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Sequencing-based polymorphism in leptin receptor gene and lack of association with postpartum anestrus in Murrah buffaloes

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Abstract: In order to detect polymorphism in exon 20 of the leptin receptor (*LEPR*) gene and its possible association with postpartum anestrus (PPA), peripheral blood samples were obtained from 40 Murrah buffaloes including 20 PPA (>120 days after parturition) and 20 normal cyclic (<60 days after parturition) buffaloes having similar postpartum estrous conditions over previous consecutive gestations. Genomic DNA was isolated, and PCR was standardized to amplify partial exon 20 of the *LEPR* gene of 413 bp. Amplified fragments of the gene were sequenced and sequence variation was detected by assessing multiple alignments. The *LEPR* gene showed polymorphism at A231G, C247A, and G347A. Chi-square test of these 3 polymorphism sites between the 2 groups did not reveal any significant association of polymorphism with PPA. Hence, polymorphism at exon 20 of the *LEPR* gene was found in Murrah buffaloes, but the role of these polymorphisms in PPA could not be established.

Key words: Buffalo, postpartum anestrus, leptin receptor gene, polymorphism

1. Introduction

Buffalo is the main dairy animal in India that contributes roughly 56.5% to the total milk output (http//faostat.fao.org/), in spite of low reproductive efficiency. Among various factors reducing its efficiency, postpartum anestrus (PPA) is the most important anomaly (1). Buffaloes do come into estrus within 59 days postpartum under good management (2,3), but nearly 35% of buffaloes experience PPA (4). Many factors acting individually or in concert have been recognized to be responsible for postpartum infertility and anestrus, making it a complex phenomenon. In ruminants, these factors include nutrients, mineral deficiencies (5,6), season (7,8), suckling (9,10), and parity (11). Although these factors are recognized as being responsible for PPA, some animals within the same herd and under similar management conditions experience normal postpartum estrus while others display delayed postpartum estrus. Hence, the genetics of an animal may play a key role. Moreover, management interventions to improve reproductive performance increase costs of rearing with only marginal gain reported in reproductive and productive performance (12,13). Therefore, appropriate management interventions must be accompanied by strategies aimed at improving genetic potential of livestock to achieve cost-effective, enhanced reproductive and productive performance.

The leptin receptor gene (*LEPR*) is reported to control energy metabolism and reproduction (14,15). PPA is highly dependent on the energy balance of domestic animals, which is related to leptin secretion and its action on the hypothalamo–pituitary–ovarian axis. Leptin acts through receptors in the hypothalamus, as well as on cells in ovarian follicles, the placenta, and lactating mammary glands (16). Expression of *LEPR* seems to be affected by high and low nutrition levels (16); it stimulates growth hormone release and interferes in luteinizing-hormone secretion (17). Thus, the present study was designed to examine polymorphism in the *LEPR* gene and its association with PPA in buffaloes.

2. Materials and methods

2.1. Experimental animals

Twenty PPA and 20 normal cyclic Murrah buffaloes were selected on the basis of their reproductive history at the animal farms of the Central Institute for Research on Buffaloes, Hisar, India, and the Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar. The animals

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had exhibited either PPA or normal cyclicity during the previous 2 or more successive gestations (Table 1). The animals were maintained as per the standard feeding and management practices at the farms.

2.2. Collection, transportation, and genomic DNA isolation from blood samples

Approximately 5 mL of jugular blood was collected from each animal. Genomic DNA was isolated from blood samples as described by Sambrook et al. (18). The quality of extracted DNA was determined by Picodrop (Picodrop Ltd., Cambridge UK) and agarose gel electrophoresis (AGE) on 0.7% w/v agarose.

2.3. DNA amplification by PCR

The 413-bp fragment of the *LEPR* gene in the genomic DNA of Murrah buffalo was amplified as described by Liefer et al. (19) as shown in Table 2 for exon 20 of *LEPR* to amplify an equivalent region in buffaloes.

2.4. Polymerase chain reaction amplification (PCR)

The reaction mixture for PCR was prepared in 0.2-mL thin-walled PCR tubes. The master mixture was prepared by adding 17.45 µL of nuclease-free water, 2.5 µL of Taq DNA polymerase buffer, 2.5 µL of 100 µM dNTPs, 0.20 µL (100 ng) of each primer, 0.15 µL of Taq DNA polymerase (Fermentas, USA), and 2.0 µL of diluted genomic DNA (50 ng) to all the tubes at 4 °C. The 25-µL reaction mixture was kept for amplification in a programmed thermocycler (AB Master Cycler Gradient, Eppendorf, Germany). The amplification conditions after standardization were initial denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 58 °C for 55 s, and extension at 72 °C for 1 min, with final extension at 72 °C for 10 min. The PCR products generated were verified by electrophoresis on a 1.5% agarose gel in 1X Tris-acetate EDTA buffer, and ethidium bromide was used as the stain for DNA visualization. All chemicals were from Fermentas.

