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Survey of mutations in prolificacy genes in Santa Ines and Morada Nova sheep

[*Pesquisa de mutações em genes da prolificidade em ovelhas Santa Inês e Morada Nova*]

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ABSTRACT

Polymorphisms in the BMP-15 gene related to Galway (*Fec*X^G) and Inverdale (*Fec*X^I) and in the BMPR-1B gene known as Booroola (*Fec*B) mutations were investigated using the Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) method, on sheep from the breeds Santa Inês (n= 574) and Morada Nova (n=282). DNA was extracted and amplified through PCR with specific primers that introduced a restriction site in association with the mutation. The PCR products were submitted to endonucleases. The experiment found no *Fec*X^G and *Fec*X^I mutations. Six samples of animals with multiple offspring/birth history presented polymorphism for *Fec*B similar to control samples, but this pattern was not confirmed by nucleotide sequencing. Although the absence of these mutations in the studied breeds, other factors related to prolificacy should be investigated to explain the inherent prolificity mechanisms.

Keywords: Galway, Inverdale, Booroola, sheep, prolificacy gene, Santa Inês, Morada Nova

RESUMO

Polimorfismos Galway (Fec*X*^{G}) *e Inverdale (*Fec*X*^{I}*), relacionados ao gene BMP-15, e Booroola* (Fec*B*)*, localizado no gene BMPR-1B, foram investigados usando-se a técnica de reação em cadeia da polimerase – polimorfismo de comprimento de fragmentos de restrição (PCR-RFLP), em ovelhas Santa Inês (n= 574) e Morada Nova (n=282). O DNA foi extraído e amplificado por PCR com iniciadores específicos, que introduziram um sítio de restrição associado à mutação, em seguida os amplicons foram submetidos à ação de endonucleases. Não foram observadas as mutações* Fec*X*^{G} *e* Fec*X*^{I} *nas amostras estudadas. Amostras de seis animais com histórico de partos gemelares apresentaram polimorfismo para* Fec*B semelhantes às amostras controle, mas esse padrão não foi confirmado pelo sequenciamento de nucleotídeos. Apesar da ausência dessas mutações nos animais das raças estudadas, outros fatores relacionados à prolificidade devem ser pesquisados para explicar os mecanismos da alta prolificidade desses animais.*

Palavras-chave: Galway, Inverdale, Booroola, ovelha, gene da prolificidade, Santa Inês, Morada Nova

INTRODUCTION

An increase in the production efficiency in herds related to gains in prolificacy is of considerable economic importance. The breed known as Santa Ines was formed naturally over the years through breeding of the native matrix with the breeds Bergamácia, Morada Nova and Somalis. The Santa Ines breed has considerable influence in the formation of commercial sheep herds in Brazil. Among those that formed the Santa Ines breed, the Morada Nova breed is cited as one of the most prolific, with its presence owing to a few persistent sheep breeders.

The discovery of mutations related to prolificacy in breeds and lineages has helped drive genetic improvement programs aimed at increasing prolificacy in herds.

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The Booroola mutation was found in the highly-conserved domain of intracellular serine-treonine-kinase of bone morphogenetic protein receptor 1B (BMPR-1B), characterized by a single change in nucleotides A to G (Q249R) resulting in a protein related with prolificacy phenotype in Merino-Booroola sheep (Mulsant *et al*., 2001, Souza *et al*., 2001, Wilson *et al*., 2001). The effect of this mutation, located in the autosomal chromosome 6, is addictive in the ovulatory rate. The alleles *Fec*B^{BB} and *Fec*B^{B+} are related to high prolificacy and the allele *Fec*B^{++} is considered wild phenotype (Davis *et al*., 2002).

Other prolific ovine lineages have been studied and mutations in the gene of bone morphogenetic protein 15 (BMP-15) were implicated (Galloway *et al*., 2000, Liao *et al*., 2004). The BMP-15 consists of a growth factor (a member of the superfamily TGFβ), which is specifically expressed in oocytes. These proteins are multifunctional and control the growth and differentiation of various cells type, having a critical role on the mammal fertility, including growth factors as Growth Differentiation Factor 9 (GDF9), localized in oocytes, and BMP-15 having receptors expressed in ovaries (Wilson *et al*., 2001). The BMP-15 regulates granulose cell proliferation and differentiation, promoting mitosis in the follicle-stimulating hormone independent phase, and inhibiting the expression of FSH receptors as well as events dependent on this hormone, thereby promoting follicle growth and avoiding premature luteinization (Otsuka *et al*., 2001, Otsuka *et al*., 2000). The BMP-15 inheritance mechanisms in sheep were revealed in studies carried out by Davis *et al* (1991) who pointed to an inheritance linked to the X chromosome for this trait, thereby creating the opportunity for studies on ovarian physiology and fertility in other mammals (Otsuka *et al*., 2001).

