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MUC1 VNTR polymorphism is not associated with early puberty in Nelore cattle (*Bos primigenius indicus*)

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Abstract

MUC1 is a transmembrane glycoprotein expressed in the male and female reproductive tracts, which plays a broad functional role in reproduction. An important feature of MUC1 is the presence of a VNTR polymorphism in the extracellular domain that is associated with the function of the molecule. In view of its role in reproduction, the aim of the present study was to analyze the effect of the length of the *MUC1* VNTR on the early puberty phenotype in Nelore cattle. A total of 230 heifers were studied, including 77 sexually precocious heifers and 153 regular heifers. The genotype was identified by PCR and the allele and genotype frequencies between the two groups (precocious or regular heifers) were compared by Fisher's exact test using the population differentiation module of the GenePop program, version 3.4. The associations between VNTR polymorphism and early puberty were analyzed as discrete data using the SAS GenMod procedure of SAS. Higher frequencies were observed for allele 1, 2, and 3, with allele 1 being predominant in both groups. No significant differences in allele or genotype frequencies were observed between precocious and regular heifers. The analyses using ANOVA and orthogonal contrasts suggest that the *MUC1* VNTR variation is not associated with early puberty in cattle. Previous studies regarding the *MUC1* polymorphism in Nelore cattle suggested that the high frequency of the 1 allele is a characteristic of Brazilian Nelore cattle.

Keywords: beef cattle, fertility trait, molecular marker.

Introduction

Early puberty or sexual precocity is an economically interesting phenotype for beef cattle because it increases the reproductive life of females. In Brazil, *Bos primigenius indicus* breeds are known for their higher heat tolerance and ability to survive on limited food resources; however, these breeds present lower reproductive efficiency (lower fertility and delayed puberty) when compared to taurine breeds (*Bos primigenius taurus;* Hiendleder *et al*., 2008).

MUC1, also know as mucin 1, is a transmembrane glycoprotein expressed in the male and female reproductive tracts, which plays a broad functional role in reproduction. MUC1 is mainly involved in cell adhesion, protection and lubrication of epithelial tissues, but is also associated with signal transduction and cell-cell interactions (Gendler *et al*., 2001). In the male reproductive tract, MUC1 is expressed by pachytene spermatocytes, arrested spermatocytes, spermatids and capacitated sperm, and is related to differentiation stage, complete spermatogenesis and male fertility status (Martinez-Conejero *et al*., 2005). In the female reproductive tract, MUC1 expression is associated with uterine health, gamete transit, prevention of ectopic pregnancy, control of the embryo implantation window, and trophoblast transendothelial migration during placentation (Eriksen *et al*., 1998; Ishiguro *et al*., 2007; Thirkill *et al*., 2007; Savaris *et al*., 2008). All these processes require the adhesion and anti-adhesion properties of MUC1.

The full-length MUC1 molecule contains three domains: a short cytoplasmic and a transmembrane domain that are highly conserved among species, and a large extracellular domain. The extracellular domain contains a variable number of tandem repeats (VNTR) consisting of repeats of 20 amino acids rich in serine, threonine and proline residues, features that permit extensive O-glycosylation of the tandem repeat domain (Pallesen *et al*., 2001). The O-glycosylation pattern is important for the adhesion and anti-adhesion properties of MUC1. The negative charge of the molecule provided by glycosylation with sialic acid promotes a steric hindrance effect, preventing the contact between cells. Alternatively, glycosylation with Lewis a and y structures promotes cell adhesion through L-selectin molecules (Carson *et al*., 2006). The VNTR polymorphism results in different levels of glycosylation and might be related to the diverse functional adhesiveness reported for humans (Costa *et al.*, 2008) and yeast (Verstrepen *et al*., 2005).