2.5. Purification of PCR products

To remove primer dimers and other PCR ingredients before custom sequencing, PCR products were purified using QIAquick gel extraction kit (QIAGEN, Germany).

2.6. Automated nucleotide sequencing and analysis

The purified PCR products were used for sequencing using an automated DNA sequencer by outsourcing to Chromous Biotech Ltd., Bangalore, India. The sequences obtained from the automated DNA sequencer were analyzed using the NCBI BLASTn online software tool after converting sequences into FASTA format (http://www.ncbi.nlm.nih.gov). Partial nucleotide sequences of the *LEPR* gene of normal cyclic and PPA animals were subjected to multiple sequence alignment along with sequences of buffaloes (Accession nos. JQ235662.1, AY177610, EU401922.1), cattle (Accession no. AJ580801.1), sheep (Accession nos. DQ788631.1, QAU62124), and goat (Accession nos. HQ834861.1, HQ83457.1) available at NCBI using Clustal W and BioEdit (version 7.1.3.0) software.

2.7. Statistical analysis

The frequency of different alleles of the *LEPR* gene was computed. Chi-square test was used to compare the genotyping frequency in cyclic and PPA animals.

3. Results

3.1. Genomic DNA isolation and its evaluation for concentration, quality, and purity

Good yield of high-molecular-weight genomic DNA was obtained from all 40 animals. The 260/280 nm absorbance ratio for all samples ranged from 1.7 to 1.9, indicating acceptable quality of genomic DNA.

3.2. Amplification of *LEPR* gene

The molecular size of the PCR amplified products was estimated to be 413 bp for *LEPR* by comparing with DNA size markers and sequencing (Figure 1).

Table 1. Physiological status of animals selected for sampling.

Physiological condition		Postpartum anestrus (n = 20)	Normal cyclic (n = 20)
First estrus Postpartum (days)	Current lactation	191.5 ± 13.3	60.6 ± 5.3
	Overall history by farm records	180.3 ± 21.4	55.2 ± 7.7

Table 2. Sequences of primers used for RFLP.

Gene	Sequences used to design primers	Accession no. of the sequence
LEPR exon 20	Forward- GCAACTACAGATGCTCTACTTTTGT Reverse- CAGGGAAATTTCCCTCAAGTTTCAA	AJ580801

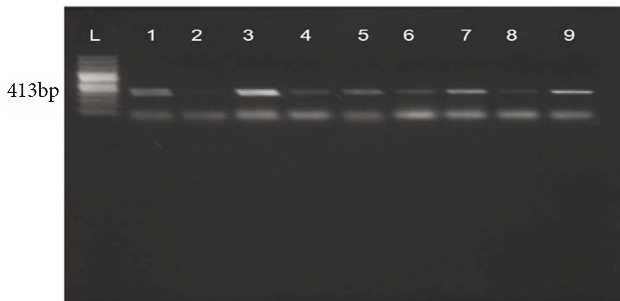

Figure 1. Amplified PCR products of partial sequences of 413-bp *LEPR* gene.
Lane L: 100-bp ladder, lanes 1–9: PCR amplified product (gel concentration: 1.5%).

3.3. Nucleotide sequencing

The sequence data showed single-nucleotide polymorphisms (SNPs) at 231 (A/G), 242 (C/A), and 347 (G/A) (Figure 2). Thus, 3 significant SNPs were identified and used for association with PPA in Murrah buffaloes.

3.4. Multiple sequence alignment of partial nucleotide sequences of *LEPR* gene

Among the species examined, variation in the *LEPR* gene of buffalo was found with goat and sheep at position 117 of the aligned sequence, where G was present in goat and sheep and A was present in buffaloes. Other polymorphisms present were not uniform or significant between buffaloes and the species listed above. Within the buffalo, a total of 3 nucleotide positions were associated with nucleotide variations across the samples (Table 3). Significant variations were found at nucleotide positions 231, 242, and 347, which were selected for further statistical analysis. No associations were found between nucleotide variations and the reproductive status of animal, i.e. PPA or cyclic.

