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Genetic variability of the regulatory region of the *FSH-β* gene and its association with reproductive traits in Nellore bulls

L.K. Silva[1], C.L. Santos[1], E.M. Barbosa[2], S.T. Filho[1], J.B. Luz[3], K.S. Alves[3], L.R. Oliveira[3] and E.S. Filho[1]

[1]Animal Health and Production Institute, Molecular Biology Laboratory, Federal Rural University of the Amazon, Belem, [2]Rural Education Department Federal University of Amapa, Mazagao, [3]Animal Production Department, Federal Rural University of Amazon, Parauapebas, Brazil

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Correspondence:
L.K. Silva
lorenakeyse24@gmail.com

Abstract

Molecular biology techniques are important tools for studying animal health and production characteristics. Thus, determining the genotypes of animals with high genetic value enables the implementation of genetic improvement programs, especially related to sperm and testicular characteristics in cattle. The objective of this study was to detect polymorphisms in the promoter region of the FSH-β gene using the Tetra-primer-PCR (T-ARMS-PCR) technique and associate these polymorphisms with seminal parameters and scrotal perimeter in Nellore bulls. Semen samples were collected and evaluated for volume, sperm motility, and vigor, and scrotal perimeter was measured in 200 bulls. DNA extractions and genotyping of the FSH-βU1 and FSH-βU2 polymorphisms were performed using the T-ARMS-PCR technique. Population genetics parameters were estimated, and associations between genotypes and semen and testicular characteristics were assessed. Both SNPs showed variability with allele frequencies close to 0.500 and an excess of heterozygotes (above 0.600). No inbreeding was detected (FIS -0.3304 and -0.2511, respectively), and deviations from Hardy-Weinberg equilibrium were observed ($P<0.05$). Only the scrotal perimeter parameter was associated with the FSH-βU1 SNP, with the GG genotype being superior to the others. Therefore, the T-ARMS-PCR technique may be applied to different SNPs directly associated with health and economically relevant characteristics in animals.

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Introduction

The development of research on reproductive traits has been a useful strategy for researchers to enhance animal husbandry. Particularly, it aims to identify sires with excellent reproductive performance capable of fertilizing multiple females during the same breeding season (1,2). These studies combined with molecular improvement methods, enable better genetic exploitation. Consequently, it becomes possible to predict an animal's behavior in a given environment and characterize the herd based on genotypes, differentiating between more and less productive animals. Brazil, being the country with the largest commercial herd worldwide, consisting of approximately 214 million heads, with around 80% being Zebu breed animals (*Bos taurus indicus*), particularly the Nellore breed, holds a significant position (3). The importance of developing methods that are easy to manipulate and cost-effective for genotyping animals can be justified. The amplification refractory mutation system by tetra-primer PCR (T-ARMS-PCR) is a method that fulfills these requirements. It employs four primers to amplify a DNA fragment containing a single nucleotide

polymorphism (SNP), representing two different allelic forms. These primers are designed to amplify fragments of different sizes, each representing one allele, which can be visualized on an agarose gel. Moreover, T-ARMS-PCR offers an inexpensive and rapid technique for genotyping a herd (4). Considering the advancements in livestock production closely tied to reproduction, one of the hormones that directly affects this characteristic is FSH (follicle-stimulating hormone). Together with LH (luteinizing hormone), it activates the hypothalamic-pituitary-gonadal axis to support spermatogenesis. FSH acts within the seminiferous tubules, stimulating Sertoli cells to produce androgen-binding protein (ABP). Secreted in the seminiferous tubules, ABP ensures the ideal concentration for germ cell maturation up to the sperm stage (5,6). Glycoprotein hormones are known to consist of two subunits: α and β. In the case of FSH, both subunits contribute to receptor binding, but it is the β subunit that determines binding specificity. Moreover, this subunit undergoes various point mutations, ranging from the promoter region to the coding region. When located in the promoter region, these mutations can create new binding sites, affecting the region's activity and gene expression itself (7). Since these mutations occur in the 5' upstream regulatory region (5'-UTR) of the gene, they can lead to changes in the primary functions of the FSH hormone, sperm production, and even sperm malformation (2,6,8).

Therefore, the objective of this study was to associate the polymorphisms detected in the promoter region of the FSH-β gene using the Tetra-primer PCR (T-ARMS-PCR) technique with seminal and testicular characteristics of Nellore cattle.

