

Molecular Characterization of Corticotrophin Releasing Hormone Gene and its Expression in Deoni Cows

N. Anand Kumar^{1*}; Dayal Nitai Das²

¹Assistant Professor, Dept. of Animal Genetics & Breeding, Veterinary College KVAFSU, Bidar-58526

²Principal Scientist, ICAR- NDRI(SRS) Adugodi, Bengaluru

*Corresponding Author

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Abstract— Improving the production performance in dairy cattle is the main concern of breeding policies, as the production traits are low to medium heritable and it is difficult to select best animals based only on phenotype. Deoni is hardy indigenous cattle with moderate milk production potential. Identification of gene polymorphism and its association with production performance of animals can be very useful for selection of superior animals. The aims of study were: (i) Molecular characterization and expression of CRH gene in Deoni cattle, (ii) Association of CRH gene polymorphism with milk yield and fat content and (iii) Breeding value estimation based on lactation performance. Genomic DNA was isolated from 152 Deoni cattle maintained at SRS of ICAR-NDRI, Bengaluru to evaluate genetic variability through PCR-RFLP method. PCR-RFLP analysis revealed monomorphism in CRH gene and was found to be highly conserved with a single nucleotide change at 19th position in all the Deoni animals compared to *Bos taurus* sequence. Association studies could not be carried out further because of lack of polymorphism. Mean phenotypic performances of AFC, FLMY, and Fat content were 44.35 ± 0.48 months, 577.64 ± 32.54 kg, and 4.61 ± 0.06 per cent, respectively. Heritability of FLMY of Deoni cattle was found to be 0.24 ± 0.20 . MPPA and breeding value for FLMY were estimated by LSA method using GLM procedure of SAS 9.3 based only on phenotypic data without incorporating the genotype information. MPPA was estimated to be 350.77 to 947.25 kg. PBV obtained through LSA method without taking genotype information were in the range from 139.32 to 1520.15 kg. Expression profiling of CRH gene was carried out in PBMC in eight Deoni cows in different lactations from the day of parturition to 60th day postpartum. Expression profiling of CRH mRNA was carried out using quantitative RT-PCR with SYBR green as fluorescent dye. Present study revealed a significant decrease of CRH mRNA expression from day 10 of calving to day 60 post calving, indicating that levels of stress is higher during parturition than during lactation. This is the first report on characterization and expression of CRH mRNA in Deoni cows.

Keywords— CRH gene, Indigenous Cattle, Deoni, FLMY, Breeding Value.

I. INTRODUCTION

In dairy enterprise the principal goal of selection is to increase the milk yield and improve its composition. Milk production genes involved in controlling milk fat, protein content and the hormones are excellent candidate genes for linkage analysis with Quantitative Trait Loci (QTL) because of their biological significance on quantitative traits of interest. It is noteworthy that QTLs for milk performance traits has been mapped in all the bovine autosomes, mostly in 6, 14 and 20 [1]

Corticotrophin - releasing hormone (CRH), also called as corticotrophin -releasing factor (CRF), is a 41 amino acid peptide deriving from a 191 amino acid precursor which is mainly synthesized in the hypothalamus, and also in other parts of brain. CRH functions as a neuropeptide hormone participating mainly in the stress response in vertebrates. CRH is involved in controlling the energy balance of an organism, which affects body weight and it also participates in modulating immune and reproductive systems [2]. CRH gene expression was found highest in hypothalamus, but the gene is also expressed in many

other places, such as ovaries, testes, placenta, uterus, liver, stomach, skin, and immune system. Gene encoding corticotrophin releasing hormone with many QTLs has been mapped in bovine chromosome 14 [3], which govern postnatal growth and it has been considered as a candidate gene for growth traits in cattle [4]. Several single nucleotide polymorphic (SNP) sites have been identified within CRH gene of *Bos taurus* cattle breeds viz. C22G which causes substitution of amino acids in signal sequence, A145G and C240G, leading to changes of amino acids in a propeptide [4] as well as two SNPs in exon 2 [5].

The present study was undertaken to determine the frequencies of alleles and genotypes with regard to gene polymorphism encoding corticotrophin releasing hormone (CRH-A145G) in Deoni cows and to study the association between genotypes and milk production traits of Deoni cows along with expression levels of CRH mRNA in postpartum Deoni cows during different days of lactation as well as its effect on milk yield and also to estimate the heritability and breeding value of Deoni cows based on First Lactation Milk Yield (FLMY).

