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Short Communication

Sequence characterization of coding regions of the myostatin gene (*GDF8*) from Brazilian Murrah buffaloes (*Bubalus bubalis*) and comparison with the *Bos taurus* sequence

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Abstract

Within about 30 years the Brazilian buffalo (*Bubalus bubalis*) herd will reach approximately 50 million head as a result of the great adaptive capacity of these animals to tropical climates, together with the good productive and reproductive potential which make these animals an important animal protein source for poor and developing countries. The myostatin gene (GDF8) is important in the physiology of stock animals because its product produces a direct effect on muscle development and consequently also on meat production. The myostatin sequence is known in several mammalian species and shows a high degree of amino acid sequence conservation, although the presence of non-silent and silent changes in the coding sequences and several alterations in the introns and untranslated regions have been identified. The objective of our work was to characterize the myostatin coding regions of *B. bubalis* (Murrah breed) and to compare them with the *Bos taurus* regions looking for variations in nucleotide and protein sequences. In this way, we were able to identify 12 variations at DNA level and five alterations on the presumed myostatin protein sequence as compared to non double-muscled bovine sequences.

Key words: buffalo, *GDF8*, myostatin.

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The water buffalo (*Bubalus bubalis*) is not only an important draft animal but is also a major meat and/or milk producer but has been underutilized as compared to other stock animals used in husbandry (Barker *et al*., 1997). Within about 30 years the Brazilian buffalo herd will reach approximately 50 million head, corresponding to the highest growth rate among all Brazilian farm animals. This growth is due to the fact that these animals are highly adaptable to tropical climates and are resistant to ecto- and endoparasites and show excellent nutritional efficiency combined with good productive and reproductive potential: characteristics that render these animals an interesting alternative protein source especially for poor or developing countries.

The role of protein factors in the development of muscle mass in cattle has been best elucidated by the study of the transforming growth factor-beta (TGF-β) superfamily of genes, which encode important factors for the regulation of embryo development and tissue homeostasis in adult animals. Within this family, growth differentiation factor 8 (GDF8) encodes the myostatin protein that is expressed during muscle development and in adult skeletal muscle (McPherron and Lee, 1997). Myostatin protein is synthesized in skeletal muscle as a 375-amino acid propeptide, which is proteolytically processed at the RSRR (amino acids 263-266: Arg-Ser-Arg-Arg) site to give rise to a 26-kDa active processed peptide. This processed mature peptide binds to a receptor to elicit biological function (Thomas *et al*., 2000).

Variation in the GDF8 gene, first discovered in mice (McPherron *et al*., 1997) and then identified in cattle (Grobet *et al*., 1997), has been shown to explain large muscle phenotypic differences between several breeds (Grobet *et al*., 1998). The GDF8 gene has been extensively investigated in bovines and a large number of alleles have been

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identified (Grobet *et al*., 1997; Kambadur *et al*., 1997; Karim *et al*., 2000). Several mutations in both the second and third exons strongly affect the phenotype and were described by (Grobet *et al*., 1998) as responsible for muscle hypertrophy (double-muscled) and have been responsible worldwide for increased meat production in several breeds.

Alignment of the myostatin protein sequence from several vertebrates (baboons, bovines, chickens, humans, mice, ovines, porcines, rats, turkeys and zebrafish) has shown a high degree of conservation among species, especially in the C-terminal region (McPherron and Lee, 1997). However, the presence of no-silent and silent changes in the coding sequences and several variations in the introns and untranslated regions have been reported (McPherron and Lee, 1997; Stratil and Kopecny, 1999).

Despite its important role in the control of muscle development the buffalo myostatin gene has never been characterized, and the objective of the work described in this paper was to characterize the myostatin coding regions from *Bubalus bubalis* and compare them to non double-muscled *Bos taurus* in an effort to locate alterations in nucleotide and protein sequences.

Whole blood samples were obtained from eight adult Murrah breed buffaloes. The blood samples were collected in Vacutainer tubes (Vacuum II, Itupeva, SP, Brasil) containing 7.5 mg of EDTA, homogenized and kept frozen on ice until needed for DNA extraction, which was performed using the Genomic Prep Blood DNA Isolation kit (Amersham Bioscience, Piscataway, NJ, USA) and 300 μL of whole blood.

