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Transcriptional profile throughout the X-inactive specific transcript (XIST) locus in the placenta of cattle

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ABSTRACT. X chromosome inactivation (XCI) compensates for the imbalance in gene expression between sexes. In mice, it is well established that the long non-coding RNA (lncRNA) X-inactive specific transcript (XIST) is essential for initiating XCI. The most well-known antisense transcript of the mouse XIST locus is TSIX, a negative modulator of XIST. However, in cattle, these events are not yet well established. In this study, we characterized the patterns of strand-specific transcription along the XIST locus in bovine fetal placenta, since understanding the regulation of gene expression in the placenta and of the transcripts involved in XCI in order to minimize

embryonic and fetal losses due to the use of assisted reproduction techniques (ARTs) is key for livestock production. Sense transcription was detected throughout the XIST locus in male and female, and antisense transcription was detected in exon 1 of female fetal cotyledons. The sense transcripts may be lncRNA XIST, while the antisense transcript identified in exon 1 is not TSIX, but rather other uncharacterized RNAs. Moreover, our results show the relevance of taking into account the possibility of antisense expression for gene expression studies, especially in non-coding RNA or pseudogenes loci, where transcription from the two DNA strands is not rare. Taken together, the results show the importance of characterization as an aid to a better understanding of XCI in cattle, considering that epigenetic reprogramming can be impaired in cattle by the use of ARTs.

Key words: X chromosome inactivation; Non-coding RNA; XIST; Antisense transcription; Sense transcription

INTRODUCTION

X chromosome inactivation (XCI) is an epigenetic mechanism that occurs during initial development in mammals to balance gene dosage between males, which have only one X chromosome and females, who have two (Lyon, 1961). The influence of long non-coding RNAs (lncRNAs) is a common feature in the regulation of XCI in different species. Loci that encode lncRNAs are usually transcribed from both DNA strands, in the sense or antisense direction, within a protein-encoding locus (Carninci, et al. 2005; Consortium et al., 2007) performing their function when the lncRNA molecules interact with transcription factors or chromatin-modifying complexes, thereby modulating the transcription of genes and regulating gene expression (Quinodoz and Guttman, 2014). X-inactive specific transcript (XIST) is one of the best-studied examples of an lncRNA that regulates gene expression and coordinates XCI (Brockdorff et al., 1991; Galupa and Heard, 2015).

XCI is controlled by a genomic region known as the X chromosome inactivation center (XIC) (Russell, 1963), in which the genes that are essential for the establishment of XCI are located, with XIST and its antisense TSIX being the main studied genes (Penny, et al., 1996). XIST triggers silencing in cis (Okamoto and Heard, 2009), recruiting several epigenetic factors to compact chromatin and silence the transcription of genes (Sado and Brockdorff, 2013), which is essential for initiating inactivation (Penny et al., 1996). In contrast, TSIX suppresses the expression of XIST, marking the future active X chromosome in cis (Lee et al., 1999; Stavropoulos et al., 2001). Furthermore, a new lncRNA, antisense of XIST, was characterized in mice, known as Xist activating RNA (XistAR), and found in exon 1 of XIST. Unlike TSIX, which suppresses XIST transcription and is expressed from the future active X chromosome, XistAR is transcribed only by the X chromosome to be inactivated, inducing the expression of XIST in cis, which is essential for XCI (Sarkar et al., 2015).

The analysis of the XIST lncRNA sequence revealed that XIST possesses blocks of repetitive tandem regions, known as A-F repeats (Sado and Brockdorff, 2013). The RepA

region, located within exon 1 of XIST, transcribes the RepA lnRNA, which is also essential for the transcriptional activation of the XIST gene to initiate the silencing of the X chromosome, and binds to PRC2 subunits, such as EZH2 and SUZ12, and recruits repressive proteins from the polycomb complex for the X chromosome to be inactivated (Zhao et al., 2008). Thus, at least in mice, two shorter lncRNAs emerging from exon 1 of XIST are essential for XIST transcription and, in turn, the initiation of X inactivation.