II. MATERIALS AND METHODS

2.1 Experimental Animals:

152 Deoni animals maintained under standard management conditions adhering to minimum standard protocols as recommended by the department of Dairying, Animal Husbandry & Fisheries, Govt. of India at Livestock Research Centre (LRC) of ICAR – NDRI SRS Bengaluru were used in this experiment. For polymorphism study cows in different age groups and in different stages of lactation which were milked twice daily were considered. Data was collected from daily milking records available in the farm and Gerber's method was used to estimate fat content from test day milk samples.

2.2 Blood Collection and DNA Extraction:

DNA was isolated from peripheral blood drawn from external jugular vein of cows into Ethylene Diamine Tetra Acetic acid (EDTA) vacutainers and processed immediately for DNA isolation. DNA was isolated using a modified high salt method described by [6]. The investigated SNP consists in changing adenine to guanine at position 145 in exon1 – *CRH- A145G* (GenBankAF340152). As a result of this transition, a change of serine to asparagine occurs at the polypeptide level. The gene fragment of 156 bp was amplified and genotyped using PCR-RFLP method. To conduct this reaction, a pair of primers proposed by [4] was used and is listed in TABLE 1.

TABLE 1
DETAILS OF THE PRIMER SEQUENCE USED FOR PCR AMPLIFICATION OF *CRH* GENE

| GENE | Primer Name | Sequence (5'-3') | Primer Length | Product Size (bp) | Reference |
|------|-------------|------------------------|---------------|-------------------|-----------|
| CRH | Forward | GCGCCCGCTAAAATGCGACTGA | 22 | 156 | [4] |
| | Reverse | CTGTGATGCCTGCCGGGCAC | 20 | | |

2.3 PCR amplification and restriction enzyme digestion:

PCR programme consisted of cycles of initial denaturation at 95 °C for 4 min, followed by 34 cycles of 94 °C for 1 min (denaturation), 59 °C for 1 min (primer hybridization), 72 °C for 1 min (product synthesis), and final extension at 72 °C for 5 min. The PCR products were digested with 4 U of the *Dde* I restriction enzyme.

2.4 Expression studies of CRH mRNA:

For expression profiling of CRH mRNA, 8 low yielding post parturient Deoni cows in different age groups and in different parity were studied. Approximately 15 ml of blood was collected by external jugular venipuncture of each animal from the day of parturition up to 60 days post-partum with an interval of 10 days in to a vacutainer containing EDTA as the anticoagulant. Soon after collection, blood samples were stored at 4 °C and immediately transferred to lab for isolation of RNA.

2.4.1 Isolation, Quantity and Quality Check of Total RN:

Ribonucleic acid was isolated from Peripheral Blood Mononuclear Cells (PBMC) using RNeasy Mini Kit (Quiagen, USA) using manufacturer's protocol. RNA was checked for quantity and integrity through agarose gel electrophoresis and quantity was measured using nanodrop spectrophotometer (Eppendorf, Germany). The purified RNA was stored at -80 °C until use.

2.4.2 Synthesis of first strand cDNA:

The first strand cDNA was synthesized from the extracted RNA using iScript® cDNA synthesis kit (Bio-rad, California). From each PBMC sample 100 ng of total RNA was reverse transcribed using 4 µl of iScript® reaction mixture and 1 µl of iScript® enzyme to make a final volume of 20 µl with nuclease free water in a sterile PCR tube on ice. This reaction mixture was incubated at 25 °C for 5 min, later at 42 °C for 30 min, after which the reaction was terminated at 85 °C for 5 min. The cDNA was stored at -20 °C.

2.4.3 Real time quantitative polymerase chain reaction:

PCR analyses were performed with three replicates per sample of each gene using the STEP ONE PLUS real time PCR system (Applied Bio systems, USA) Fast SYBR Green master mix (Applied Bio systems, USA) and gene specific primers (**Table 2**). Primers were designed using the Primer3 programme with an annealing temperature of 60 °C and amplification size of less than 250 bp. B-actin was used as endogenous control. Thermal cycling was carried out as per manufacturer's protocol (95 °C, 20 s; followed by 40 cycles of 95 °C for 3 s; and 60 °C for 30 s) and a melt curve of 95 °C (15 s), 60 °C (1 min) and 95 °C (15 s). The specificity of each PCR product was determined by a melt curve analysis and amplicon size determination by agarose gel (0.2 %). Negative control which consisted of all components of qRT-PCR mix except cDNA was used. The relative quantification of gene expression changes were recorded after normalizing for β – actin gene expression computed using the $2^{-\Delta\Delta C_T}$ method in which C_T value from controls served as calibrator.