Materials and methods

Ethical approve

All procedures carried out were in accordance with Concea/MCTI, the Brazilian Guide for the Production, Maintenance or Use of Animals for Teaching or Scientific Research Activities and approved by the Animal Use Ethics Committee of the Federal Rural University of the Amazon with number 006/2017.

Animals' origin, testicular biometrics and semen collection

The collection was carried out at the Nellore Quality farm, located in the rural region of the city of Parauapebas-PA (Brazil), Latitude: 6 ° 4 '15' 'South, Longitude: 49 ° 54' 15 " West, and the initial laboratory procedures performed at the Biotechnology Laboratory in Animal Reproduction of Carajás - LABRAC at the Parauapebas Campus of the Federal Rural University of the Amazon. Were evaluated 200 sires of Nellore breed, with an average age of 24 months, with an average body weight of 550 kg, kept in paddocks formed by *Brachiaria brizanta* grass, receiving concentrated supplementation, based on soybean meal, ground corn, mineral supplement and water *ad libitum*. Before the beginning of the experiment, the animals had measured testicular perimeter and were adapted to the semen collection procedure and, after this period, a single semen collection was performed from each animal.

Semen collect method was throught of eletroejaculation (Neovet®, Eletrojac). Where the animal's tail was deflected laterally, and then, the electrode was lubricated with mineral oil and introduced into the rectum of the animal, keeping it in the ventral position. The first drips were discarded and the remainder was drained into graduated tapered tubes (15 mL), previously identified, where the volume of the ejaculate was determined in the graduated tube itself.

The tubes containing the seminal material were wrapped in aluminum foil, to avoid exposure to light, and kept in a water bath at 37 ºC. Sperm motility and vigor evaluations were performed using a phase contrast microscope (OLYMPUS®, mod. CX 21). Motility and vigor were determined with 10 µL of semen between slide and coverslip, motility being calculated by the percentage (0 to 100) of sperm with movement and vigor was assessed based on the quality of the straight-progressive movement and its speed in a scale of 1 to 5 (9). After carrying out the analyzes, the tubes containing the semen samples were kept in thermal boxes containing ice. Then, laboratory analyzes of molecular biology were carried out.

DNA extraction

DNA extraction was performed by saline method (10), using the following soluctions: PBS (KCl 2,7 mM; KH2PO4 1,5 mM; NaCl 137 mM; Na2HPO4 8 mM; com pH 7,0); Semen lise buffer (2-mercaptoetanol 2%; Tris−HCl 10 mM, with pH 8,0; NaCl 100 mM; EDTA 10 mM, with pH 8,0; SDS 0,5%); Proteinase K (20 mg/mL); NaCl (5M); Absolute Ethanol; Ethanol 70%.

Design of primer and genotyping by T-ARMS-PCR

Two wide primers were used as described by (8) to amplify the 5'URR of FSH-β gene for two SNPs: FSH-βU1 (-782G >A) and FSH-βU2 (-1727T>A). We used the GenBank FSH-β gene sequence access number M83753 to design internal primers to detect the specific alleles for those SNPs through T-ARMS-PCR (11). Schemes about T-ARMS-PCRs are represented in figure 1 and all primer sequences are in table 1.

The reactions were carried out in a total volume of 15 µL with the following reagent conditions: 1x PCR buffer, 1 mM of each dNTP, 2 mM of MgCl2, 10 pmol/µL of each primer, 1 U of Taq DNA polymerase (Invitrogen/Carlsbad, California, USA), and 25 ng of genomic DNA. The PCR temperature and time conditions were as follows: an initial 6 min denaturation temperature at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C (FSH-βU1) and 54 °C (FSH-βU2) for 45 s, extension

temperature at 72 °C for 30 s, and a final extension temperature of 6 min at 72 °C, using the 2720 Thermal Cycler (Applied Biosystems).

Genotype identification by electrophoresis

The genotypes of the FSH-BU1 and FSH-BU2 SNPs were characterized through electrophoresis in 3% agarose gel submerged in 1X TAE buffer solution (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA, pH 8.0). The standard electrophoresis performance was set at 100V for 60 minutes. Subsequently, band patterns were established using GelRed dye and visualized under a UV light transilluminator. All fragments were referenced against a 100 bp molecular weight ladder.