In comparison to mice, little is known about the process of XCI in cattle, especially regarding the role of lncRNAs within this process. The bovine XIST gene is known to undergo alternative splicing and is capable of generating three variants, X1 (XR_001495594.2), X2 (XR_001495596.2), and X3 (XR_001495595.2), which are generated depending on the tissue and sex. Mendonça et al. (2019) characterized the profile of DNA methylation and expression of the XIST locus during the initial development of cattle, providing insights into the transcriptional profile of XIST in livestock. Nevertheless, little is known about the ncRNAs that participate in XCI in cattle. Assisted reproductive techniques (ARTs), such as *in vitro* embryo production and cloning, are commonly used in cattle production and are essential for animal improvement and biotechnology. Nevertheless, ART efficiency is low, especially cloning (Chavatte-Palmer et al., 2012; Siqueira et al., 2020). Xue et al. (2002) showed for the first time that in conventional breeding cattle the XCI patterns are normal, however, aberrant and irregular patterns are identified in clones that died. The use of ARTs can alter the epigenetic profiles of embryos and, consequently, impair the XCI process, compromising the quality of the embryos. Mann et al., analyzed the methylation and expression of the imprinted gene in the placenta of mice soon after preimplantation development in vitro and suggested that tissues that have a trophectoderm origin are not able to recover genomic imprints and that the mechanisms involving imprinting are less developed in the placenta than in the embryo (Mann et al., 2004). Among the various problems generated in the context of ARTs, placental changes are very frequent, generating embryonic and fetal losses throughout the gestational period (Rivera, 2020). Therefore, the placenta should be an important focus of study aimed at minimizing these losses and understanding the regulation of gene expression and function of the placenta is essential to improve ART protocols. In the present study, we used the XIST locus as a model to improve our understanding of the mechanisms of gene regulation of lncRNAs in the placenta of cattle. Thus, the aim of this study was to characterize the transcriptional profile of the XIST locus in the fetal placenta of cattle.

MATERIAL AND METHODS

Obtaining the biological material

Samples of placental tissue (fetal cotyledon) from pregnancies produced by artificial insemination using sexed semen were collected from two births of Nellore animals (one female and one male) immediately after delivery. Sterile scissors and tweezers were used to obtain the fetal cotyledons, which were placed in sterile 1.5-mL microtubes. Then, RNAlater (Ambion, Austin, TX, USA) was added to a volume necessary to cover all the collected fragments and then packed in ice until transported to the laboratory. Samples were stored at -80ºC for later RNA extraction.

Total RNA extraction and treatment with DNase

Total RNA was isolated using the PureLink™ RNA Mini Kit (Ambion, Austin, TX, USA). Firstly, 100–200 mg of the samples were macerated in liquid nitrogen. The resulting powder was transferred to a 2.0-mL microtube, to which 1 mL of Trizol™ Reagent (Invitrogen, Carlsbad, CA, USA) was added to every 100 mg of macerated tissue. The following steps of the protocol were performed according to the manufacturer's recommendations, including treatment with 10 U of DNase during RNA isolation. Finally, the purified total RNA samples were quantified, and the samples were again treated with RQ1 RNase-Free DNase® (Promega, Madison, W1, USA) at 37°C for 30 min, using 2 U of the enzyme for each 1 µg of total RNA to ensure that the total RNA samples were free from contamination with genomic DNA. The stopping solution was added following an incubation step at 65°C for 10 min to inactivate the enzyme.

cDNA Synthesis

Total RNA (1 µg) was subjected to complementary DNA synthesis using the GoScript™ Reverse Transcription System (Promega, Madison, W1, USA). Gene-specific primers (forward or reverse) for exons 1, 4, and 6 of the XIST gene were used at a final concentration of 0.5 µM. The XIST *Bos taurus* sequence has six exons and three isoforms of alternative splicing can be found: X1 (XR_001495594.2), X2 (XR_001495596.2), and X3 (XR_001495595.2). The specific primers for exon 1, XIST1 and XIST1.1, were designed from the XIST RNA sequence of cattle. XIST1 was found in variants X2 and X3, while XIST1.1 was only observed in variant X2. The XIST4 (exon 4) and XIST6 (exon 6) primers were designed using the reverse and complementary sequences of XIST *GenBank* XR_001495596.2 for the three variants, namely X1, X2, and X3. Primers were designed using IDT Primer Quest software (http://www.idtdna.com/Scitools/Applications/Primerquest/). The primer sequences are listed in Table 1.