After quantification and dilution of the DNA samples, the regions corresponding to the three exons of the putative buffalo GDF8 gene were amplified by PCR using primer pairs designed from *Bos taurus* DNA sequences (Table 1) and a *B. taurus* DNA sample as amplification control. Each 25 μL reaction contained 50 ng of sample DNA, 0.4 μM of each primer, 1X PCR buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP and 1 U of Platinum *Taq* DNA polymerase (Invitrogen, São Paulo, SP, Brazil). Amplification reactions were carried out in a PTC100 thermocycler (MJ Research, Inc., Watertown, MA, USA), with 4 min denaturation at 94 °C, 35 cycles of 94 °C for 1 min, 54 °C for 1 min and a 72 °C extension for 1 min, and a final extension at 72 °C for 4 min.

Sequencing was performed in a final volume of 10 μL containing 50 ng of the PCR product, 2 μL of Big Dye™ Terminator (Applied Biosystems, Foster City, CA, USA), 1X sequencing buffer (400 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0) and 6 pmol of primer. Each PCR product was sequenced using the 3' and 5' primers. Reaction parameters were kept at 94 °C for 1 min, and 25 cycles of 94 °C for 10 s, 55 °C for 10 s, and 60 °C for 4 min. Sequencing was performed with an ABI 3100 automated sequencer (Applied Biosystems). The resulting sequences were aligned using the BioEdit program (BioEdit v5.0.9) and the obtained consensus sequences were used to compare it with GenBank bovine sequences using the BLAST algorithm (Altschul *et al*., 1990). The nucleotide sequences of exons 1, 2 and 3 of the GDF8 gene were deposited in GenBank under the accession numbers AY254098, AY363177 and AY363178 respectively.

Amplification of DNA yielded fragments of about 620, 550 and 500 bp for exons 1, 2 and 3, respectively, very similar to those observed for the *B. taurus* DNA control. Sequencing of these amplified products and subsequent alignment of the sequences with the *B. taurus* sequence (AF320998) permitted a detailed comparative analysis of the buffalo DNA coding region and the presumed protein sequence. Nucleotide changes were observed in all three exons of the gene, with the alterations only comprising point mutations but no insertions or deletions. The 1128-bp coding region showed 98.94% similarity and the presumed amino acid sequence showed 98.67% identity to the bovine protein.

The eight buffaloes sequenced did not show polymorphisms among themselves but 12 nucleotide variations were found when the buffalo myostatin gene was compared with the bovine myostatin gene. Exon 1 showed three alterations, the first was an A → G transition located at position 301 from the start codon and was responsible for the substitution of serine with glycine at position 101 of the protein chain. The second substitution was an A → C transversion at position 348 of the nucleotide sequence leading to a glutamate being replaced by aspartame at position 116 in the protein sequence. The third substitution was an A → G transition at position 349 which resulted in threonine being replaced by alanine at position 117 in the buffalo presumptive myostatin sequence. Exon 2 revealed the presence of

Table 1 - Sequence of the forward (F) and reverse (R) primers used for the amplification and sequencing of buffalo myostatin exons.

Exon	Primers (5' to 3')	Reference
1	F - ATTCACTGGTGTGGCAAGTTGTCTCTCAGA R - CCCTCCTCCTTACATACAAGCCAGCAG	Grobet *et al*., 1998
2	F - GTTCATAGATTGATATGGAGGTGTTCG R - ATAAGCACAGGAAACTGGTAGTTATT	Grobet *et al*., 1998
3	F - TGAGGTAGGAGAGTGTTTTGGG R - TCGAAATTGAGGGGAAGACC	Kambadur *et al*., 1997

five alterations, which included four silent transitions (T414C, G483A, G639A and C702T) and one A → C transversion at position 421 responsible for a Lys141Gln substitution in the amino acid sequence. Four changes were observed in exon 3, three being silent transitions (A822G, T1020C and C1083T) and one (A824G) leading to a His275Arg substitution in the C-terminal region of the protein. All alterations in the presumed myostatin amino acids sequence as compared to bovine sequence are shown in Figure 1.

The observation of amplified fragments of the expected sizes and subsequent analysis of the obtained sequences confirmed the amplification of the regions of interest, demonstrating the complete transferability of the primer pairs developed for *B. taurus* to *B. bubalis*. This is important in terms of reducing the costs of genome analysis of closely related species, as has been widely demonstrated for a large number of organisms, especially plants (Katzir *et al*., 1996; Lübberstedt *et al*., 1998).