In the *MUC1* gene, the sequence that forms the repeat of the VNTR consists of 60 nucleotides. In humans, the allelic variation in the VNTR ranges from 25-125 repeats and has been associated with female infertility due to embryo implantation failure (Horne *et al.*, 2001), an

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increased risk of gastric cancer (Carvalho *et al.*, 1997), severe acne (Ando *et al.*, 1998), and increased adhesion of *Helicobacter pylori* to gastric cells (Costa *et al.*, 2008). In *Bos primigenius taurus* and *Bos primigenius indicus* cattle, this variation ranges from 10-27 repeats and has been associated with variations in the mucin of milk fat globules (Rasero *et al.*, 2002), milk yield (Hens *et al*., 1995), growth, fertility, and carcass traits (Souza *et al.*, 2010).

In the present study, we evaluated the influence of *MUC1* VNTR variability on early puberty, testing the hypothesis that VNTR length affects fertility performance in Nelore cattle, in order to develop molecular markers related to this trait for application in marker-assisted selection.

Materials and Methods

Sample

The sample consisted of 230 Nelore heifers (*Bos indicus*) including 77 sexually precocious heifers and 153 non-precocious heifers from the same farm, year and season of birth. The animals were kept under the same conditions of feeding and management and all heifers were exposed to reproduction, irrespective of their weight and body condition score. The age at puberty was determined by exposing the females to multiple sire lots at the age of 12-16 months and determining the presence of pregnancy 60 days after the end of a 90-day breeding season by ultrasound examination. Early puberty is a binary trait and was defined as the presence of pregnancy determined by ultrasound examination 60 days after the end of the breeding season (1 - success or 0 - failure). Failed heifers were classified as presenting late puberty and were selected from the general herd as regular heifers for the breeding season of the next year.

DNA extraction and PCR

Genomic DNA was extracted from peripheral blood by precipitation with NaCl using standard techniques (Olerup and Zetterquist, 1992). The animals were genotyped for the *MUC1* gene by the polymerase chain reaction (PCR). The forward and reverse primer sequences (5'-CGCAGA ACT ACG CCA GTT TCC-3' and 5'-AGA GCGGGT GGT CAT GGA TG-3') were based on the bovine sequence deposited in GenBank (AF399757) and adapted from the primer sequences that flank the repetitive VNTR region of bovine MUC1 published by Rasero *et al*. (2002). About 100 ng of genomic DNA was mixed with 1.5 pmol of each primer in a total volume of 25 µl containing 200 µM of each dNTP, 0.75 mM $MgSO_4$, 0.5 U of Platinum Pfx Taq DNA polymerase, 1X Platinum Pfx Amplification Buffer, and 1X PCR Enhancer Solution (Invitrogen, Carlsbad, CA, USA). PCR was performed in a Whatman Biometra TGradient Thermocycler under the following conditions: denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 40 s, 58°C for 40 s, 68°C for 1 min and 30 s, and a final extension step at 68°C for 5 min. The amplified fragments were separated by 1.5% agarose gel electrophoresis prepared with 0.5X Tris-borate-ethylenediaminetetraacetic acid (EDTA) buffer. The gel was photographed and allele lengths were estimated using the Kodak Digital Science1D Image Analysis software. A 1-kb Plus DNA ladder was used as molecular weight standard.

Statistical analyses

The allele and genotype frequencies of VNTR *MUC1* between the groups "precocious" and "regular" heifers were compared by Fisher's exact test (*F*-test) using the population differentiation module of the GenePop software, version 3.4 (Raymond and Rousset, 1995). P-values ≤0.05 were considered to be significant.

The associations between VNTR polymorphisms and early puberty were analyzed as discrete data using the SAS GenMod procedure of SAS (Statistical Analysis System). Early puberty was assumed to have a binomial distribution (1 or 0) and was applied a logit link function using the procedure PROC GENMOD of SAS software. In order to avoid the dilution of the genotype effects, we grouped the genotypes according to the presence of the allele with more repeats, thus genotype 1/1 was grouped in class 1; genotype 1/2 and 2/2 were grouped in class 2; genotype 1/3, 2/3, and 3/3 were grouped in class 3; and genotype 1/4 and 4/4 were grouped in class 4. Since just one animal in the sample presented the genotype 5/5, this was included in the class 4.

