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Research Article

PCR-RFLP OF BUBALINE LEPTIN RECEPTOR (LEPR) GENE TO ESTABLISH ASSOCIATION WITH POSTPARTUM ANESTRUS

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ABSTRACT

The aim of this study was to detect polymerase chain reaction based restriction fragment length polymorphism of leptin receptor (LEPR) gene and to establish its association with postpartum anestrus (PPA) in buffaloes. Sixty (n=60) genetically unrelated Murrah buffaloes were selected and divided into two groups, 30 animals with PPA (n1) and other 30 with normal oestrous cycle (n2) after parturition. Selection and division of animals were done on the basis of farm records. Genomic DNA was extracted from peripheral blood and amplification of partial sequence of LEPR gene (413 bp) was done by PCR followed by restriction enzyme digestion of the products. Digestion of PCR product using *Alu*I and *Mbo*II enzymes have manifested monomorphic pattern of restriction fragments with "aa" and "bb" genotypes respectively. Hence, it was not possible to study association of LEPR gene with PPA in buffalo.

Key words: Anestrous, LEPR gene, polymorphism, postpartum

Buffalo contributes to India's economy in terms of milk and meat. Reduced reproductive efficiency of buffalo contributed by various factors like silent estrus, postpartum anestrus (PPA), infertility etc. leads to economic shrinkage in buffalo farming. Cut down reproductive efficiency cause by PPA is one of the major factors as compared to others (Samad *et al*., 1984). Under good management practices, buffaloes exhibit estrus within 60 days of postpartum (Balhara *et al*., 2013), but around 20-50% buffaloes still exhibit PPA condition (Kumar *et al*., 2014). Various factors like nutrients, mineral deficiencies season (Khattab *et al*., 1995), suckling (Honnapagol *et al*., 1993), parity (Devraj and Janakiraman, 1986), dystokia etc. are responsible for PPA in ruminants. These may not only be the potential causes contributing to PPA because under similar management conditions there is coexistence of normal as well as PPA animals within the same herd. Thus the involvement of genetic factor with PPA in buffaloes may be speculated.

The leptin receptor (LEPR) is a member of the cytokine I family of receptors and signal transducers and is indirectly related to PPA (Chilliard *et al*., 2005). Leptin receptors are also found on ovary, placenta and lactating mammary glands. Moreover, expression of LEPR gene also changes with nutrition (Chilliard *et al*., 2005). The LEPR regulates growth hormone release and luteinizing-hormone secretions (Kadokawa *et al*., 2006). Thus it has been hypothesised that the genetic make-up of LEPR could be a deciding factor of PPA in buffalo. The present study was designed to examine polymerase chain reaction (PCR) base restriction fragment length polymorphism (RFLP) in LEPR gene and its association with PPA in buffaloes.

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MATERIALS AND METHODS

Animal Selection and Sample Collection: Murrah buffaloes (n=60) were selected on the basis of their reproductive history available at the Animal Farms of Central Institute for Research on Buffaloes (CIRB), Hisar and Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, India. The animals were divided into two groups, PPA (n1; 30) and normal cyclic (n2; 30). According to history, the animals grouped in PPA exhibited anestrus period of more than 120 days in last three or more consecutive parturition and the normal group showed sign of estrus within 60 days of postpartum for minimum of three continuous parturition. Moreover, from the reproductive history it was ensured that animals under study were not genetically related, i.e., they were neither full sib nor half sib. The animals were maintained under standard management and feeding conditions at their respective farms. All animal experimental procedures were approved by Institutional Animal Ethical Committees of CIRB and LUVAS, Hisar. Approx. 5 ml of venous blood sample was collected centrifuge tube containing 0.25

ml of 0.5 M EDTA as an anticoagulant. The blood samples were gently mixed with anticoagulant and transported to the laboratory at 4^{0}C for immediate processing.