Table 1. Primer sequences, concentrations, and amplicon length for XIST.

Name		Primer Sequence (5'-3')	Primer Concentration	Amplicon length (bp)
XIST1	F:	TTTACCCAACGGGGTCATGG	200 nM	139
	R:	AGGGGGTACACAGCAGGTAT		
XIST1.1	F:	CTGCCGTGTGCATTTCCTG	200 nM	172
	R:	AGCACGCCATCTTTAACAATG		
XIST4	F:	GAGTTGTTGCTCAGTGGTAG	200 nM	96
	R:	CAGTGTTAGTGACCCATTCC		
XIST6	F:	GGACCAGACTCCACCAAGAAA	200 nM	159
	R:	GAAATGGGCCTAGTCTAAAGG		

F (forward); R (reverse); nM (nanomolar); bp (base pair).

As a positive control, cDNA synthesis was performed using Oligo$(dT)_{15}$ and random primers. As a negative control, to demonstrate the absence of contamination by genomic DNA, cDNA synthesis was performed using Oligo$(dT)_{15}$ and random primers in the absence of the reverse transcriptase enzyme (RT-). Amplicons corresponding to the cDNAs synthesized using XIST1 and XIST1.1 forward primers refer to antisense

transcripts, whereas those corresponding to the cDNAs synthesized using the reverse primers refer to sense transcripts. On the other hand, the specific primers for exons 4 (XIST4) and 6 (XIST6), which were designed from the reverse and complementary sequence of XIST, showed the opposite result. Amplicons corresponding to the cDNAs synthesized using the forward primers refer to sense transcripts, while those synthesized using reverse primers refer to antisense transcripts (Figure 1).