The following non-linear model was used: $y_{ij}=\mu+ G_i+ e_{ij}$, where y_{ij} is the phenotype (precocious or regular) of the ij^{th} animal, μ is the general mean of the trait, G_i is the fixed effect of the i^{th} *MUC1* VNTR genotype class, and e_{ij} is the random effect associated with the ij^{th} observation. The early puberty means of each class were compared with the grouping of the other three classes by orthogonal contrasts, resulting in four orthogonal contrasts.

Results

The primers amplified five fragments of the VNTR *MUC1* locus. Estimation using the Image Analysis software permitted to correlate the fragments with the alleles of 1036, 1175, 1323, 1526 and 1894 bp described by Souza *et al*. (2007; Fig. 1). The estimated number of repeats was 13, 15, 18, 21, and 27, respectively. To simplify, we called the allele 1036 bp of allele 1, the allele 1175 bp of allele 2, allele 1323 bp of allele 3, allele 1526 bp of allele 4, and allele 1894 bp of allele 5.

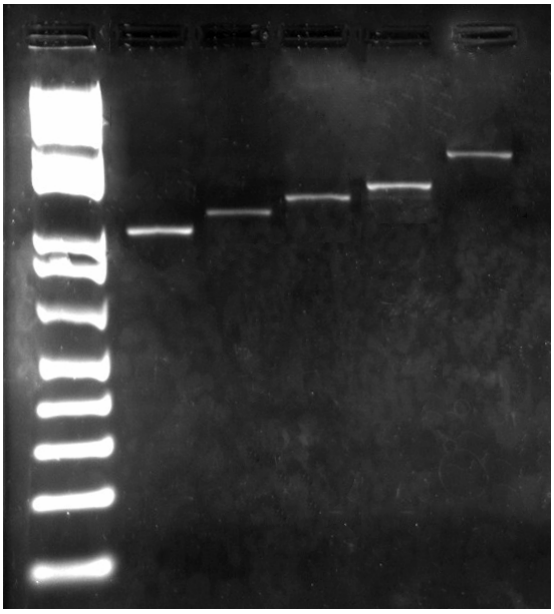

Figure 1. Gel electrophoresis showing the PCR products of five DNA samples. Lanes from left to right: 1-kb Plus DNA ladder, genotypes 1/1, 2/2, 3/3, 4/4, and 5/5. Sizes are estimated by the Kodak Digital algorithm.

Alleles containing short VNTRs were the most frequent in the sample as a whole, with allele 1 being the most prevalent, followed by alleles 2 and 3. Alleles 4 and 5 were the least frequent. Although allele 5 was the least frequent, interestingly, the homozygous genotype 5/5 was found in this sample (Table 1).

Table 1. Allele, genotype frequencies and number of animals in the genotypic classes of the *MUC1* polymorphism.

Allele	Frequency	Genotype	Frequency (n)
1	0.802	1/1	0.700 (160)
2	0.085	1/2	0.090 (21)
3	0.082	1/3	0.080 (20)
4	0.025	1/4	0.035 (8)
5	0.006	2/2	0.035 (8)
		2/3	0.010 (2)
		3/3	0.035 (8)
		4/4	0.010 (2)
		5/5	0.005 (1)

Allele frequencies were similar in sexually precocious and regular heifers, with allele 1 being the most prevalent in both groups (frequency >0.79). In the group of regular heifers was possible to observe higher frequency of the allele 2, but the *F*-test revealed no significant differences in allele frequencies between sexually precocious and regular heifers (P = 0.37; Table 2). Genotype 1/1 was the most frequent in the two groups (frequency >0.68). The genotypes 1/2 and 2/2 presented higher frequencies in the group of regular heifers than in sexually precocious heifers, but the *F*-test revealed no significant differences in genotype frequencies between precocious and regular heifers (P = 0.67; Table 2).

Since MUC1 is a transmembrane protein that differs in the length of the extracellular domain and long alleles may overlap short alleles, we formed 5 classes based in the presence of the allele with more repeats of the genotype to avoid the dilution of the effects between all 9 genotypic classes. The variance analysis (ANOVA) showed no correlation between the classes and early puberty (P > 0.05). Although no significant value was obtained, the orthogonal contrast (comparing each class with the grouping of the other three) showed that class 2 (constituted by genotypes 1/2 and 2/2) had a value more close to significance (P = 0.19) than the other classes (Table 3).

