 1 of all the animals were determined by direct sequencing.

RESULTS

The α -LA gene had 4 exons and 3 introns and was a length of 2784 bp in buffaloes of concern (*Bubalus bubalis*). The SNFs observed in the sequencing of the gene are presented in Table 2. The information on complete nucleotide sequence is not presented as they are attainable from many databases (GenBank Acc:AF194373).

All eight buffaloes from each breed were screened for polymorphisms in the coding regions of α -LA gene. In comparison of the sequences obtained with the sequence of α -LA gene AF194373, all buffaloes belonging to both the breeds had a nucleotide substitution at position 864 (C \rightarrow T) (Table 2). Moreover, one of the South Kanara buffalo showed nucleotide substitution at position 1264 (A \rightarrow G). The polymorphism at position 864 (C \rightarrow T) resulted in substitution of an amino acid P \rightarrow S while the observed polymorphism at 1264 was a silent mutation in the coding region of the gene. No SNPs were observed in other three exons.

DISCUSSION

The primary amino acid sequence of bovine α -LA was first determined by Gordon et al., (1968) and Brew et al., (1970).

Consequently, many reports on complete sequence of bovine alpha-lactalbumin gene using various species and techniques has been appeared in literature (Bell et al., 1970; Hurley and Schuler, 1987; Vilotte et al., 1987; Wang et al., 1989; Bleck and Bremel, 1993; Pike et al., 1996; Yamamoto N., 2000 and Dhinakar and Kumanan, 2000, Dayal et al., 2007) and inter- as well as intra species comparisons among nucleotide sequences were made.

In Holstein Friesian cattle, Bleck and Bremel (1993) identified three single bp polymorphisms for α -la within the 5' flanking region at position +15, +21 and +54 to the mRNA transcription start point. The +15 and +21 variations were in the zone encoding the 5'-untranslated region (5'UTR) of the mRNA sequence, while the +54 polymorphism was a silent mutation in the coding region of the gene. The α -la (+15) A and α -la (+15) B alleles are characterized respectively by an adenine and a guanine in position +15. The α -la (+15) A variant is associated with greater milk, protein and fat yields, while α -la (+15) B allele is associated with higher protein and fat percentages. The (+15) polymorphism was also noticed in Taiwan Holstein (Mao, 1994), Italian Fresian and Italian Red Pied (Vrech *et al.*, 1997), Swedish Red and White (Lunden and Lindersson, 1998). Ramesha et al., (2002) screened six indigenous Cattle breeds of Southern India (Amrithmahal, Krishna Valley, Hallikar, Deoni, Ongole and Malnad Gidda) and Holstein Crosses (HF x Sahiwal) for Single Nucleotide Polymorphisms (SNPs) in the coding sequence of the bovine alpha lactalbumin gene. SNPs at four positions (772, 775, 792 and 857) in exon 1 and three positions at 1231, 1264 and 1335 in exon 2 were observed. They further interpreted the effect of these SNPs in the mature protein based on the crystal structure of bovine α -LA.

In the current study, two single nucleotide substitutions were detected in exon1 of α -LA gene. Such a SNP (substitution at position 864 (C \rightarrow T) α -LA gene) found in all the samples belonging to the both buffalo breeds (Table 2) as the sequences were compared to sequence of α -LA gene of buffaloes in NCBI gene bank AF194373. This polymorphism at position 864 results in substitution of an amino acid P \rightarrow S. The alteration in amino acid sequence usually leads in structural diversity of the α -LA which it turn may cause amendment in renneting properties of milk (Altonen, and Antila, 1987; Kreuzer et al., 1996; Dayal et al., 2007), milk components and cheese-producing ability (Aleandri et al.,

1990; Lawrence et al., 1993; Lodes et al., 1996), milk clotting chemistry (Johnson, M.E. 1988; Brown, and Ernstrom. 1988), coagulation properties of milk (Davoli et al., 1990). It may also influence mastitis resistance ability of cow (Atroshi et al., 1982), lactation traits (Haenlein et al., 1987), reproductive performance of dairy heifers (Lin et al., 1987; Lin et al., 1992). On the other hand, the SNP observed in all the buffaloes under study indicates a possibility of Indian buffaloes are riverine buffaloes which they differ from the buffaloes of China. NCBI gene bank AF194373 α -LA gene sequence for buffaloes was submitted based on studies using buffaloes from China.

One South Kanara buffalo in this study showed substitution at position 1264 (A→G). The polymorphism at position 864 (C→T) results in substitution of an amino acid P→S while the observed polymorphism at 1264 was a silent mutation in the coding region of the gene. No SNPs were observed in other three exons.

Eight buffalo breeds have been reported in India alone with many other minor breeds. Now low Solid Not Fat (SNF) is a major problem in the milk of crossbred cattle. However, it is observed that buffaloes produce milk with higher SNF as compared to crossbreds. It is well established that the quality and quantity of milk protein including α -LA differ between the species, breeds as well as individuals within the breed. Present investigation on SNPs in α -LA gene reveals potential diversity in milk yield and composition which it might be traced intended to considering technological benefits of such diversity. The new and previously published information on alpha-lactalbumin sequences is analyzed in relation to the evolutionary history of the alpha-lactalbumin line as well as the relationship of structure to function in these proteins (Shewale et al., 1984 and Dayal et al., 2007).

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