DNA purification and sequencing

For confirmation and validation of the genotypes, purifications and sequencing of the three genotypes for both SNPs were performed. The purifications were carried out using the QIAquick PCR Purification Kit (Qiagen, USA) following the manufacturer's recommendations. The quantifications of the purified samples were performed using the BioDrop μLITE spectrophotometer (BioDrop). The purified PCR products were sequenced using both the Forward and Reverse primers with the BIG DYE Kit (Invitrogen, California, USA) on an ABI 3500 XL automatic DNA sequencer (Applied Biosystems). The generated sequences were edited using the Finch TV version 1.4.0 software (Geospiza Research Team, USA) and compared to the reference sequence deposited in Genbank using the BLAST system (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Subsequently, they were aligned using the Bioedit software (12).

Statistical analysis

Allelic and genotypic frequencies were estimated, as well as deviations from Hardy-Weinberg equilibrium, were tested by comparing genotypic frequencies using the chi-square test. The inbreeding coefficients F_{IS} were calculated following the parameters (13). All these analyses were performed using the GENEPOP computational program (14). Regarding seminal characteristics, they were analyzed using the PROC MIXED application of SAS Analytics Software (OnDemand version), assuming normal data distribution. ANOVA was used, along with the Tukey test, to assess the comparison between averages at a significance level of 0.05.

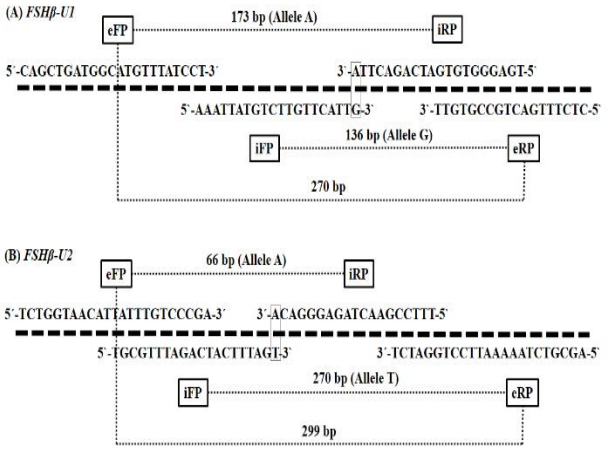

Figure 1: Design of the T-ARMS-PCR technique for genotyping the FSH-βU1 and FSH-βU2 SNPs in Nellore bulls. Each reaction is performed with four primers, two external (eFP and eRP) and two internal (iFP and iRP). The external primers generate a band around the SNP, while the internal primers combine with the external primers to determine the presence of the allele. The SNPs are highlighted at the 3' ends of the inner primers (iFP and iRP).

Table 1: Primer sequences for AMRS-PCR to genotype the FSH-βU1 e FSH-βU2 polymorphisms

SNPs	Primer sequences (5'-3')	Sizes	Temperatures
FSH-βU1	External Forward Primer (eFP) CAGCTGATGGCATGTTTATCCT External Reverse Primer (eRP) CTCTTTGACTGCCGTGTT Internal Forward Primer (iFP) AAATTATGTCTTGTTCATTG Internal Reverse Primer (iRP) ATTCAGACTAGTGTGGGAGT	A (173 bp); G (136 bp); Total: 270 bp	55°C
FSH-βU2	External Forward Primer (eFP) TCTGGTAACATTATTTGTCCCGA External Reverse Primer (eRP) TCTAGGTCCTTAAAAATCTGCGA Internal Forward Primer (iFP) TGCGTTTAGACTACTTTAGT Internal Reverse Primer (iRP) ACAGGGAGATCAAGCCTTT	T (270 bp); A (66 bp); Total:299 bp	54°C

Results

All animals analyzed for both SNPs were genotyped based on the band patterns resulting from T-ARMS-PCR technique. Therefore, three bands will always be revealed for a bi-allelic system. In figure 2/A are represented the genotypes GG, GA, and AA with their respective band patterns for the FSH-βU1 SNP, and in figure 2/B are

represented the genotypes TT, TA, and AA with their respective band patterns for the FSH-βU2 SNP. The sensitivity of the technique was confirmed through DNA sequencing analyses of both genotypes in both SNPs.

