Nucleotide Sequencing and PCR-RFLP of Insulin-like Growth Factor Binding Protein-3 Gene in Riverine Buffalo (*Bubalus bubalis*)

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ABSTRACT : Insulin-like growth factor binding protein-3 (IGFBP-3) gene is a structural gene associated with the growth and development of the animals. The present investigation was carried out to unravel nucleotide sequence and polymerase chain reaction-restriction fragment polymorphism (PCR-RFLP) of IGFBP-3 gene in buffalo. Genomic DNA was isolated from a total of 157 animals belonging to Murrah, Surti, Jaffarabadi and Nagpuri breeds of Indian riverine buffalo. A 655 bp of IGFBP-3 gene was amplified in all the breeds and amplicons were digested with *Hae* III, *Taq* I and *Msp* I restriction enzymes. On digestion with *Hae* III yielded single restriction pattern of 8 fragments of sizes 201, 165, 154, 56, 36, 19, 16 and 8 bp in all the animals studied. Similarly *Taq* I and *Msp* I also revealed single restriction pattern yielding fragments of sizes 240 and 415 bp and 145 and 510 bp, respectively. This shows non-polymorphic nature of restriction sites in buffalo. Nucleotide sequencing of 587 bp of IGFBP-3 gene in Murrah buffalo was done and submitted to the GenBank (Accession No. AY304829). Nucleotide sequencing revealed an addition of 4 bases in the intronic region as compared to cattle. ***(Asian-Aust. J. Anim. Sci. 2004. Vol 17, No. 7 : 910-913)***

Key Words : Buffalo, IGFBP-3, Sequencing, PCR-RFLP, Polymorphism

INTRODUCTION

India possesses more than half of the world buffalo population and account for 55% of the country's total milk production. Indian buffaloes are hardy, thrive well on poor quality nutrition and are also good converter of inputs into milk as compared to cattle (Tomer, 1999; Biswas et al., 2003). Buffalo is also emerging as a good source of meat apart from its better milk production. However, their inherent potentials for growth and production have not been exploited due to inadequate information about genetic basis and the breeding strategies.

Insulin-like growth factor binding protein-3 (IGFBP-3) gene is a structural gene responsible for the multiple effects of insulin-like growth factors (IGFs) (Bale and Conover, 1992). IGF-I and IGF-II are a couple of hormones involved in the process of mammalian growth and regenerative processes besides having active role in mammary gland development (Hossner et al., 1997) and also has a role in muscle protein synthesis in young chickens (Kita et al., 2002).

Cattle IGFBP-3 gene has been cloned and sequenced and found to be 8.9 kb in length (Spratt et al., 1991). Polymorphic studies and nucleotide sequencing of IGFBP-3 gene have been reported in cattle (Maciulla et al., 1997; Haegeman et al., 1999; Sun et al., 2002). However, no such study has been reported in buffalo so far. Considering the importance of buffaloes in Indian context, there is a need to identify a molecular marker for growth performance of the animals. Based on above observations, present study has been undertaken to find out polymorphism and sequence the buffalo IGFBP-3 gene.

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Received October 29, 2003; Accepted March 29, 2004

MATERIALS AND METHODS

Experimental animals

This study was conducted on four breeds of riverine buffalo involving 54 animals of Murrah breed maintained at Cattle and Buffalo farm, IVRI, Izatnagar (UP); 25 of Surti breed maintained at Gujarat Agricultural University, Anand (Gujrat); 32 animals of Jaffarabadi breed maintained at cattle breeding farm, Gujarat Agricultural University, Junagarh (Gujarat) and 46 animals of Nagpuri breed from the adjoining fields of Nagpur district in Maharastra which is the breeding tract of this breed.

Collection of blood samples

About 15 ml of venous blood was collected from the jugular vein into a sterile 15 ml polypropylene centrifuge tube containing 0.5 ml of 2.7% EDTA solution as an anticoagulant. It was then brought to the laboratory in ice box and stored at 4°C till the isolation of DNA.