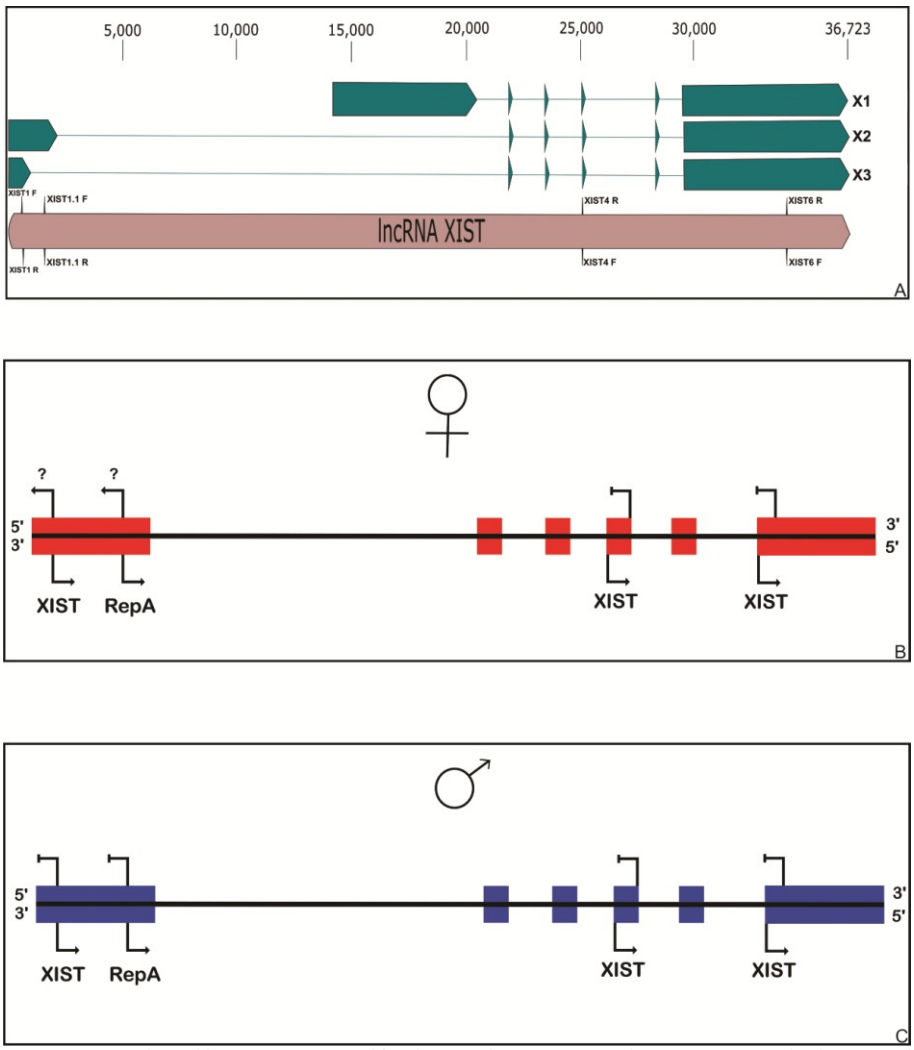

Figure 1. (A) Representation of lncRNA XIST and its three variants. XIST1 primers are present in the X2 and X3 variants; XIST1.1 primers only in the X2 variant. Both primers pairs are found in exon 1 of XIST. The XIST4 and XIST6 primers are present in all three variants, in exon 4 and exon 6, respectively. Figure adapted from the Geneious Prime program. **(B)** Summary of results obtained in female fetal cotyledon. Red bars represent exons 1, 2, 3, 4, 5, and 6, respectively. The sense strand shows that there was transcription in exons 1 (XIST and RepA), 4 (XIST), and 6 (XIST), and the antisense strand indicates that there was transcription only in exon 1 (XistAR/other(s)). **(C)** Summary of results obtained in male fetal cotyledon. The blue bars represent exons 1, 2, 3, 4, 5, and 6, respectively. The sense strand shows that there was transcription in exons 1 (XIST and RepA), 4 (XIST), and 6 (XIST); however, no antisense transcription was detected (upper part of the Figure).

Strand-Specific Reverse Transcription Polymerase Chain Reaction (SS-RT-PCR)

cDNA was used for real-time PCR. SS-RT-PCR analyses were performed on a 7500 Fast Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using the GoTaq® qPCR Master Mix (Promega, Madison, USA). The amplification conditions were as follows: 95°C for 2 min and 30 s, followed by 50 cycles of denaturation at 95°C for 15 s, and primer annealing and extension at 60°C for 1 min. Unlike XIST1 primers, the annealing and extension conditions were 58°C and 72°C for 1 min, respectively. Sense and antisense expression were analyzed for exons 1 (XIST1 and XIST1.1), 4 (XIST4), and 6 (XIST6) of the XIST locus. The SS-RT-PCR technique used in this study is presented in Figure 2. Amplicons were subjected to electrophoresis at a constant electrical current of 50 mA in agarose gel 2.0% stained with SYBR Safe in 0.5× TBE buffer. The molecular weight marker 1 Kb Plus DNA Ladder® (Invitrogen, Carlsbad, CA, USA) was used. Amplicons were cut from the gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), according to the manufacturer's recommendations. Samples were then quantified using a NanoDrop® (ND-1000) spectrophotometer (Thermo Scientific, Asheville, NC, USA) and stored at -20°C until use.

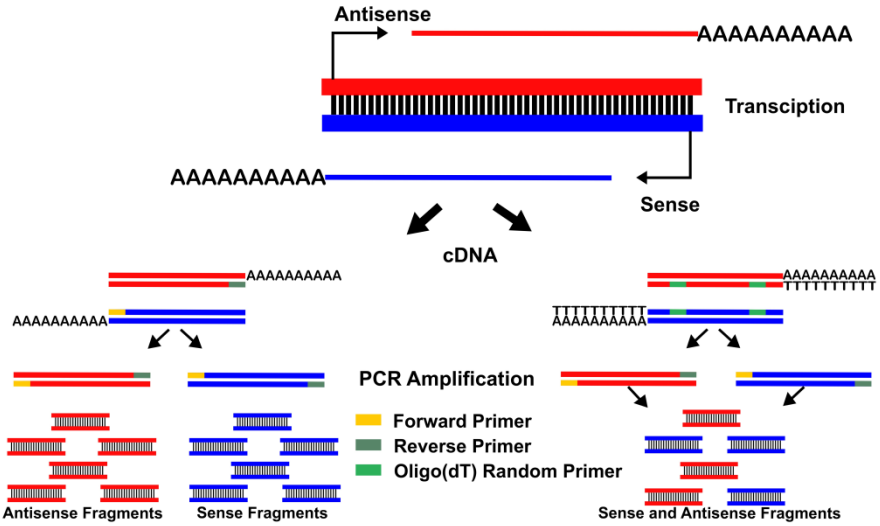

Figure 2. Representation of the SS-RT-PCR technique that was used to analyze the sense and antisense transcripts along the XIST locus. The red strip represents the antisense transcripts and the blue strip the sense transcripts. Both show a polyA tail at the 3'-end and are part of the same locus. In addition to specific primers (yellow and gray bars), Oligo(dT) and random primers (green bars) are also used to make cDNA synthesis. Samples are then amplified by real-time PCR.