The BMP-15 gene was denominated *Fec*X (Galloway *et al*., 2000), and the *Fec*X^G gene mutation (Galway lineage) is characterized by an exchange of nucleotides (Q239Ter) that results in the insertion of a premature stopping point in the transcription of the protein. In heterozygosis, this mutation has been associated to an increase in ovulation in Cambridge and Belclare sheep (Hanrahan *et al*., 2004).

Another mutation in this gene was observed in Inverdale lineage in prolific Romney sheep (Davis *et al*., 1991). The Inverdale mutation is characterized by a single change of nucleotides resulting in different aminoacids (V31N) (Liao *et al*., 2003). Rams bearing the mutation (*Fec*X^{IY}) breed with heterozygote females (*Fec*X^{I+}) produced homozigote daughters (*Fec*X^{II}) with no functional ovaries, while heterozygote ones presented one more ovulation than wild females (*Fec*X^{++}) (Davis *et al*., 1992).

This increase in ovulation rate may be due to the reduced action of BMP-15 as a FSH inhibitor and consequently greater number of follicle producing estrogen, with LH receptors (Otsuka *et al*., 2001).

According to Davis (2004) the commercial use of markers for this trait consists of retaining prolific female offspring resulted of breeding between rams with this mutation and ewes without it.

The existence of sheep lineages with high prolificacy, which exhibit mutations in specific genes, raises the question as to whether other prolific breeds, including native breeds, have a heretofore unknown genetic proximity. As the *Fec*X^G mutation has been found in unrelated breeds (Belclare/Cambridge and Small-Tailed Han) (Davis *et al*., 2006b), it is interesting to study the possible existence of *Fec*X^G , *Fec*X^I or *Fec*B^B in other prolific breeds, such as Santa Inês and Morada Nova, using these markers.

Thus, the aim of the present study was to detect polymorphism in a single nucleotide from the BMPR-1B (*Fec*B^B) and BMP-15 (*Fec*X^G and *Fec*X^I) genes in sheep from the Santa Inês and Morada Nova breeds, which have considerable importance in Brazilian production.

MATERIALS AND METHODS

Eight hundred and fifty six animals were selected from the semi-arid region of the Brazilian Northeast. All animals had at least three parturitions, divided into groups of one offspring/parturition (Santa Inês, n=393 and Morada Nova, n=170) or two or more offspring/parturition (Santa Inês, n= 181 and Morada Nova, n=112). Blood samples (5mL) were collected from each sheep, with sodium

citrate used as anticoagulant. The genomic DNA was extracted from the leukocytes using the modified phenol-chloroform method (Sambrook *et al*., 1989) and stored at a temperature of -20°C.

Methodology that amplifies the region of the gene using primers with single mismatches to generate products with forced restriction site in association of mutation was employed to identify mutation.

The primers used to identify the *Fec*X^G mutation inserted a restriction site to *Hinf*I (G/ACT) in animals bearing no mutation – F: 5'CACTGTCTTCTTGTTAC TGTATTTCAATGAGAC3' and R: 5'GATGCAATACTGCCTGCTTG3' (Hanrahan *et al*., 2004).

The amplification conditions were: denaturation at 95°C/ 5min, followed by 30 denaturation cycles at 95°C/ 45sec, annealing at 63°C/ 45 sec and extension at 72°C/1min, with a final extension at 72°C/10 min. DNA digestion was performed for a final volume of 15µL (8.5µL of ultrapure water, 1.5µL of enzyme buffer and 10 U of the *Hinf* I enzyme and 5µl of the amplified DNA (PCR product). The digested DNA was visualized in 8% polyacrylamid gel. The individuals of the wild type (*Fec*X^{++}) had fragments of 111 bp and 30 bp; heterozygotic individuals (*Fec*X^{G+}) had fragments of 141 bp, 111 bp and 30 bp; and homozygotic individuals (*Fec*X^{GG}) only had the uncut fragment of 141 bp.

To study this mutation were used primers that introduced a restriction site for the endonuclease *XbaI*; 5'-GAAGTAACCAGTGTTCCCTCCACCCTTTTCT – 3' e 5'-CATGATTGGGAGAATTGAGACC-3' (Davis *et al*., 2006b).

The PCR reactions were performed in 20µL final volume, containing: 2,5µL 10x PCR buffer; 3,0µL of 50mM $MgCl_2$ 1µL of each primer (10 pmol), 3,5µL of 2mM dNTP; 0,2µL *Taq*-polimerase (5U/µL), 11,8µL ultrapure water and 2,0µL of DNA (50ng). The conditions of amplification were: 35 cycles of denaturation at 94ºC/ 30 sec, annealing at 60ºC/40sec and e extension at a 70ºC/ 30sec, and a final extension at 72ºC/4min. The amplicon was submitted to endonuclease *Xba*I cut (8,5µL ultrapure water, 1,5µL of buffer and 10 U *XbaI* I). Products containing the *Fec*X^I mutation yield 124 bp and 30 bp fragments, whilst non-carrier products remain uncut at 154 bp.