Isolation of DNA and Partial Amplification of LEPR Gene by PCR: From whole blood samples genomic DNA was extracted by the Phenol: Chloroform method (Sambrook and Russel, 2001). The quality and quantity of genomic DNA was accessed in Picodrop (Picodrop Ltd., Cambridge UK). Using forward 5'-GCAACTACAGATGCTCTACTTTTGT-3' and reverse 5'-CAGGGAAATTTCCCTCAAGTTTCAA-3' primers, partial amplification of exon 20 of LEPR gene (413 bp) was done. The reaction volume of 25 µL for each sample was prepared in 0.2 mL thin walled PCR tubes. The master mix contained 17.45 µL of nuclease-free water, 2.5 µL of buffer, 2.5 µL of 100 µM dNTPs, 0.20 µL (100 ng) of each primer, 0.15 µL of Taq DNA polymerase (Thermo Scientific, USA), and 2.0 µL of diluted genomic DNA (50 ng). The amplification was done in a programmed thermocycler (AB Master Cycler Gradient, Eppendorf, Germany) with initial denaturation at 95^{0}C for 3 min then 35 cycles of denaturation at 95^{0}C for 45 sec, annealing at 58^{0}C for 55 sec and extension at 72^{0}C for 1 min, followed by a final extension step at 72^{0}C for 10 min. The amplified fragments were resolved in 1.5% agarose gel containing ethidium bromide and visualized under UV transilluminator.

Sequencing of LEPR Gene and Identification of Restriction Sites: Using QIAquick gel extraction kit (QIAGEN, Germany) the PCR products were purified. The purified products were sequenced using an automated DNA sequencer from Chromous Biotech Ltd., Bangalore, India. The sequences obtained were analyzed using the NCBI BLAST online software tool (http://www.ncbi.nlm.nih.gov) and submitted to NCBI (Accession no. KM368261.1). Online software NEBcutter V2.0 (http:/nc2.neb.com/NEBcutter2) was used to predict restriction sites with respective restriction enzymes (RE) in the partial sequence of LEPR gene.

RE Digestion: On the basis of restriction pattern predicted by NEBcutter V2.0 the restriction enzymes *Alu*I and *Mbo*II (Thermo Scientific, USA) were selected randomly and used to digest the partial amplified product of LEPR gene. Total reaction volume of 20 µL was prepared in 0.2 mL tube for both the enzymes, containing 1ìl of each restriction enzyme, 2.0 µL buffer, 2 µL NFW and 15 µL of PCR product. The 20 µL reaction mix was kept for digestion in incubator at 37^{0}C overnight. Completing the digestion, heat inactivation of the enzymes was done at 65^{0}C for 20 min. The RE products were resolved by electrophoresis on 3% agarose gels containing ethidium bromide at 85 V for 1 h. The digested samples were visualized under UV transilluminator.

RESULTS AND DISCUSSION

The amplified product of LEPR gene showed a specific band of 413 bp in 1.5% agarose gel. The PCR products of 413 bp from both PPA and normal samples digested by *Alu*I showed monomorphic band pattern (aa) of 266 bp and 147 bp in 3% agarose gel (Fig. 1 A and B). Similarly, monomorphic band pattern (bb) of 227 bp and 186 bp was observed in both PPA and normal buffaloes when digested with *Mbo*II enzyme (Fig. 2 A and B). It indicated no polymorphism and association of LEPR gene with PPA in buffalo.

In this study partially amplified exon 20 of LEPR gene was subjected to RE digestion to detect RFLP and to establish its association with PPA. The RFLP pattern between PPA and normal cyclic group indicated no polymorphism and hence its association with PPA in buffaloes could not be established. The most probable reasons of monomorphism may be due to small number of animals in this study or as the investigated sequence was an exonic region which may be highly conserved.

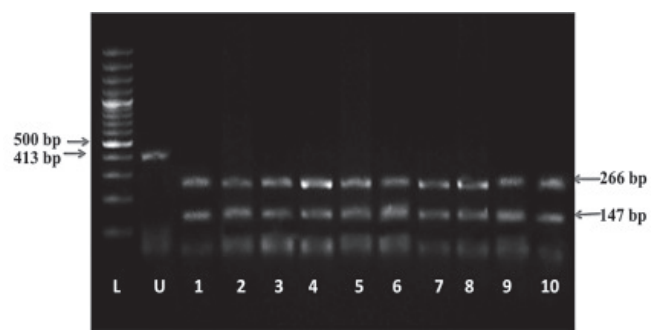

Fig 1A. Agarose gel electrophoresis (3% agarose gel) of RE digested product of partial sequences of LEPR gene with AluI in PPA animals. Lane L=100 bp DNA, Lane U=undigested PCR product ladder, Lanes 1-10=RE digested product.