TABLE 2
DETAILS OF PRIMERS USED IN RT – PCR

| Primer Name | Primer Sequence | Length (bp) | Product Size (bp) |
|-------------|------------------------|-------------|-------------------|
| CRH Forward | AAGCAGCAGTCGCCTTTCT | 19 | 156 |
| CRH Reverse | CCTTGGTCATTTCCAAGACTTC | 22 | |

2.5 Data adjustment and statistical analysis:

The recorded FLMY data were adjusted for various non –genetic factors like period of birth, lactation number of animal, period of calving, season of calving and age at first calving (AFC) before estimating the heritability. The model used for study was:

$$Y_{ij} = \mu + F_i + e_{ij} \quad (1)$$

Where:

Y_{ij} = Phenotypic data of j^{th} observation in the i^{th} subclass

μ = Overall population mean of the traits (FLMY and Fat %)

F_i = Fixed effect of non-genetic factor of i^{th} subclass

e_{ij} = Random error, assumed to be normally and independently distributed with mean zero and constant variance, i.e NID (0, σ_e^2)

Heritability was estimated by paternal half sib correlation method (intra – sire correlation among daughter) as described by [7] using the following model:

$$Y_{ij} = \mu + S_i + e_{ij} \quad (2)$$

Y_{ij} = Adjusted data of the j^{th} progeny under the i^{th} sire.

(I = 1, 2, 3, ..., s; j = 1, 2, 3, ..., n_i)

μ = Population mean

S_i = Effect of ith sire

S = number of sires

n_i = number of progeny under ith sire

e_{ij} = Random error, assumed to be normally and independently distributed with mean zero and constant variation *i.e.* NID (0, σ_e^2)

And the formula used was:

$$h^2 = 4 * t = 4 * \frac{\sigma_s^2}{(\sigma_s^2 + \sigma_e^2)} \quad (3)$$

Most Probable Producing Ability (MPPA) was calculated before estimating the breeding value of FLMY using the model:

$$\text{MPPA of cow} = \mu + h^2(Y_i - \mu) \quad (4)$$

Where,

μ = Population mean

h² = Heritability of first lactation milk yield of Deoni cattle.

Y_i = Phenotypic value of FLMY of ith cow

Generalized Linear Model was used to predict Breeding Value (BV) through Least Square Analysis (LSA) method. The mixed linear model was chosen without incorporating molecular information of the loci of CRH gene. The model is as follows:

$$Y_{ijklmnox} = \mu + S_i + POB_j + LND_k + POC_l + SOC_m + CAFC_n + CFLL_o + e_{ijklmnox} \quad (5)$$

Where,

Y_{ijklmnox} = xth cow of ith sire born in jth period at kth lactation of its dam, calving at mth season of lth period under nth and oth group of AFC and FLL

μ = Population mean

S_i = ith sire (i = 1, 2, 3, ... 7)

POB = jth period of birth (j = 1 and 2)

LND = kth lactation number of dam (k = 1, 2, 3, ... , 5)

POC = lth period of calving (l = 1, 2, 3 and 4)

SOC = mth season of calving (m = 1, 2 and 3)

CAFC = nth class of age at first calving (n = 1, 2, 3, ... , 6)

CFLL = oth class of first lactation length (o = 1, 2, 3, ... , 6)

III. RESULTS AND DISCUSSION

Molecular characterization through PCR–RFLP analysis of CRH gene exhibited monomorphism in 152 Deoni samples revealing two fragments *viz.*, 120 bp and 36 bp (**Figure 1**). DNA sequencing samples revealed a change in nucleotide at 19th position from ‘T’ to ‘C’ in all Deoni (*Bos indicus*) cattle as compared to reference sequence of *Bos taurus* species (**Figure 2**). Due to lack of presence of SNP in exon 2 of CRH gene, exonic region was found to be conserved and probably gene is fixed in the present population studied. [8] reported the presence of SNP in exon 2 of CRH gene in Jersey breed of *Bos taurus* cattle affecting the milk production traits. Due to absence of polymorphism in CRH gene exon 2, the association study could not be carried out. Rat [9] and ovine [10] *CRH* also consist of two exons with the coding region in exon 2.