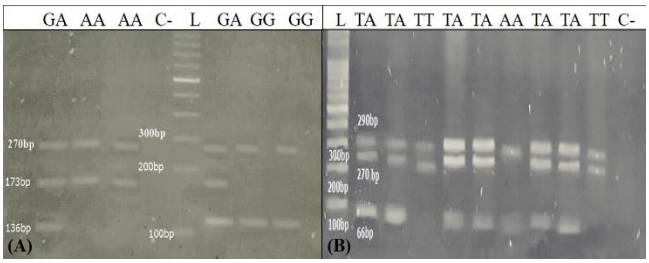

Figure 2: Images of agarose gels genotyping of FSH-βU1 (A) and FSH-βU2 SNPs (B) in Nellore bulls. In the image, the genotypes are represented by their respective band patterns, with the 100 bp molecular ladder (L) and a negative control (C-) serving as references.

Genetic analyses at the population level demonstrate that the Nellore bull herd exhibits genetic variations according to allelic and genotypic frequencies. Table 2 shows the results of the population analysis. For the FSH-βU1 SNP, the mutant allele A was more frequent than the wild-type allele G, while the opposite was observed for the FSH-βU2 SNP, where the wild-type allele T was more frequent than the mutant allele A. Excess of heterozygotes and absence of inbreeding (negative F_{IS}) were also observed. Hardy-Weinberg proportions showed significant deviations, providing strong evidence of selection favoring heterozygous genotypes in both SNPs.

The associations analysis of SNP effects with seminal quality and quantity characteristics, as well as scrotal circumference, are presented in table 3. Only scrotal circumference shows significant differences ($P<0.05$) among genotypes, where animals with the GG genotype exhibit a higher average scrotal circumference compared to those with the GA and AA genotypes.

Table 2: Population analysis of the Nellore bull herd

SNPs (Position)	Alleles	Observed Genotypes	Experted Genotypes	F_{IS}	HWP
FSH-βU1					
G → A	G= 0.481	GG= 0.155	GG= 0.231	-0.3304	0.0001
(-782)	A= 0.519	GA= 0.652	GA= 0.501		
		AA= 0.193	AA= 0.268		
FSH-βU2					
T → A	T= 0.513	TT= 0.200	TT= 0.263	-0.2511	0.0029
(-1727)	A= 0.487	TA= 0.627	TA= 0.501		
		AA= 0.173	AA= 0.236		

F_{IS}= Endogamy coefficient; HWP= Hardy-Weinberg Probability.

Table 3: Association of SNP genotypes with sperm parameters and scrotal perimeter in Nellore bulls

Genotypes	Volume (mL)		Motility (%)		Vigour (1-5)		Scrotal perimeter (cm)	
FSH-βU1	Average	SD	Average	SD	Average	SD	Average	SD
GG	4.379	1.550	74.218	10.633	3.343	0.865	34.093a	2.441
GA	5.352	2.090	74.950	8.291	3.288	0.692	33.132b	1.832
AA	4.480	1.922	75.434	7.372	3.200	0.645	32.434b	1.823
FSH-βU2								
TT	5.183	1.749	76.207	8.200	3.366	0.718	32.875	1.659
TA	5.073	2.100	75.000	9.028	3.281	0.749	33.206	2.157
AA	4.913	1.749	73.206	8.203	3.166	0.702	33.386	1.932

SD= Standard deviation; Different bold lowercase letters in the same column of the scrotal perimeter average indicate a significant difference at 0.05.

Discussion

The standardization of the T-ARMS-PCR technique for genotyping the FSH-βU1 and FSH-βU2 SNPs in Nellore cattle was highly successful, demonstrating simplicity in execution and interpretation of results. Therefore, this genotyping method can also be applied to other SNPs in different genes that are of great importance in the health and production of economically important animals or in species conservation (15-17).

Studies on the genetic diversity of cattle breeds with high reproductive and productive indices enable genetic improvement programs for other breeds (18). However, assisted reproduction allows for the concentration of

desirable alleles in the animal production system. Nevertheless, it is still possible to find high frequencies of undesirable alleles that influence genes related to health and economically important traits in cattle on a global scale (19).

Locis with significant excess of heterozygotes can be caused by selection, population history, or sexual linkage. However, it is not possible to confirm the intensity of any evolutionary force acting on the population entirely, as genetic selection can lead to concordance or deviation from HWE deviations, depending on whether the selection favors heterozygotes or homozygotes. This is especially true considering that genotypic frequencies are affected only by selection within a generation in a population, which is why evaluating only HWE tests usually provides little information for candidate gene studies in animal production (20).