Isolation of genomic DNA

The genomic DNA was isolated by phenol-chloroform

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   1 tcttgtggat gtgggggtgg ggccacctgg ccctgggtat ccagagatca cagggtcacc
  61 attactcaag agcccagcag ttactccagt ggtcctgctg atgcactgag cagctgtgag
 121 cccctgctta cagaagggat attgaccctc ccctatggca gagatcccag gagaatcagt
 181 gcactgctct ccaggcctcg gctgggcaga gcagtgttct cacaaagctg gcctcttttt
 241 gttcacttgg cctctgagtg tcctggcctg tgtatccctg tcccagtcct gtagcttgcc
 301 ctggggaatc acaagagaga caggggggctg tggttggcat ctgcacagga acagtgacaa
 361 ctaaatcaga caaaagatac tcgaggagca cgtggtcagt cccctgggtg ttacagggtt
 421 ttatcagaca cagagttccc aggtaaccca tgcctccttc ccaggggccc tgccgccggg
 481 aaatggaaga cacgctgaac cacctgaagt tcctgaacat gctcagcccc aggggcatcc
 541 acattcccaa ctgcgacaag aagggcttct acaagaaaaa gcaggtg
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Figure 1. A 587 bp sequence of Buffalo IGFBP-3 gene (Accession No. AY304829).

extraction method as described by Sambrook et al. (1989). The purity of genomic DNA was assessed by spectrophotometry. Samples showing OD ratio (260 nm/280 nm) between 1.7 and 1.9 were retained for further analysis and others were reprocessed. Concentration of DNA was calculated by using OD value at 260 nm by the following formula: DNA concentration (μg/ml)= OD_{260}×dilution factor×50.

PCR amplification

A region of IGFBP-3 gene spanning over a part of exon 2, complete intron 2, exon 3 and a part of intron 3 was amplified by using forward primer P_3 (5'-CCA AGC GTG AGA CAG AAT AC -3') and reverse primer P_4 (5'-AGG AGG GAT AGG AGC AAG AT-3') (Maciulla et al., 1997). For amplification, 50 μl of PCR reaction was prepared by adding 20 pM of each primer, 50 μM of each dNTPs, 1.5 mM $MgCl_2$, 10×PCR assay buffer, 200 ng DNA template and 1 U *Taq* DNA polymerase.

The amplification was carried out using a pre-programmed thermal cycler (PTC-200, M J Research) with the following conditions: initial denaturation of 5 min at 94°C followed by 35 cycles of denaturation at 94°C, annealing at 60°C and extension at 37°C each of 1 min and finally the final extension of 5 min at 72°C. The PCR products were checked by agarose gel electrophoresis using 1.5% agarose gel in 1×TAE buffer at 6 volts/cm for one hour. The amplified product was visualized under UV transilluminator.

Restriction enzyme analysis

The amplified PCR products were digested separately with three restriction enzymes viz. *Hae* III, *Taq* I and *Msp* I. About 15 μl of PCR product was digested with 5 units of restriction enzyme using suitable 10×RE buffer. *Hae* III and *Msp* I digestion was carried out at 37°C and *Taq* I digestion at 65°C for 3 h. The RE digested PCR products were electrophoresed in agarose gel containing ethidium bromide as staining agent in 1×TAE buffer for 2-3 h at 6 volts/cm. *Hae* III digests were electrophoresed in 4% agarose and *Taq* I and *Msp* I digests were electrophoresed in 2% agarose gel. The digested products were visualized and documented under gel documentation system.

DNA sequencing

PCR products were run in 1% agarose gel and the product band was eluted using gel elution kit (GIBCO BRL) for purification. The purified PCR products were sequenced using the automated dye-terminator cycle sequencing method with Ampli *Taq* DNA polymerase in ABI PRIZM 377 DNA sequencer (Perkin-Elmner).

RESULTS AND DISCUSSION

Nucleotide sequencing of 587 bp of IGFBP-3 gene in Murrah buffalo was done and submitted to the GenBank (Accession No. AY304829) (Figure 1). This was the first report of sequencing of buffalo IGFBP-3 gene. The length of the amplified product of IGFBP-3 was 655 bp in all the breeds of buffalo studied (Figure 2). In contrast, a 651 bp of amplified product was found in cattle using the same set of primers in our laboratory (Accession No. AY306011) (Shukla, 2001). There were some insertions of nucleotides in the intronic region of buffalo IGFBP-3 gene causing an increase in the length of PCR product to 655 bp as compared to 651 bp of cattle. These insertions could be utilized as a maker for species differentiation/ identification.