Myostatin shares several characteristics with the TGF-β superfamily, *e.g.* a sequence of hydrophobic amino acids close to the N-terminus that functions as a secretion signal, an RSRR sequence-containing a proteolytic processing site and a characteristic pattern of cysteine residues in the C-terminal region (Jeanplong *et al*., 2001). Mutations that disrupt this bioactive region or the structure of the cysteine knot can lead to functional knockouts, two copies of dysfunctional myostatin leading to the extreme double-muscled phenotype.

Our result shows a high degree of conservation in buffalo myostatin compared to the bovine protein, especially in the C-terminal region. The similarity and identity between the nucleotide and amino acid sequences from *B. taurus* and *B. bubalis* were, as expected, high due to the close proximity of the species which belong to the family Bovidae.

Of the five alterations observed, only the His275Arg modification, localized at the protein C-terminal region, could possibly represent a biological effect. This is due to the fact that the other alterations were localized before the proteolytic site and are excluded from the active protein.

Using the program Loopp v 3.0, (http://ser-loopp.tc.cornell.edu/loopp.html), a fold recognition program based on the collection of numerous signals, merging them into a single score, and generating atomic coordinates based on an alignment into a homologue template structure it was possible to create 9 high confidence 3D models of the GDF8 protein based on proteins such as the 'cystine-knot cytokines'. The models with the best score were based on the structure of PDB (Protein Data Bank): 2TGI e 1ES7. A visual analysis of the generated models indicated that the alteration of His275Arg could be in the ligation region with the myostatin receptor and this implies important consequences considering that histidine and arginine represent basic amino acids. Despite this observation, in principle this alteration seems not to affect the activity of myostatin as suggested by the results of some studies comparing carcasses of *Bos* and *Bubalus* specimens (Moran and Wood, 1986; Gazzeta, 1993; Müller *et al*., 1994; Lourenço Jr. *et al*., 1997; Rodrigues *et al*., 2001). One hypothesis to explain this could be that the alteration in the active protein could be correlated with an alteration in the receptor as a compensatory mechanism.

In summary, in this paper we report the first characterization of the myostatin coding regions from buffaloes and the identification of 12 nucleotide changes and five al-

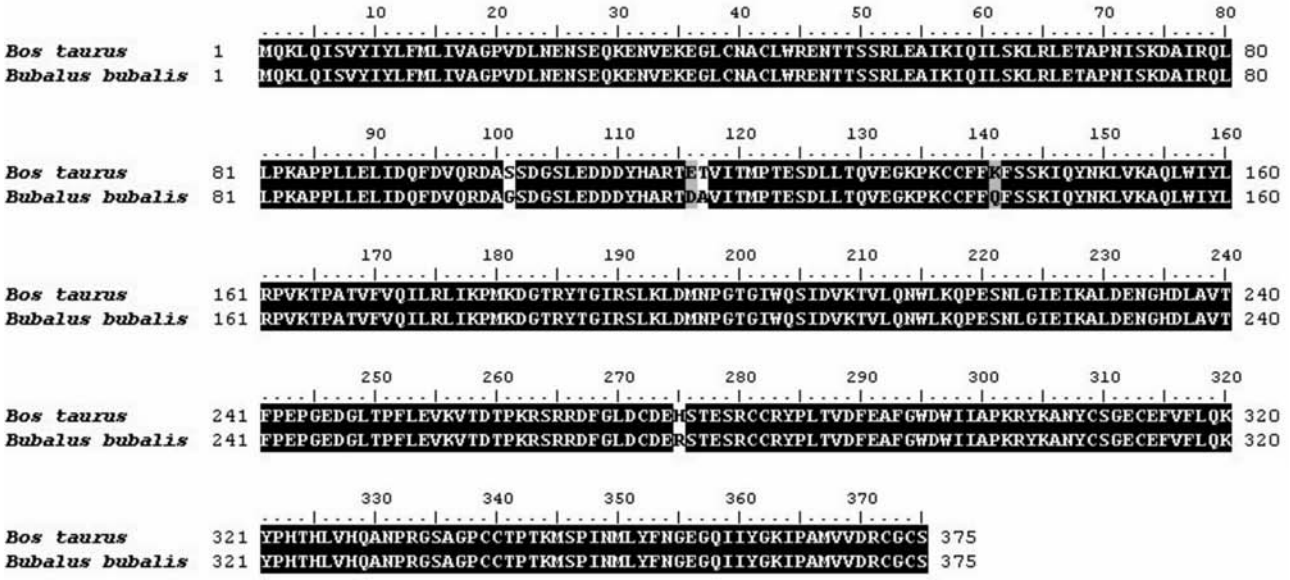

Figure 1 - Comparative alignment of the presumed myostatin amino acid sequence from non double-muscled *Bos taurus* (AF320998) and *Bubalus bubalis*. Shaded amino acids indicate matching sequence consensus.

terations in the presumed myostatin protein sequence as compared to non double-muscled bovine sequences.