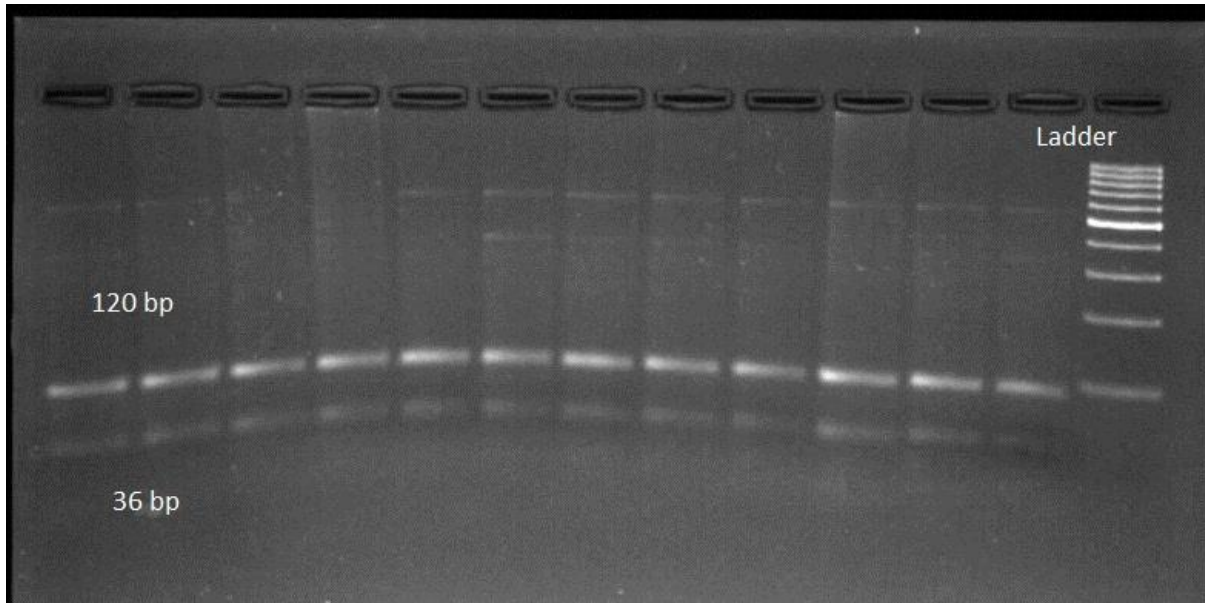


FIGURE 1: Restriction enzyme digestion of CRH gene

BLAST sequence of exon 2 of CRH gene revealed 99 per cent identity with *Bos taurus* (AF340152.1), 98 per cent with *Bubalus bubalis* (XM006044572.1) and 97 per cent identity with both *Ovis aries* (XM015097787.1) and *Capra hircus* (XM013969156.1). Upon amplification of porcine CRH by [11], they determined that the porcine coding sequence was 84 per cent and 85 per cent identical to sheep and rat sequences respectively; the porcine amino acid sequence is 74 per cent and 75 per cent identical to the sheep and rat amino acid sequence, respectively.