Polymorphisms identified in the gene promoter region tend to modify the gene's own genetic expression, directly affecting how the genotype is expressed with the phenotype (7,8). In fact, the authors discovered that mutations indicated in this region affect the binding sites of transcription factors, resulting in changes in transcription and consequently causing differences in the coding region sequence (exon 3) when investigating polymorphisms in the FSH-β gene (8).

Furthermore, also identified mutations in the 5' - URR that generated a new cis-regulatory element in FSH-β in cattle, where transcript levels in bulls carrying the genetic mutations were significantly lower than in bulls without these mutations (21). Therefore, even though in this present study only one reproductive trait was significant (scrotal circumference), the mutations found in the gene's regulatory region may be affecting gene expression.

Conclusion

The T-ARMS-PCR technique proved to be efficient in identifying the genotypes of the FSH-βU1 and FSH-βU2 SNPs in Nellore cattle. The SNPs exhibited variations that revealed population diversity with an excess of heterozygosity. However, only the scrotal circumference trait showed a significant association with the FSH-βU1 SNP, with the GG genotype having the highest average. Therefore, this genotyping technique can be employed for the studied SNPs, as well as other SNPs related to animal health and production.

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Conflict of interest

All authors and involved institutions in this research declare that there is no conflict of interest.

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التباين الوراثي للمنطقة التنظيمية لجين FSH-β وارتباطه بالصفات الإنجابية في ثيران نيلور

لورينا كيز سيلفا[١]، سينتيا لوانا سانتوس[١]، إليزابيث ماتشادو باربوسا[٢]، سيباستياو تافاريس فيلهو[١]، جانينا باروس لوز[٣]، كاليأندرا سوزا ألفيس[٣]، لويس رينان أوليفيرا[٣] و إدنالدو سيلفا فيلهو[١]

[١]معهد الصحة والإنتاج الحيواني، مختبر البيولوجيا الجزيئية، الجامعة الفيدرالية الريفية في منطقة الأمازون، بيليم، [٢]قسم التعليم الريفي جامعة أمابا الفيدرالية، مازاجاو، [٣]قسم الإنتاج الحيواني، جامعة الأمازون الريفية الفيدرالية، باراوابيباس، البرازيل

الخلاصة

التقنيات البيولوجية الجزيئية هي أدوات مهمة لدراسة صحة وخصائص الإنتاج الحيواني. وبالتالي، فإن تحديد الأنماط الوراثية للحيوانات ذات القيمة الوراثية العالية يمكن من تنفيذ برامج التحسين الوراثي، خاصة تلك المتعلقة بالنطف وخصائص الخصية في الماشية. كان الهدف من هذه الدراسة هو الكشف عن تعدد الأشكال في المنطقة المروجة لجين FSH-β باستخدام تقنية T-Tetra-primer-PCR (ARMS-PCR) وربط هذه الأشكال المتعددة بمعايير السائل المنوي ومحيط كيس الصفن في ثيران نيلور. تم جمع عينات السائل المنوي وتقييمها من حيث الحجم وحركة النطف ونشاطها، وتم قياس محيط كيس الصفن في ٢٠٠ ثور. تم إجراء عمليات استخراج الحمض النووي والتنميط الجيني لتعدد الأشكال FSH-βU1 و FSH-βU2 باستخدام تقنية T-ARMS-PCR. تم تقدير المعايير الجينية وتقييم الارتباطات بين الأنماط الجينية وخصائص السائل المنوي والخصية. أظهر كلا SNPs تباينا مع ترددات أليل قريبة من ٠,٥٠٠ وفائض من الزيجوتات غير المتجانسة (أعلى من ٠,٦٠٠). لم يتم الكشف عن زواج الأقارب (FIS 0.3304- و -٠,٢٥١١ ، على التوالي)، ولوحظت انحرافات عن توازن هاردي واينبرغ (P<0.05). ارتبطت معلمة محيط كيس الصفن فقط بـ FSH-βU1 SNP، مع تفوق النمط الجيني GG على الآخرين. لذلك ، يمكن تطبيق تقنية T-ARMS-PCR على SNPs المختلفة المرتبطة مباشرة بالخصائص الصحية والاقتصادية ذات الصلة في الحيوانات.

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