The 655 bp of amplified fragment of buffalo IGFBP-3 gene comprised of last part of exon 2, complete intron 2,

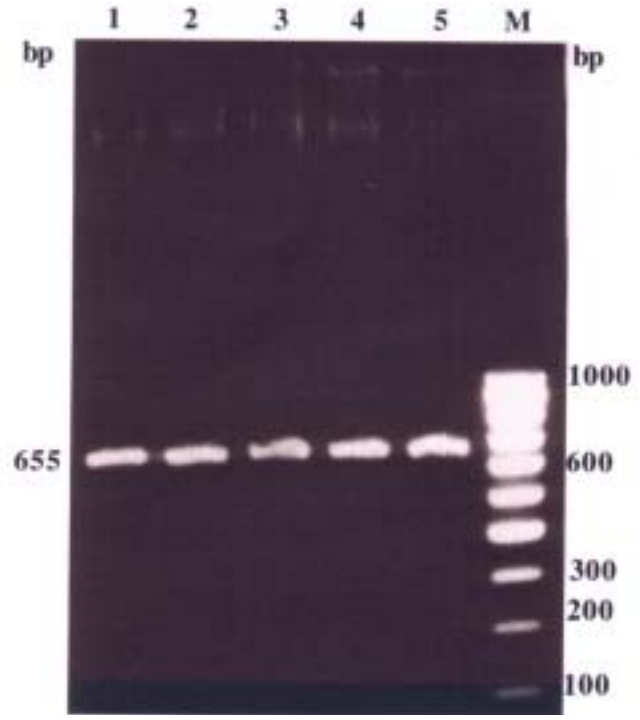

Figure 2. PCR products of buffalo IGFBP-3 gene. Lane M : Molecular size marker (100 bp DNA ladder). Lane 1-2: Murrah breed. Lane 3: Surti breed. Lane 4: Jaffarabadi breed. Lane 5: Nagpuri breed.

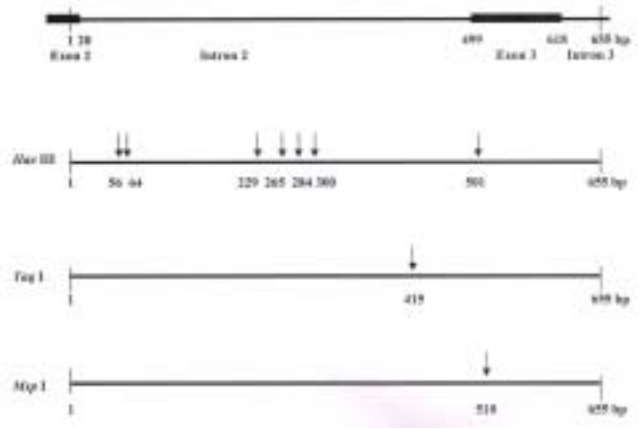

Figure 3. Diagrammatic representation of exon-intron regions and *Hae* III, *Msp* I and *Taq* I restriction sites on IGFBP-3 gene.

exon 3 and a part of intron 3 (Figure 3). The exon-intron regions were assigned on the basis of the available reports in cattle (Spratt et al., 1991). To study the polymorphism, the sequence was then analyzed for restriction sites of various enzymes. The restriction sites of *Hae* III, *Taq* I and *Msp* I enzymes in IGFBP-3 gene are presented in Figure 3. Digestion of the PCR product with *Hae* III revealed only one type of restriction pattern yielding 8 fragments of sizes 201, 165, 154, 56, 36, 19, 16 and 8 bp (Figure 4). This pattern was assigned as AA genotype. However, three genotypes were identified in exotic cattle with restriction