Cloning of PCR products and plasmid DNA extraction

After purification, amplicons from each group (XIST1, XIST1.1, XIST4, and XIST6) were inserted into the TOPO TA Cloning® vector (Invitrogen), according to the manufacturer's instructions. Transformation was performed in DH5α cells by thermal shock, and the product was spread in petri dishes (90 × 15 mm) containing agar with

ampicillin (100 µg/mL), 40 µL of X-Gal (Sigma) at 20 mg/mL, and 4 µL of IPTG 0.1 M (Sigma), followed by the inversion of the plates and incubation in an oven at 37°C for 14 h and at 4ºC for two hours. Two white colonies from each group were selected for cultivation in Luria-Bertani (LB) liquid medium supplemented with 100 µg/mL ampicillin. A sterile wooden toothpick was used to remove the colony from the growth plate and to deposit it in a 15-mL tube containing 3 mL of LB medium. The tubes were agitated at 250 rpm at 37°C for 16 h in an agitator (New Brunswick Scientific Co., NJ, USA). Half of the contents of the tubes were used for plasmid extraction, following the protocol of the PureYield™ Plasmid Miniprep System (Promega, Madison, USA). The plasmid DNA was quantified using a NanoDrop® (ND-1000) spectrophotometer (Thermo Scientific, Asheville, NC, USA) and stored at -20ºC until use.

Sequencing of plasmid DNA and analysis of the sequences

Plasmid samples containing the DNA of interest were digested with 5 U of EcoRI enzyme at 50 U/µL, 1× H-buffer, and 5 µL of DNA; the reaction volume was adjusted to 10 µL using deionized water. The samples were placed in a water bath at 37°C overnight. The digested products were subjected to electrophoresis on a agarose gel 2.0% stained with SYBR Safe 10 mg/mL to confirm the presence of fragments of interest. Plasmid DNA samples (80 ng/µL) containing the inserts were sequenced by the Sanger method using the M13 reverse universal primer. The quality of the chromatograms was analyzed using CHROMAS® 2.4.4. The sequences obtained were compared with the corresponding sequences deposited in *GenBank* using the BLASTN tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

RESULTS

The identity of the products was confirmed using three methods: (1) amplicon size (139 bp for XIST1, 172 bp for XIST1.1, 96 bp for XIST4, and 159 bp for XIST6); (2) melting temperature (Tm) (~81°C for XIST1, ~84°C for XIST1.1, ~80°C for XIST4, and ~77°C for XIST6); (3) sequencing.

Characterization of the sense and antisense gene expression of female cotyledons by SS-RT-PCR for the XIST locus

For the characterization of the sense and antisense transcription of female cotyledons, the positive controls (cDNA synthesized with oligo(dT)/random primers) validated the primer-specific amplifications and the negative controls (RT- and PCR in the absence of cDNA) confirmed the absence of contamination by genomic DNA.

In exon 1, the amplification of the positive control and cDNAs synthesized using the XIST1 forward and reverse primers produced an amplicon of 139 base pairs (Figure 3). Furthermore, in exon 1, using the XIST1.1 primers, the amplification of cDNAs was performed using the XIST1.1 forward and reverse primers, producing an amplicon of 172 base pairs (Figure 3).