4. Discussion

Polymorphism has been reported in the *LEPR* gene, which includes short tandem repeats (STRs) BM7225 at 101.7 cM, BMS 694 at 94.6 cM, and BMS 2145 at 93.8 cM in cattle as reported by Kappes et al. (20). One mutation, T945M, at exon 20 on position 115 as T/C was reported by Liefer et al. (19) in the *LEPR* gene of cattle (Accession no. AJ580801). In the present study, SNPs were detected at 231, 247, and 347 in the aligned sequence: nucleotide A or G at 231, C or A at 247, and G or A at 347. However, no significant association of these 3 polymorphisms was found with PPA in the buffalo population studied. No such association between *LEPR* gene polymorphism with PPA has been reported yet. However, studies of *LEPR* gene polymorphism association with other characters are available; for example, Komisarek (21) reported that daughters of bulls with the TC genotype at T945M of the *LEPR* gene had a lower age of first insemination compared to daughters of CC homozygotes in Holstein-Friesian cattle. Almedia et al. (22), in a study of 3 beef cattle breeds, observed that STR BM7225*98 was present only in the Charolais breed, and BMS 694*149 and BMS 694*153 were seen in the Brangus-Ibage breed. They further reported that these variations, along with variation at T945M, had

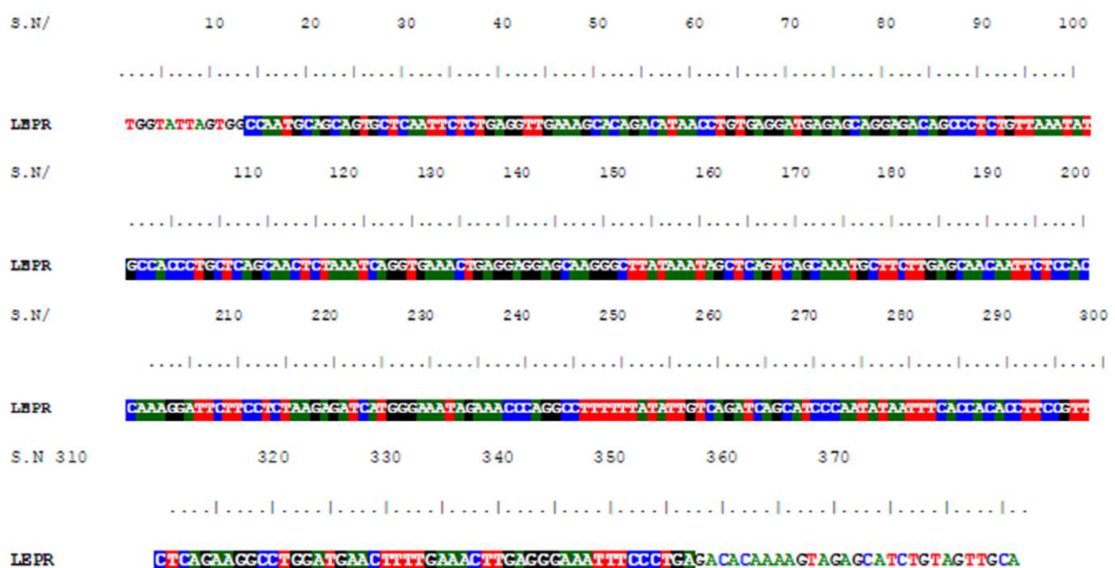

Figure 2. Sequence after alignment of partially amplified *LEPR* gene (S.N = serial no.).

Table 3. Frequency of different nucleotides studied in partial sequences of *LEPR* gene and chi-square analysis of association of nucleotide frequency with anestrus.

Position		Normal	Frequency	PPA	PPA frequency	Total	P-value
231	A	12	0.30	17	0.43	29	
	G	8	0.20	3	0.07	11	
						40	0.15
247	C	16	0.40	17	0.43	33	
	A	4	0.10	3	0.07	7	
						40	1.00
347	G	13	0.33	18	0.45	31	
	A	7	0.17	2	0.05	9	
						40	0.13

PPA = Postpartum anestrus, A = adenine, G = guanine, C = cytosine.

no association with postpartum cow milk production. Liefer et al. (19) reported that animals with genotype CC had significantly higher circulating leptin concentrations than those with the CT genotype during late pregnancy, but they found no correlation during lactation in Holstein-Friesian cows. The expression of *LEPR* varies with plane of nutrition in ruminants (16). Komisarek and Dorynek (23) reported that the TT genotype had the lowest value of milk fat and milk protein percentage across family analysis in Jersey cows.

In summary, 3 SNPs in the *LEPR* gene were found, indicating that the *LEPR* gene of buffalo is polymorphic, but these polymorphisms were not associated with PPA in buffaloes.

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