

Sequencing and Analysis of the Myostatin Gene (GDF-8) in *Bubalus bubalis* Young Animals to Determine the Existence of Possible Mutations Expressed in Double Musculature Phenotype

María Angélica Reyes Páez¹, Natalia Johana Rojas Tovar¹, Geovanny Mendoza-Sánchez¹, Marcela Fernández Muñoz², Boris Julián Sepúlveda Molina³, Andrés Felipe Santander Torres¹, Jesús Alfredo Berdugo Gutiérrez^{4,*} and Jhon Didier Ruiz Buitrago⁵

¹Programa de Medicina Veterinaria, Facultad de Ciencias Agropecuarias, Universidad de la Salle, Bogotá Colombia

²Grupo de Investigación REMEAT Reproducción y Mejoramiento en Animales Tropicales Programa de Medicina Veterinaria, Facultad de Ciencias Agropecuarias, Universidad de la Salle, Bogotá Colombia

³Biotecnología y Genética S.A (Biotecgen S.A)

⁴BIOGEM Facultad de Ciencias Agrarias, Universidad Nacional de Colombia, Sede Medellín Colombia

⁵Grupo de Investigación INCA-CES. Facultad de Medicina Veterinaria y Zootecnia de la Universidad CES. Medellín Colombia

Abstract: Since the 19th century, the presentation of bovines with disproportionate muscle development have been associated with mutations that inhibit the action of the myostatin gene, it is referred to as double muscle mutation, which is common in some European *Bos taurus* breeds but it is not reported in buffaloes *Bubalus bubalis*. This study aims to evaluate if the phenotype observed in 6 young buffaloes with disproportionate muscle development has the same myostatin mutation reported in cattle. DNA was obtained from the blood of the animals of the Murrah breed. First, second and third exon was amplified end point PCR; the fragments were sequenced using capillary electrophoresis. Holstein cattle (*Bos taurus*) was used as control for normal phenotype. The results obtained from the comparison of the sequence of the myostatin gene show that the observed double-muscled phenotype did not show differences from normal controls. Interspecific variation was demonstrated by comparing exons two and three of the gene, finding 12 variations between the *Bos taurus* and *Bubalus bubalis* species in the evaluated fragments. It is necessary to study physiology, and the animals to explain the phenotype observed in buffaloes.

Keywords: Myostatin, mutation, double muscle, muscle hyperplasia, *Bubalus bubalis*.

INTRODUCTION

The occurrence of hereditary defects in cattle is estimated worldwide from 0.2% to 3%, and their knowledge depends on the frequency they are studied and described [1]. Congenital anomalies in bovines usually cause abortion or neonatal death leading to considerable reproductive losses [2].

Congenital defects in buffaloes have been diagnosed in Brazil; sometimes this diagnosis is based only on the similarity with diseases already described in cattle, lacking epidemiological, pathological, and molecular studies that identify the defective agent or gene. Besides, diagnostics considering clinical signs only at the moment when the animal is examined, without the accompaniment throughout its development and use techniques that prove the definitive diagnosis.

Excessive muscle development is a hereditary syndrome of several species of mammals caused by the alteration of the expression of a single autosomal gene and its regulatory elements [3]. In cattle, this syndrome is named double musculature (DM), which was described for the first time in 1807 by Culley [4] in the Shorthorn cow breed. After this first report, double musculature syndrome has been reported in many European breeds such as Belgian Blue, Piemontese, Marchigiana, Asturian Valley, Charolais, Limousine, Maine Anjou [5]. Animals with the syndrome are characterized by a 20% increase in muscle mass, due to generalized skeletal muscle hyperplasia [6], other authors have been described it as muscle hypertrophy [7].

The observed phenotype is caused by a mutation in the myostatin gene, also named growth differentiation factor-8 (GDF8), belonging to the family of β -transforming factor (*TFG- β*), localized at the bovine chromosome 2 (BTA2) [8]. Myostatin acts as a negative extracellular regulator. It is essential for the proper regulation of skeletal muscle mass [7,9]; it has been demonstrated that inactive myostatin is

*Address correspondence to this author at the BIOGEM, Facultad de Ciencias Agrarias, Universidad Nacional de Colombia, Sede Medellín, Carrera 65 No 59a 110. BI 50 of 231 Medellín, Colombia; Tel: 57 5898335; E-mail: jaberduog@unal.edu.co

responsible for the exaggerated muscle growth characterizes this syndrome [10]. Animals that present this condition are characterized by an increased body musculature, decreased subcutaneous and intramuscular adipose tissue, and smaller organ size [11], which are usually associated with other problems such as decrease of fertility, dystocia, susceptibility to stress, and low viability of the calves [3,12].