Figure 3. SS-RT-PCR using XIST1, XIST1.1, XIST4, and XIST6 primers (exons 1, 4, and 6) at the XIST locus in the female fetal cotyledon. **(A)** Amplification curves and Ct values (threshold cycle) from cDNA synthesized with oligo(dT) and random primers (C+); cDNA using forward (XIST1, XIST.1.1, XIST4, and XIST6 F) and reverse (XIST1, XIST1.1, XIST4, and XIST6 R) gene-specific primers and cDNA without the presence of reverse transcriptase (C-). Samples are in duplicates. **(B)** 2% agarose gel electrophoresis, demonstrating the 1 kb Plus DNA Ladder-Invitrogen marker (lane 1), amplifications of C+ (lanes 2-3), primers F (lanes 4-5), primers R (lanes 6-7), C- (lanes 8-9), and PCR- (lane 10). Amplicon size is 139 base pairs for the XIST1, 172 bp for XIST1.1, 96 bp for XIST4, and 159 bp for XIST6. **(C)** Melting curves of C+ amplicons (2-3), forward primers (4-5) and reverse primers (6-7).

In exon 4, the positive control and cDNAs were amplified using the XIST4 forward and reverse primers, producing an amplicon of 96 base pairs (Figure 3). The agarose gel bands of the samples that used the cDNA synthesized with the XIST4 reverse primer were not very well defined and were slightly larger than 96 bp (Figure 3).

In exon 6, the positive control, and the samples that used cDNAs synthesized using the XIST6 forward primer, producing an amplicon of 159 base pairs were amplified. However, PCR using cDNA synthesized using the XIST6 reverse primer was not amplified (Figure 3).

All amplicons were sequenced and the BLASTN tool was used. XIST1 exon 1 amplicons showed 99% identity with the X2 and X3 sequences of the bovine *XIST* transcript (Figure S1). XIST1.1 exon 1 amplicons showed 100% identity with the X2 sequence (Figure S2). These results confirm that there was sense (referring to cDNA synthesized with reverse primers) and antisense (from cDNA synthesized with forward primers) expression in exon 1 of the XIST gene in female fetal cotyledons.

The XIST4 forward exon 4 amplicon showed 100% identity with the three variants of bovine XIST (X1, X2, and X3) (Figure S3). The XIST6 forward exon 6 amplicon also showed 100% homology with all three variants (Figure S4). However, the sequencing results of the samples that used cDNA synthesized with the reverse primer of XIST4 did not align with any XIST sequence deposited in GenBank. However, it showed 93% identity with the sequence "Ovis canadensis isolate 43U from chromosome 17" (Figure S3). As the XIST6 reverse primer was not amplified, no sequencing was performed. Thus, the results indicate that while the sense transcripts were expressed (referring to cDNA synthesized with the forward primer), there was no expression of the antisense transcripts (from cDNA synthesized with the reverse primer) in exons 4 and 6 of the XIST gene in female cotyledons.

Characterization of the sense and antisense gene expression of male cotyledons by SS-RT-PCR for the XIST locus

Regarding the results described below, of fetal cotyledon in male, they were found to be similar to those in female, except that no antisense transcripts were detected for exon 1 (XIST1 and XIST1.1) (Figure 4). The cDNAs synthesized using oligo(dT) and random primers, referring to the positive control, validated the primer-specific amplification and the absence of amplification of the negative controls (RT- and PCR in the absence of cDNA) confirmed that there was no contamination by genomic DNA or cDNA in none of the samples (Figure 4).

In XIST exon 1, the cDNA was amplified using specific primers XIST1 forward and reverse primers. Although all samples were amplified, only the positive control and those using the XIST1 reverse primer showed a correct amplicon of 139 base pairs. Amplicons produced by the XIST1 forward primer were lower in size and showed a slightly lower melting temperature compared to the positive controls and amplicons using cDNA synthesized using reverse primer (Figure 4). In exon 1, using XIST1.1, cDNAs synthesized with the specific XIST1.1 reverse primer were found to be amplified, with an amplicon of 172 base pairs. However, cDNA samples synthesized with the XIST1.1 forward primer were not amplified (Figure 4).