As the positive control for the *Fec*B mutation, DNA samples of Booroola animals were used (kindly provided by Dr. Carlos Jose Hoff de Souza/Embrapa Sul).

The primers inserted a restriction site to *Ava*II (G/GACC) in animals bearing the Booroola mutation – F: 5'-CCAGAGGAACAATAGCAAAGCAAA; *R:* 5'-CAAGATGTTTTCATGCCTCATCAACACGGTC (Davis *et al*., 2002). The amplification conditions were initial denaturation at 94°C/2 min, 8 cycles of denaturation at 94°C/15 sec, annealing at 62°C/30 sec, and extension at 72°C/30 sec, followed by 30 cycles of denaturation at 94°C/15 sec, annealing at 60°C/30 sec, and extension at 72°C/15 sec, with final temperature at 8°C. The PCR product was digested with *Ava*II (8.5µL of ultrapure water, 0.8U of enzyme, 1.5µL of buffer and 5µL of DNA). After electrophoresis, no digested fragment presented 190 bp, and digested fragment showed 160 bp and 30 bp. Heterozygote animals should present both fragments of 30, 160 and 190 bp.

The products of endonuclease reactions were analyzed in 2% agarose gel using blue green loading dye I (LGC- Biotecnologia)**.** The data on the bands found in each group of sheep breed, for each mutation, were analyzed for frequency and related to the prolific phenotype.

The amplified fragments of BMPR-1B were separated on "Low Melting Point" 1,5 % agarose gel at 4v/ cm for 2 hours and re-extracted using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare), according to manufacturer instructions. Each DNA fragment was ligated into the pGEM-T Easy vector (Promega, Madison, WI USA) at the ratio of 3:1 (insert:vector), at 4°C overnight, according to the manufacturer's instructions. The fragment was then transformed into *Escherichia coli* EletroMAX DH10B competent cell (Invitrogen) using a micro pulser electroporator ECM 399 Electroporation System (BTX Harvard Apparatus, Cat 45-0000). Positive clones of transformed cells were identified based on blue-white selection of recombinant vectors, and confirmed by colony PCR using vector primers

(T7 and Sp6) showing the 190 bp insert fragments upon gel analysis. Three positive clones of each genotype (biological triplicates) were selected for mini preparation of plasmid DNA according to modified alkaline lysis plasmid mini-prep method (Sambrook *et al*., 1989). The authenticity of the fragments was confirmed by sequencing of insert-containing plasmids performed from both directions for three times using the BigDye Terminator v3.1 Cycle Sequence Kit (Applied Biosystems, Foster City, CA-USA), in an Automatic ABI 3130XL sequencer (Applied Biosystems, Foster City, CA-USA).

The chromatograms were passed through a base-calling process using phred (Ewing *et al*., 1998), which assigned quality values above 20 to the bases. Sequences alignment and generation of each consensus was performed by using Clustal W, running under BioEdit, version 5.0.9 (Hall, 1999). The consensus sequence respective to each genotype was analyzed by Blast (Basic Local Alignment Search Tool) against nucleotide collection, that consisted of GenBank, EMBL, DDBJ, PDB and RefSeq sequences, using nucleotide sequences (BlastN).

RESULTS

The Santa Inês and Morada Nova ovine breeds have been reported to be prolific in the semi-arid region conditions of northeastern Brazil. In the visited herds, 39,7 % of Morada Nova females had at least one multiple-offspring birth, and 31,5% of Santa Inês females had scores of multiple-offspring births. The prolific index of these animals was 2.44 and 2.13 for the Santa Inês and Morada Nova breeds, respectively (Table 1).

Table 1. Mean number of offspring/birth for Morada Nova and Santa Inês breeds with a history of multiple-offspring births

	Breed	
	Morada Nova	Santa Inês
Total animals	282	574
No. of animals with multiple offspring/birth	112	181
Average of offspring/birth (multiples)	2.13	2.44

No mutations referring to the Galway *Fec*X^G gene were found in the studied breeds. PCR-RFLP revealed that all wild bands were cut by the *Hinf* I enzyme (G/ACT), resulting in fragments of 111 bp, which characterizes an absence of mutation for the *Fec*X^G gene.

All analyzed samples for *Fec*^{I} mutation showed uncut fragments after PCR-RFLP, in this case characterizing the absence of Inverdale mutation.

Otherwise, the *FecB* mutation revealed polymorphism in six samples from Santa Inês females with litter size of 2,49 in average. In these samples was observed two bands (190 and 160 bp) similar to heterozygote positive control (Figure 1).