The animals that have the mutation have meat with some desirable characteristics: leaner meats, reduced lipid content, less bone, and greater water retention at the muscular level, which results in more tender meat [12]. On the other hand, regarding the effect of the mutation on the meat production industry, there are controversies, especially because of the effect over the animal production system of the problems mentioned above.

In Colombia, in the last five years, there has been information from some breeders that belongs to the Colombian Buffalo Breeders Association (ACB) related to the presentation of DM in buffaloes (*Bubalus bubalis*) based on the phenotype of the animals. However, there are no reports that study this phenotype and also its possible genetic origin. This study aims to analyze if the observed phenotype in young buffaloes corresponds to the same mutation described for DM in cattle, and it is true to analyze the pedigree of the animals looking for carriers of the mutation.

MATERIAL AND METHODS

Population and Sample Collection

Six buffaloes of (*Bubalus bubalis*) from the Murrah breed and their crosses with 6 to 15 months, were included according to their phenotypic characteristics: animals compatible with double musculature, mild hypertrophy who presented muscle tremors associated with hypermuscularity, and normal animals as control (Figure 1). An animal of the Holstein breed of the *Bos taurus* specie was also selected as a control for the amplification of the Myostatin gene. 4 mL of blood was taken directly from the coccygeal vein in an EDTA anticoagulated tube (Vacutainer Becton Dickinson, USA), homogenized and kept refrigerated (5°C) [13] and sent to the University of La Salle, molecular biology laboratory for processing.

DNA Processing and Sequencing

All procedures were performed at the molecular genetics' laboratory of the Universidad de la Salle,



Figure 1: Different phenotypes of the Animals used in this study. A. DM phenotype. Mild DM phenotype. C. Normal buffaloes.

Bogotá, Colombia. DNA was extracted using DNeasy Blood and Tissue Kit (QIAGEN Corporation, USA) following manufacturer instructions, and the obtained DNA was kept at -20°C until PCR was performed. First, second and third exon of the myostatin gene was amplified using the primers designed for *Bos Taurus* previously described by [13], with minor modifications. For the amplification the kit GoTaq® Green Master Mix (Promega, Corporation; USA) was used, in a C1000 thermocycler (BIO-RAD, CA, USA, with the following conditions: 4 minutes of denaturalization, and 35 cycles of 1 minute 94°C, 54°C one minute, an extension of 1 minute at 72°C and a final extension of 4 minutes at 72°C [13]. PCR amplification products were electrophoresed in a 2% agarose gel. PCR products were sequenced by capillary electrophoresis using ABI3730XI (Applied Biosystem) at Macrogen, Corea. The sequences of each one of the exons 1 (AY854495.1), 2 (AY854496.1) and 3 (AY854497.1) of the individuals were compared with the *Bos taurus* and *Bubalus bubalis* whole gene sequence (AF320998), and (DQ091762.1) respectively. Alignment were performed using BioEdit Sequence Alignment Editor y Molecular Evolutionary Genetics Analysis (MEGA Version 6).

Approved by the ethics committee of the School of Veterinary Medicine of La Salle University on 8th, October 2013.

Table 1: Primer Sequences and PCR Conditions for Xon Amplification

Exon	Primer	Fusion temperature	Average	Annealing Temperature (Ta)	Final Tm
1	ATCACTGGTGTGGCAAGTTGTCTCTCAGA	61,6	62,2	67,2	67
	CCCTCCTCCTTACATACAAGCCAGCAG	62,8			
2	GTTTCATAGATTGATATGGAGGTGTTTCG	56,7	54,95	59,95	62
	ATAAGCACAGGAACTGGTAGTTATT	53,2			
3	TGAGGTAGGAGAGTGTTTTGGG	54,8	53,3	58,3	54
	TCGAAATTGAGGGGAAGACC	51,8			

RESULTS

Although the amplification protocol of the study of Mota *et al.* was applied [13], the fusion temperature used in this only allowed the correct amplification of exon 3, due to this, this temperature was modified for exons 1 and 2 for achieving the correct amplification Table 1.

All the obtained fragments have the expected size exon 1 620pb, exon 2 550pb, and exon 3 500pb. Sequencing analysis of the exon 1 was avoided due to technical reasons Figures 2, 3.