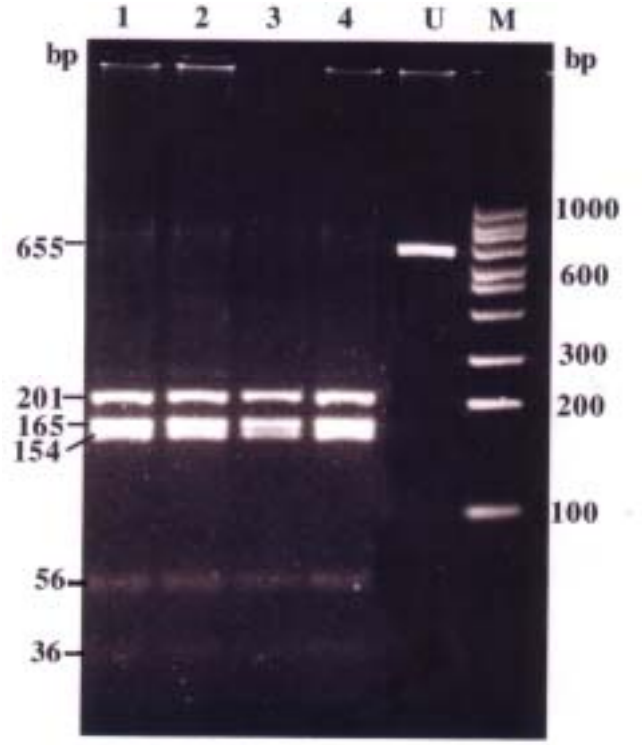

Figure 4. *Hae* III restriction pattern of buffalo IGFBP-3 gene. Lane M: Molecular size marker (100 bp DNA ladder). Lane U: Undigested PCR product. Lane 1: AA genotype (Murrah breed). Lane 2: AA genotype (Surti breed). Lane 3: AA genotype (Jaffarabadi breed). Lane 4: AA genotype (Nagpuri breed).

fragments of sizes 199, 164, 154, 56, 36, 18, 16 and 8 bp (AA genotype); 215, 164, 154, 56, 36, 18 and 8 bp (BB genotype) and 215, 199, 164, 154, 56, 36, 18, 16 and 8 bp (AB genotype) (Maciulla et al., 1997). All the buffaloes have intact *Hae* III restriction site at the base no. 266 (Figure 1) indicating absence of polymorphism at this site. However, the corresponding site in cattle showed polymorphism due to absence of this site.

Since *Hae* III showed absence of polymorphism, other enzymes i.e. *Taq* I and *Msp* I were tried. The reason behind selecting *Taq* I was its position in the intron 2 of 655 bp fragment of IGFBP-3 gene. It was assumed that *Taq* I site could show polymorphism as mutations are relatively more common in introns than exons. Though introns do not code for any protein, it may play a role in splicing of mRNA and its stability. All the animals screened showed the presence of a *Taq* I site, characterized by a single homozygous genotype possessing two fragments of sizes 415 and 240 bp (Figure 5). No homozygote possessing only 655 bp fragment or heterozygote with three fragments of sizes 655, 415 and 240 bp were found.

The *Msp* I site (C$^{\downarrow}$CGG) on 655 bp fragment is located in the exon 3. The site involves two adjacent codons, both coding for Arginine. Since four bases of the site comprise two wobble bases (1st C and 2nd G of C$^{\downarrow}$CGG) of the two