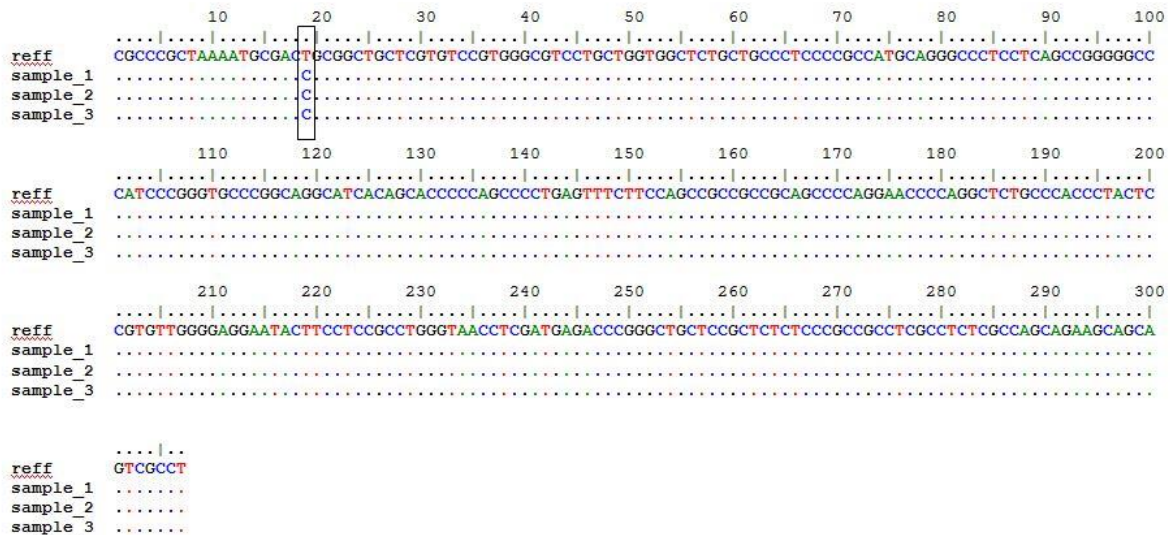


FIGURE 2: Clustal W-Alignment of CRH gene in Deoni breed

Heritability of first lactation milk yield in Deoni cattle was calculated using paternal half – sib correlation (PHC) method. Data on First Lactation Milk Yield (FLMY) and sire information of 152 Deoni cows was used to calculate heritability and was estimated as 0.24 ± 0.20 . Previous report on heritability of FLMY in Deoni cattle was reported as 0.38 ± 0.29 by [12] which was higher than the present findings. Lower estimate of heritability value may be attributed due to more number of observation considered in this study which provided more accurate estimate of heritability. Estimated heritability of FLMY in different breeds were 0.13 ± 0.12 and 0.1 ± 0.43 in Harijana and Tharparkar cattle as reported by [13] and [14] respectively. Reports on higher estimates of heritability in PHC method was observed in Sahiwal cattle as 0.48 ± 0.15 [14]. Predicted breeding values (PBV) of cattle were obtained through LSA method without taking genotype information and were found to be in the range from 139.32 to 1520.15 kg with CV value of 79.59 per cent. Breeding values of 7 sires used in the herd are tabulated and

ranked in the **Table 3**. Sire no. 3 used in the herd was ranked first followed by sire no. 30 and sire no. 24 respectively based on the breeding value, Sire no. 48 was found to be having the least breeding value among all the sires.

TABLE 3
BREEDING VALUE OF SIRES

| Sire no. | No. of Daughters | Breeding value | Rank |
|----------|------------------|-----------------|------|
| 1 | 14 | 565.96 ± 96.44 | 5 |
| 2 | 13 | 601.31 ± 92.93 | 4 |
| 3 | 36 | 693.53 ± 64.18 | 1 |
| 24 | 16 | 609.15 ± 92.73 | 3 |
| 26 | 40 | 544.70 ± 63.22 | 6 |
| 30 | 8 | 641.45 ± 103.09 | 2 |
| 48 | 25 | 533.22 ± 74.78 | 7 |

To the best of our knowledge this is the first study to report expression of CRH mRNA. In the present study we investigated the expression of CRH mRNA in Deoni animals over a period of 60 days from the day of calving, with an interval of every 10 days. Primer concentration was optimized for the gene under study using 0.25 picomolar (pM), 0.5 pM, 0.75 pM and 1.0 pM concentrations, and 0.75 (pM) concentration was found to be ideal for amplification of CRH mRNA. Housekeeping gene, β – actin was selected for comparative quantitation studies. The primer efficiency ranged between 96 per cent to 104 per cent. The present study revealed that CRH mRNA increased from day of parturition (Day 0) to Day 10 and then a decreasing trend was observed till day 60 post-partum in low yielding Deoni cows and milk yield also dropped over the same period in the animals studied. The decreasing levels of CRH mRNA suggests decreased stress levels after parturition which may be due to low milk production ability of Deoni breed of cows and involution of uterus post-partum.

Mean daily milk yield of animals studied for expression profiling was 1.8 ± 0.2 kg with highest yield being 2.5 kg on 10th day post-partum which gradually declined significantly over the lactation length and the lowest production was observed as 0.5 kg on day 60 post-partum.

Among the eight animals studied one animal exhibited highest expression of CRH mRNA on day 30 post-partum whereas; in all other animals peak level of expression was observed on 10th day post calving. This indicated that among low yielding Deoni cows, first 10 days post-partum period is more important and which may help in deciding selection of better yielder if more number of observations are included for expression study.

IV. CONCLUSION

CRH gene primarily being a stress regulator was found to be highly conserved in the Deoni breed of *Bos indicus* cattle. The lack of polymorphism in CRH gene made it difficult to flag it as a candidate gene for production traits in indigenous dairy cattle breeds. Further studies on same gene in other zebu breeds of cattle are to be carried out to ascertain the polymorphic nature of gene. Expression studies of CRH mRNA revealed higher level of stress in Deoni animals during the parturition than the lactation stress. A positive correlation was found among the expression Relative Quantitation (RQ) values and the daily milk yield of Deoni animals, in which both of them revealed a declining trend from the day of calving to day 60 post calving. Heritability of the FLMY was estimated to be 0.24 ± 0.20 and bulls used in the herd were ranked based on the Breeding values of FLMY calculated only on adjusted phenotype records excluding the genotypes. The study suggests scope for further evaluation of same gene in different *Bos indicus* breeds of cattle yielding higher quantum of milk to undertake appropriate breeding strategies to decrease the stress levels in lactating animals.

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