Figure 2, 3 of exon 1. Lanes: 1. DNA ladder 100 – 1000 bp; 2. Negative Control (NE1); 3. Affected animal double muscling (aE1); 4. Affected animal mild hypertrophy (bE1); 5. Affected animal, mild hypertrophy (eE1); 6. Suspicious Animal of muscular hypertrophy (fE1); 7. Normal animal (kE1); 8. Normal animal; 9Positive control normal *Bos taurus* animal (mE1).



Figure 2: PCR Products from Exon 1 of the myostatin gene.

It was found that there is no variation between nucleotides in the exon 2 and exon 3 in all animals

studied, as consequence, there is no mutation described in cattle in the analyzed fragments. A total of 8 nucleotide sequences with a total of 476 positions were included, where 100% equality was found among all the analyzed buffaloes.

Based on the reported sequence of myostatin gene from GenBank (DQ091762.1), after the comparison of the exon 2 and exon 3 sequences, 5 variations were found for the exon 2 (C2306T, C2313A, G2375G, A2531G y T2594C) and seven for exon 3 (G4748A, G4750A, C4946T, T5009C, A5056G, T5058C y A5081C).

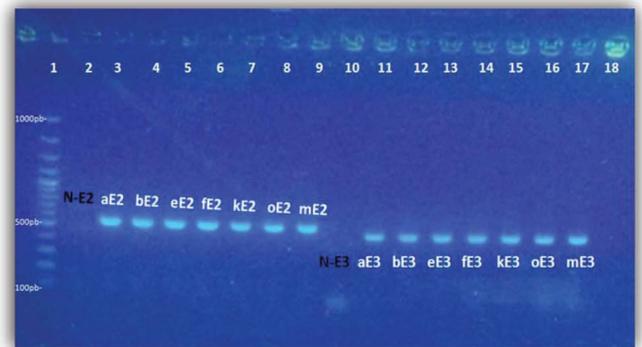


Figure 3: PCR Products from Exon 2 and Exon 3 of the myostatin gene. Seven samples from exon 2. Lanes: 1. DNA ladder 100 – 1000 bp; 2. Negative Control exón 2 (NE2); 3. Double muscling animal (aE2); 4. Double muscling animal (bE2); 5. Affected animal mild hypertrophy (eE2); 6. Affected animal mild hypertrophy (fE2); 7. Normal phenotype (kE2); 8. Normal phenotype (oE2); 9. Positive control normal *Bos taurus* animal (mE2); 10. Negative Control (NE3); 11. Double muscling animal (aE3); 12. Double muscling animal (bE3); 13. Affected animal mild hypertrophy (eE3); 14. Affected animal mild hypertrophy (fE3); 15. Affected animal mild hypertrophy (kE3); 16. Normal phenotype (oE3); 17. *Bos taurus* animal (mE3).

DISCUSSION

Marco-Longo *et al.*, studied the presentation of congenital abnormalities from 106 buffaloes in the Rio Grande do Sul region in Brazil from 1978 to 2009 and report that only two cases observed, were diagnosed

has hereditary myotonia representing the 25 % of the abnormalities using histopathological analysis [14].

The analysis if any mutations in the myostatin gene explain the observed muscle hypertrophy compatible with double musculature, in six buffaloes (*Bubalus bubalis*) comparing the sequences obtained in exons 2 and 3 with the current information available in the literature was unsuccessful.

The fusion temperatures for exons 1 and 2 were standardized; therefore, all the exons were successfully amplified (Table 1). At the sequencing level, it was not possible to analyze the sequence of exon 1 due to the presence of overlapping peaks in the Electropherogram. As Grobet *et al.*, reported [10] the mutations that directly affect the expression of the myostatin gene are not found in this region, amplification of exon 1 is avoided in this study. The observed sequences of the exon 2 and exon 3 show no differences with the compared *Bos taurus* and *Bubalus bubalis* normal myostatin gene reference sequences. Consequently, no one of the animals with the observed phenotype has the mutation reported for double musculature in cattle.

Few data exist regarding this phenotype in buffaloes, in 2003, Mota *et al.* [15] reported two variations in the exon 3 (A940G y A942G), which lead to a substitution of histidine for arginine in the amino acid sequence at the C-terminal level of the protein, proposing the hypothesis that the mutation in the bioactive region of the gene could generate a decrease in the negative control of myostatin gene causing differentiated muscular development, as observed for other bovine breeds such as the Belgian Blue White and Piedmontese [6, 16, 17]. Later on, same researchers evaluating eight buffaloes with similar phenotypes but different breeds (Bhadawuari, Murrah y Jaffabaradi) don't found any difference in exon 2 and 3, as in our study.