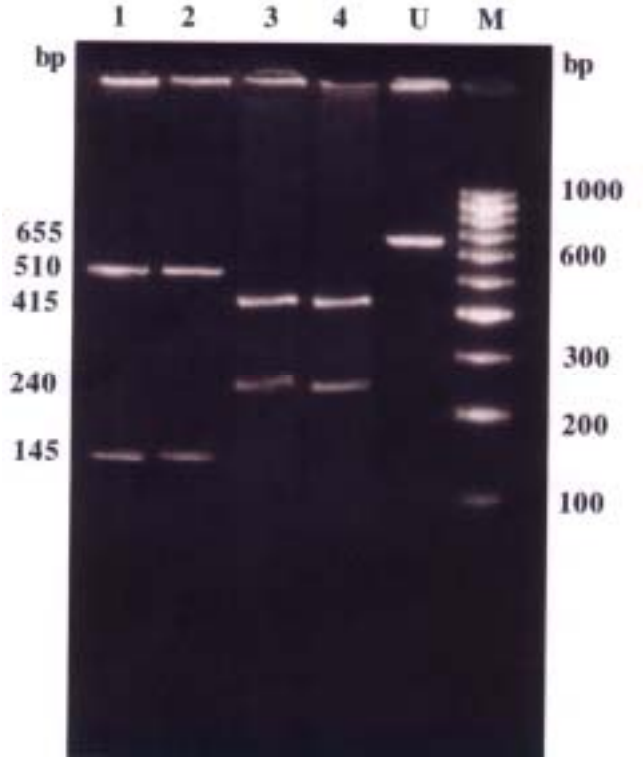

Figure 5. *Msp* I and *Taq* I restriction patterns of buffalo IGFBP-3 gene. Lane M: Molecular size marker (100 bp DNA ladder). Lane U: Undigested PCR product. Lane 1-2: AA genotype with *Msp* I. Lane 3-4: AA genotype with *Taq* I.

codons, there is a high possibility of occurrence of mutation for this site at the wobble base. So the *Msp* I enzyme was employed to check for the presence or absence of the site and thereby to determine the genotypes. All the animals revealed presence of *Msp* I site yielding two fragments of sizes 145 and 510 bp (Figure 5). Thus the *Msp* I site was also non-polymorphic as was in the case of *Hae* III and *Taq* I. The findings of present study with *Taq* I and *Msp* I could not be compared as this aspect has not been reported in any species of livestock.

In conclusion, the PCR-RFLP studies with *Hae* III, *Taq* I and *Msp* I indicated single restriction pattern in all the breeds of buffalo. The nucleotide sequencing revealed an addition of four bases in the intronic region of buffalo IGFBP-3 gene as compared to cattle. These findings may be utilized as a marker for species differentiation/ identification.

REFERENCES

Bale, L. K. and C. A. Conover. 1992. Regulation of Insulin-like growth factor binding protein-3 messanger ribonucleic acid expression by insulin-like growth factor I. Endocrinol. 131:608-614.

Biswas, T. K., T. K. Bhattacharya, A. D. Narayan, S. Badola, Pushpendra Kumar and A. Sharma. 2003. Growth Hormone gene polymorphism and its effect on birth weight in cattle and buffalo. Asian-Aust. J. Anim. Sci. 16:494-497.

Haegeman, A., A. Van-Zeveren and L. J. Peelman. 1999. A new mutation in the bovine insulin-like growth factor binding protein-3. Anim. Genet. 18:213-222.

Hossner, K. L., R. H. McCusker and M. V. Dodson. 1997. Insulin-like growth factors and their binding proteins in domestic animals. Anim. Sci. 64:1-15.

Kita, K., T. Shibata, M. Aman Yaman, K. Nago and J. Okumura. 2002. Response of muscle protein synthesis to the infection of insulin-like growth factor-I and fasting in young chickens. Asian-Aust. J. Anim. Sci. 15:1760-1764.

Maciulla, J. H., H. M. Zang and S. K. DeNise. 1997. A novel polymorphism in the bovine insulin–like growth factor binding protein-3 (IGFBP-3) gene. Anim. Genet. 28:375.

Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. Molecular cloning: A laboratory manual. Ed 2nd, Vol 3. Cold Spring Harbour Laboratory Press. New York.

Shukla, A. 2001. PCR-RFLP studies on insulin-like growth factor binding protein 3 (IGFBP-3) gene in cattle. M.V.Sc. thesis submitted to the Indian Veterinary Research Institute (Deemed University), Izatnagar, Bareilly, U.P., India.

Spratt, S. K., G. P. Tatsuno and A. Sommer. 1991. Cloning and characterization of bovine insulin like growth factor binding protein-3 (IGFBP-3) gene. Biochem. Biophys. Res. Commun. 177:1025-1032.

Sun, W. B., H. Chen, C. Z. Lei, L. S. Zan, X. Q. Lei, R. B. Li, H. Chen and S. M. Geng. 2002. Polymorphism of insulin-like growth factor binding protein-3 gene and its relationship with beef performance of Qinchuan cattle. Anim. Biotech. Bulletin 8:95-99.

Tomer, O. S. 1999. National seminar on “Challenges-Dairying in the next millennium”, Karnal, India. Indian Dairyman, 51:9-14.