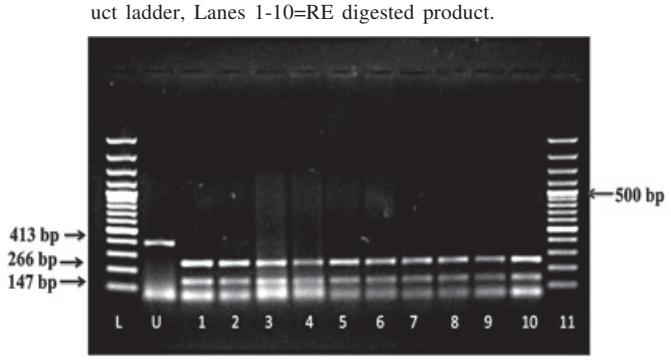

Fig 1B. Agarose gel electrophoresis (3% agarose gel) of RE digested product of partial sequences of LEPR gene with AluI in normal cyclic animals. Lanes L and 11=100 bp DNA ladder, Lane U=Undigested PCR product Lanes 1-10=RE digested product.

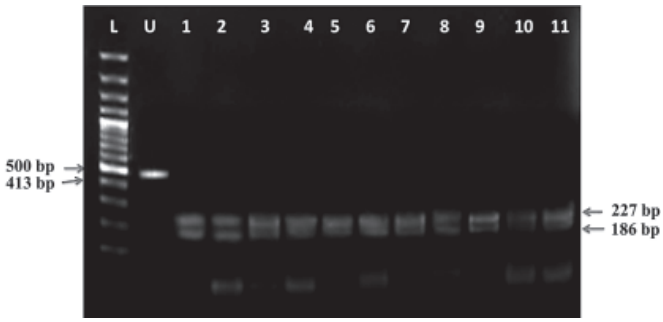

Fig 2A. Agarose gel electrophoresis (3% agarose gel) of RE digested product of partial sequences of LEPR gene with MboII in PPA animals. Lane L=100 bp DNA ladder, Lane U=Undigested PCR product Lanes 1-11=RE digested product.

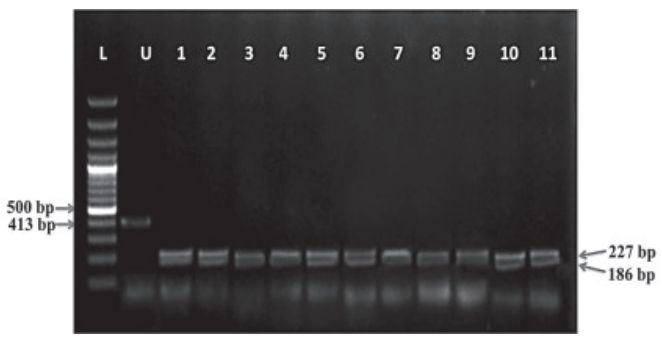

Fig 2B. Agarose gel electrophoresis (3% agarose gel) of RE digested product of partial sequences of LEPR gene with MboII in normal cyclic animals. Lane L=100 bp DNA ladder, Lane U=Undigested PCR product, Lanes 1-10=RE digested product.

Till date, there are scanty reports on polymorphism and association of LEPR gene with PPA in buffalo or other ruminants but polymorphism and its association with other traits in cattle have been well documented (Komisarek and Dorynek, 2006). It is a well-established fact that disturbance in energy balance status of ruminants leads to PPA which is controlled by leptin through its receptor on the hypothalamus, the centre of energy homeostasis (Chilliard *et al*., 2005). This indicates that there should be some relation between LEPR gene and PPA in buffalo. Hence, the LEPR gene was selected to establish the involvement of genetic factor causing PPA in buffalo. Moreover, the exon 20 was investigated because of earlier studies conducted in cattle. Although, in this study no polymorphism and association was observed but single nucleotide polymorphism has been reported in LEPR gene of buffalo but the role of these polymorphisms in PPA could not be established (Kumar *et al*., 2014). Polymorphism in LEPR gene of cattle has been reported in short tandem repeats (STR) BM7225 at 101.7 cM, BMS 694 at 94.6 cM and BMS2145 at 93.8 by Kappes *et al*. (1997). Moreover, one mutation T945M at exon 20 on position 115, as T/C is reported by Liefer *et al*. (2004) in LEPR gene of cattle. To detect polymorphism and to establish relation with PPA extensive studies considering the whole LEPR gene segment in ample population are needed. Only then, potential of LEPR gene as a marker for PPA could be established.

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