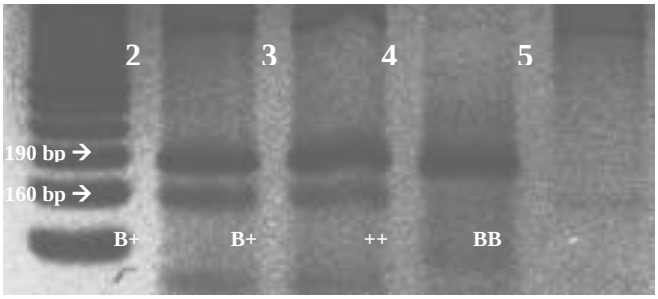

Figure 1. PCR products digested with *Ava* II. 1: DNA ladder 50 bp; 2: Santa Inês heterozygote (B+); 4: wild genotype (++); 3 and 5: positive controls for Booroola-Merino, B+ (190 and 160 bp) and BB (160 bp), respectively.

To confirm this mutation, the amplified fragments were submitted to cloning and sequencing, but the sequences did not show the expected change in the nucleotide (A-G), that was present in the positive control.

DISCUSSION

This study was performed in order to search mutations in the BMP15 and BMPR-1B genes in prolific Santa Inês and Morada Nova ewes. Due to the extensive breeding system, breeders do not prize prolific females, citing management difficulties in the pasture breeding system. Thus, there is a need to offer further information to breeders with regard to the benefits in terms of production gains when there is efficient management of prolific females. Although this trait has not been well accepted in the extensive breeding system, 39.7% of the Morada Nova, and 31.3% of Santa Inês females had history of at least one multiple-offspring birth. Thus, prolificacy is an inherent characteristic of these breeds.

In the present study, the PCR-RFLP method cited by Hanrahan *et al*. (2004) was employed for the investigation of the mutation referring to the BMP-15 gene (*Fec*X^G loci), which was not found in the Santa Inês and Morada Nova breeds. Although the relevance of these studies, the results are conflicting. These mutations were not found during a study performed in 21 of the most prolific breeds and lineages in the world. Among these breeds, the *Fec*X^I mutation (Inverdale) was not observed in the Small-Tailed Han, in which the *Fec*B mutation was detected (Davis *et al*., 2006a). However, studies carried in China described the Asian Small-Tailed Han breed as having simultaneous mutations (*Fec*B and *Fec*X^G) (Chu *et al*., 2007).

The occurrence of the Booroola gene was reported in Indian sheep of the Kendrapada breed with a history of prolificacy when investigating the BMPR-1B (*Fec*B) and BMP15 (*Fec*X^G) simultaneously (Kumar *et al*., 2008). As in the present study; however, no mutations related to the *Fec*X^G gene (Galway) were found.

Regarding studies carried out in Brazil on larger genes linked to prolificacy, a new single nucleotide polymorphism (SNP) located in the position 345 inside GDF-9 gene in the Santa Inês breed, that was named *Fec*G^E, may be related to the high ovulation frequency that is characteristic of this breed (Silva *et al*., 2010). No studies have been carried out investigating the *Fec*X gene in the breeds analyzed here and no surveys have been noticed for this gene in any Brazilian breeds. Therefore, there is a need for further studies with the aim of investigating the possible existence of other mutations related to the prolificacy of the Santa Inês breeds as well as its precursors, such as the Morada Nova breed.

As no mutations related to the *Fec*X^G gene were found in the present study, the use of surveys related to these mutations as a marker for prolificacy with the methodology employed here is not an adequate tool for commercial use in improvement programs for this trait in the breeds studied. However, studies on phylogeny in sheep based on the investigation of common polymorphisms between breeds or lineages should be taken into consideration. For example, the Booroola gene has been chosen for studies on binding maps. Thus, such studies on breeds of Brazilian sheep with a history of prolificacy are important.

Binding maps are an important tool for the identification of genes associated to production traits in large animals. Selection programs for domestic animals based on phenotype measurements have improved, and an understanding of the structure and function of the genomes of domestic animals offered by genetic maps allows the opportunity to identify the variations responsible for the differences in production performance. Molecular markers linked to inheritable traits will enable a more efficient selection of elite animals and will eventually allow the identification of genes that influence genetic expression (Crawford *et al*., 1994).

In the other hand, more recently, mRNA assays performed to study the gene expression in ovaries of prolific animals, have demonstrated the relationship between the number of antral follicles/ovarian mass and the abundance of BMP15 mRNA in goats follicles (Pramod *et al*., 2013).

Despite the absence of the studied mutations in the Santa Inês and Morada Nova breeds, which have a history of prolificacy, other markers

should be investigated that are particular to these breeds and may explain the passing down of this trait to descendents.

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