References

Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403-410.

Barker JSF, Tan SG, Selvaraj OS and Mukherjee TK (1997) Genetic variation within and relationships among populations of Asian water buffalo (*Bubalus bubalis*). Anim Genet 28:1-13.

Gazzetta MCRR (1993) Avaliação das carcaças de búfalos *Bubalus bubalis e* bovinos Nelore *Bos indicus*, terminados em confinamento. MSc Thesis, Universidade Estadual Paulista 'Júlio de Mesquita Filho, Jaboticabal.

Grobet L, Royo Martin LJ, Poncelet D, Pirottin D, Brouwers B, Riquet J, Schoeberlein A, Dunner S, Ménissier F, Massabanda J, Fries R, Hanset R and Georges M (1997) A deletion in the myostatin gene causes double-muscling in cattle. Nat Genet 17:71-74.

Grobet L, Poncelet D, Royo Martin LJ, Brouwers B, Pirottin D, Michaux C, Ménissier F, Zanotti M, Dunner S and Georges M (1998) Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle. Mamm Genom 9:210-213.

Jeanplong F, Sharma M, Somers WG, Bass JJ and Kambadur R (2001) Genomic organization and neonatal expression of the bovine myostatin gene. Mol Cell Biochem 220:31-37.

Kambadur R, Sharma M, Smith TPL and Bass JJ (1997) Mutations in myostatin (GDF-8) in double muscled Belgian Blue and Piedmontese cattle. Genom Res 7:910-916.

Karim L, Coppieters W, Grobet L, Valentini A and Georges M (2000) Convenient genotyping of six myostatin mutations causing double-muscling in cattle using a multiplex oligonucleotide ligation assay. Anim Genet 31:396-399.

Katzir N, Danin-Poleg Y, Tzuri G, Karchi Z, Lavi U and Cregan PB (1996) Length polymorphism and homologies of microsatellites in several Cucurbitaceae species. Theor Appl Genet 93:1282-1290.

Lourenço Jr. JB, Sá TDA, Simão Neto M, Camarão AP and Lourenço AV (1997) Environmental effects on cattle and buffaloes in cultivated pasture of Marajó Island, Brazil. Proceedings of 5th World Buffalo Congress, Caserta, Italy, pp 424-427.

Lübberstedt T, Dussle C and Melchinger AE (1998) Application of microsatellites from maize to teosinte and other relatives of maize. Plant Breed 117:447-450.

McPherron AC and Lee SJ (1997) Double muscling in cattle due to mutations in the myostatin gene. Proc Natl Acad Sci 94:12457-12461.

McPherron AC, Lawler, AM and Lee SJ (1997) Regulation of skeletal muscle mass in mice by a new TGF-superfamily member. Nature 387:83-90.

Müller L, Aguirre LF, Restle J and Perobelli Z (1994) Carcass and meat quality of cattle and buffalo. Proceedings of 4th World Buffalo Congress, São Paulo, Brasil, pp 130-132.

Moran JB and Wood JT (1986) Comparative performance of five genotypes of Indonesian large ruminants. III. Growth and development of carcass tissues (cattle breeds; buffaloes). Aust J Agric Res 37:435-447.

Rodrigues VC, Andrade IF, Sousa JCD, Neto AI and Rodrigues VN (2001) Avaliação da composição corporal de bubalinos e bovinos através do ultra-som. Ciênc Agrotec 25:1174-1184.

Stratil A and Kopecny M (1999) Genomic organization, sequence and polymorphism of the porcine myostatine (GDF8; MSTN) gene. Anim Genet 30:462-470.

Thomas M, Langley B, Berry C, Sharma M, Kirk S, Bass J and Kambadur R (2000) Myostatin, a negative regulator of muscle growth, functions by inhibiting myoblast proliferation. J Biol Chem 275:40235-40243.

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