One possible explanation from the results obtained herein is that the observed phenotype is not double muscling, but the congenital myotonia of the buffalo, previously known as congenital buffalo hyperplasia [18, 19], which is an autosomal recessive disease, reported in Murrah breed [19, 20], this could not be confirmed in this study. The clinical signs that characterize hereditary myotonia are muscle contractions in the body in general, which occur when animals are stimulated to come out of the resting state. The affected buffaloes have stiffness, slow movement, and effort; however, the stiffness is less visible after

exercise, which indicates a warming phenomenon. The lesions are characterized by increasing the semitendinosus, semi-membrane muscle and skin area, making the adipose tissue smaller and thinner [18]. These characteristics, together with the muscle tremors, were observed in the animals of this study.

This condition is attributed to a mutation in the chloride channel gene (CLCN1), which causes a decrease in chloride conductance at the sarcolemma level, causing hyperexcitability of the muscle membrane, delaying muscle relaxation after contraction [20].

The myostatin gene is conserved between species, it has 100% of identity at the C-terminal region between mammals, as Mc Pherron and Lee demonstrated [17] in mice, rats, humans, pigs and cattle, suggesting that the high degree of conservation of the sequences of these animals leads to conserving the function of the gene. To date, in all vertebrates, it has been described that the genomic organization of the myostatin gene has three exons of similar size separated by two introns and whose size is conserved in birds and mammals. As confirmed by Tanta *et al.* [21]. comparing the size of the exons as well as the introns of the *Bos Indicus*, *Bos taurus*, *Bubalus bubalis*, *Ovis aries* and *Homo sapiens*. The size of the exons was the same in all the species., but in the sequence of the introns of the *Bubalus bubalis* species, they are closer to that of the *Bos taurus*., the similarity is expected as buffalo and cattle belong to the same family, only different genus.

This paper contributes to the knowledge of hereditary disorders of buffaloes, the molecular analysis of the sequenced exons 2 and 3 of the myostatin gene in the observed buffaloes with increased muscle mass, none of the individuals in the study had a mutation described in cattle as double muscle. Next research must be performed if the observed phenotype is associated with congenital Myotonia of the buffalo.

In conclusion, the observed phenotype doesn't correspond to the reported mutations of myostatin gene of double-muscling phenotype; more research is needed to establish if the observed buffalo phenotype is the same at the physiological and histopathological level.

REFERENCES

- [1] Leipold HW, Dennis SM. Congenital defects affecting bovine reproduction. In: Morrow D.A. (Ed.), Current Therapy in Theriogenology: Diagnosis, treatment and prevention of