Table 2. Allele, genotype frequencies and number of animals of the *MUC1* polymorphism in sexually precocious and regular Nelore heifers.

Sexually precocious heifers (n = 77)				Regular heifers (n = 153)			
Allele	Frequency	Genotype	Frequency (n)	Allele	Frequency	Genotype	Frequency (n)
1	0.824	1/1	0.727 (56)	1	0.791	1/1	0.680 (104)
2	0.058	1/2	0.052 (4)	2	0.098	1/2	0.111 (17)
3	0.098	1/3	0.104 (8)	3	0.075	1/3	0.078 (12)
4	0.020	1/4	0.039 (3)	4	0.030	1/4	0.032 (5)
		2/2	0.026 (2)	5	0.006	2/2	0.039 (6)
		2/3	0.013 (1)			2/3	0.007 (1)
		3/3	0.039 (3)			3/3	0.032 (5)
						4/4	0.014 (2)
						5/5	0.007 (1)

Table 3. ANOVA and orthogonal contrasts results for binomial sexually precocious and regular Nelore heifers.

Source*	DF	Chi-Square	P-value
Classes	3	3.23	0.36
Orthogonal contrasts			
Classes	DF	Chi-Square	P-value
1 vs. other classes	1	0.74	0.39
2 vs. other classes	1	1.72	0.19
3 vs. other classes	1	1.53	0.22
4 vs. other classes	1	0.07	0.78

*class 1: genotype 1/1; class 2: genotype 1/2 and 2/2; class 3: genotype 1/3, 2/3 and 3/3; class 4: genotype 1/4, 4/4 and 5/5.

Discussion

The role of MUC1 transmembrane glycoprotein in mammalian reproduction is well known. In the male reproductive system, MUC1 has been suggested to play a role in the enlargement of the space between Sertoli cells and germ cells, in the acceleration of detachment and transit of delayed and abnormal germ cells, and in glycocalyx formation on the sperm surface (Martinez-Conejero *et al.*, 2005). MUC1 expression is generally associated with normal spermatogenesis (Franke *et al*., 2001; Seo *et al.*, 2005).

In the female reproductive tract, MUC1 also plays a role in many processes associated with fertility and uterine health. The high extension of the molecule and the negative charges provided by sialic acid promote a certain degree of viscosity between the uterine epithelium and spermatozoa, preventing their attachment to basal endometrial cells (Eriksen *et al*., 1998). This anti-adhesion property of MUC1 also promotes uterine health by reducing the binding of some types of microorganisms and thus preventing infection of the uterine tissue and pyometra (Ishiguro *et al*., 2007).

MUC1 is also involved in the process of embryo implantation. In mice, pigs and sheep, progesterone controls the expression of the *MUC1* gene in uterine tissue during the receptive phase. High levels of progesterone inhibited the expression of MUC1 throughout the surface of the uterine epithelium in these animals (Braga *et al*., 1993; Bowen *et al.*, 1996; Johnson *et al*., 2001). In humans and rabbits, progesterone up-regulates MUC1 and down-regulation occurs at the site of embryo implantation. In this case, MUC1 expression has been suggested to be controlled by the embryo (Hoffman *et al.*, 1998). These findings suggest that down-regulation of MUC1 during the receptive phase is important for the first contact between trophoblastic cells and the luminal epithelium (Meseguer *et al*., 1998).

Early puberty is a reproductive trait that may be affected by many processes that occur in the reproductive tract and are associated with MUC1. However, no association between MUC1 polymorphism and early puberty was observed in the present study. The observation of similar allele frequencies in pregnant and non-pregnant heifers suggests that this polymorphism is not associated with the phenotype of early puberty.

In conclusion, despite the well-known role of *MUC1* VNTR length in human reproduction, its role in bovine reproduction remains unclear and more investigations are necessary. An alternative to explore the function of *MUC1* VNTR in bovine reproduction is to establish the role of *MUC1* polymorphisms in the process of embryo implantation by studying recipient cows that present embryo implantation failure.

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