- reproductive diseases in small and large animals. W.B. Saunders Company, Philadelphia 1986; 177-199.
- [2] Jolly RD. Screening for genetic diseases in cattle. *Aust Vet J* 2002; 80(5): 284-285. <https://doi.org/10.1111/j.1751-0813.2002.tb10843.x>
- [3] Arthur PF, Makarechian M, Price MA. Incidence of dystocia and perinatal calf mortality resulting from the reciprocal crossing of double-muscled and normal cattle. *Canadian Vet Journal* 1988; 29: 163-167.
- [4] Miranda M, Amigues Y, Boscher M, Menissier F, Corte's O, Dunner S, Anim J. Simultaneous genotyping to detect myostatin gene polymorphism in beef cattle breeds. *Breed Genet* 2002; 119: 361-366. <https://doi.org/10.1046/j.1439-0388.2002.00352.x>
- [5] Royo MLF. Secuenciación y análisis del gen de la miostatina bovina. Tesis Doctoral Universidad Complutense de Madrid, Facultad de Medicina Veterinaria. Madrid 2002.
- [6] Grobet L, Royo-Martin LJ, Poncelet D, Pirottin D, Brouwers B, et al. A deletion in the myostatin gene causes double-muscling in cattle. *Milan, Italy: Nat Genet* 1997; 17: 71-74. <https://doi.org/10.1038/ng0997-71>
- [7] Moreno V, Sifuentes A, De La Rosa X, Pereyra B. Evaluación de regiones polimórficas del gen de la miostatina en ganado beefmaster. *Arch Med Vet* 2008; 40: 59-63. <https://doi.org/10.4067/S0301-732X2008000100008>
- [8] Charlier C, Coppieiers W, Farnir F. The mh gene causing double-muscling in cattle maps to bovine Chromosome 2. *Belgica: Mamm Genome* 2002.
- [9] McPherron AC, Lawler AM, Lee SJ. Regulation of skeletal muscle in mice by a new TGF-beta superfamily member. *Nature* 1997; 387: 83-90. <https://doi.org/10.1038/387083a0>
- [10] Grobet L, Poncelet D, Royo LJ, Brouwers B, Pirottin D, Michaux C, Menissier F, Zanotti M, Dunner S, Georges M. Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle. *Mamm Genome* 1997; 9: 210-213. <https://doi.org/10.1007/s003359900727>
- [11] Vermorel M, Ortigues I, Vernet J, Geay Y, Jailler R, Jailler R, Baumont R, Hocquette JF, Deswysen AG. Energy metabolism in normal and double-muscled BelgianBlue calves in relation with body composition and organ size. In *Energy Metabolism of Farm Animals*; Aguilera, J.F., Ed., Consejo Superior de Investigaciones: Granada, Spain 1997; pp. 209-211.
- [12] Ménissier F. General survey of the effect of double muscling on cattle performance. *Curr Top In Vet Med And Anim Sci* 1982; 23-53. https://doi.org/10.1007/978-94-009-7550-7_2
- [13] Mota LSLS, Curi RA, Palmieri DA, Borges AS. Sequence characterization of coding regions of the myostatin gene (GDF8) from Brazilian Murrah buffaloes (*Bubalus bubalis*) and comparison with the *Bos taurus* sequence. *Genet Mol Biol* 2006; 29: 79-82. <https://doi.org/10.1590/S1415-47572006000100016>
- [14] Marcolongo-Pereira C, Schild AL, Soares MP, Vargas Jr, SF, and Riet-Correa F. Defeitos congênitos diagnosticados em ruminantes na Região Sul do Rio Grande do Sul. *Pesquisa Veterinária Brasileira* 2010; 30(10): 816-826. <https://doi.org/10.1590/S0100-736X2010001000003>
- [15] Mota LSLS, Curi RA, Palmieri DA, Borges AS. Mutação no exon 3 do gene da miostatina de búfalos (*Bubalus bubalis*). Resumos do 49º Congresso Brasileiro de Genética Água de Lindóia, SP 16 a 19 de Setembro 2003. www.sgb.org.br (Resumen).
- [16] Kambadur R, Sharma M. Mutations in myostatin (GDF8) in Double-Muscled Belgian Blue and Piedmontese Cattle. Research, Ruakura, Hamilton, New Zealand; 3 US Department of Agriculture (USDA)/Agricultural Research Service (ARS). Meat Animal Research Center, Clay Center. Nebraska 1997; 68933-0166 7:910-915 ©1997. <https://doi.org/10.1101/gr.7.9.910>
- [17] McPherron AC, Lawer AM, Lee SJ. Double muscling in cattle due to mutations in the myostatin gene. *Proc Natl Acad Sci USA* 1997; 94: 12457-12461. <https://doi.org/10.1073/pnas.94.23.12457>
- [18] Barbosa JD, Tury E, Pfeifer Barbosa IB, Cunha Dias VR. Hiperplasia muscular congênita (Doppellender, Double Muscling, Culard) em búfalos no Estado do Pará, Brasil. *Ciênc Vet Trop* 1999; 2: 50-52. <https://doi.org/10.1016/j.nmd.2012.11.008>
- [19] Borges AS, Barbosa JD, Resende LAL, Mota LSLS, Amorim RM, Carvalho TL, Garcia JF, Oliveira-Filho JP, Oliveira CMC, Souza JES, Winand NJ. Clinical and molecular study of a new form of hereditary myotonia in Murrah water buffalo. *Neurom Disord* 2013; 23: 206-213.
- [20] Borges AS. Estudo clínico, morfológico e molecular da miotonia hereditária em búfalos da raça Murrah. Tese de Livre Docente em Clínica de Equinos e Ruminantes, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu 2008; p. 94.
- [21] Tantia MS, Vijn RK, Mishra B, Bharani KT. Sequence of gdf 8 (myostatin) gene in *Bubalus bubalis*. *Animal Biotechnology* 2007; 18: 177-181. <https://doi.org/10.1080/10495390601115526>

Received on 01-12-2020

Accepted on 28-12-2020

Published on 01-01-2021

DOI: <https://doi.org/10.6000/1927-520X.2021.10.01>© 2021 Páez *et al.*; Licensee Lifescience Global.

This is an open access article licensed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted, non-commercial use, distribution and reproduction in any medium, provided the work is properly cited.