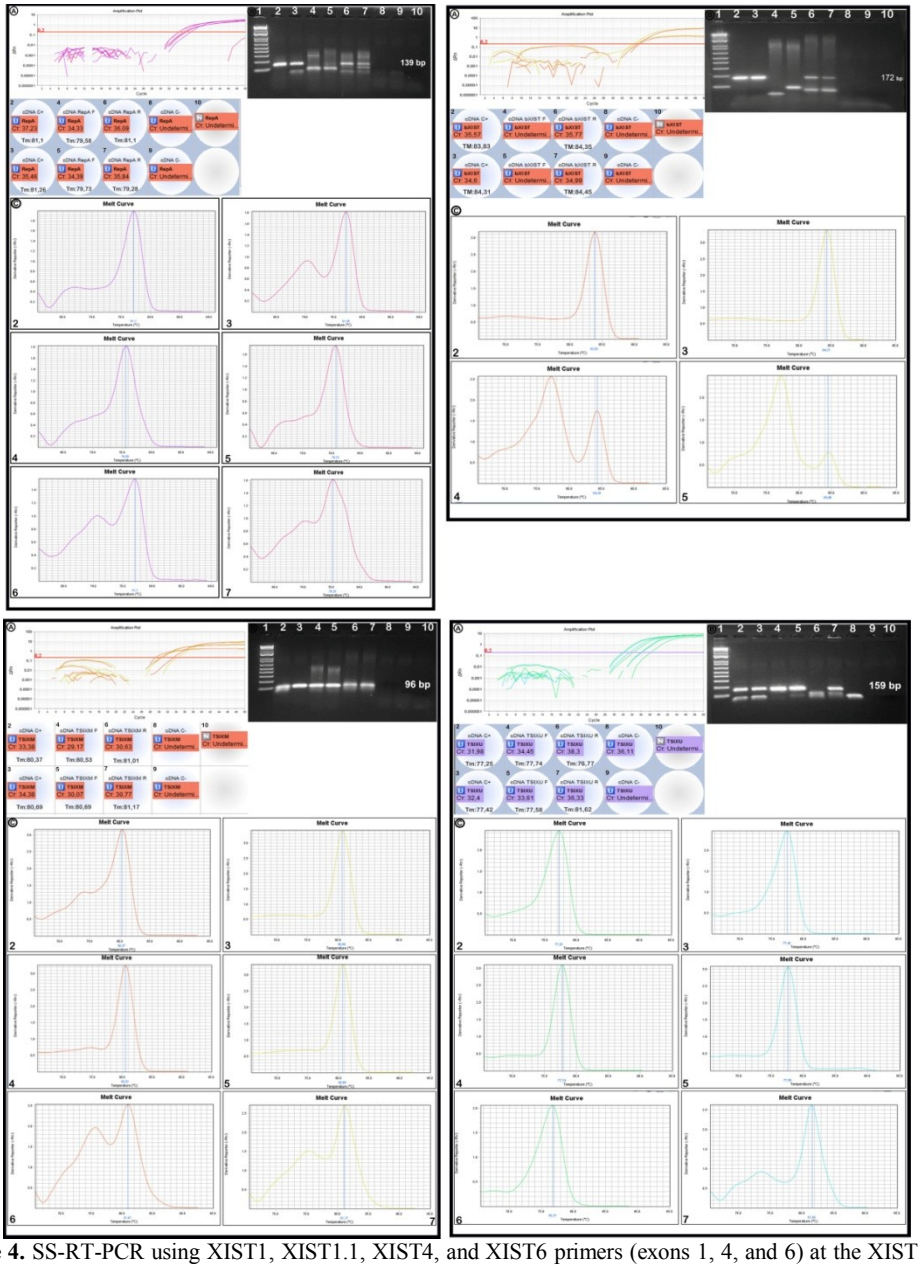

Figure 4. SS-RT-PCR using XIST1, XIST1.1, XIST4, and XIST6 primers (exons 1, 4, and 6) at the XIST locus in the male fetal cotyledon. **(A)** Amplification curves and Ct values (threshold cycle) from cDNA synthesized with oligo(dT) and random primers (C+); cDNA using forward (XIST1, XIST1.1, XIST4, and XIST6 F) and reverse (XIST1, XIST 1.1, XIST4, and XIST6 R) gene-specific primers and cDNA without the presence of reverse transcriptase (C-). Samples were performed in duplicate. **(B)** 2% agarose gel electrophoresis, demonstrating the 1 kb Plus DNA Ladder-Invitrogen marker (lane 1), C+ amplifications (lanes 2-3), forward primers (lanes 4-5), reverse primers (lanes 6-7), C- (lanes 8-9), and PCR- (lane 10). Amplicon size is 139 base pairs for the XIST1, 172 bp for XIST1.1, 96 bp for XIST4, and 159 bp for XIST6. **(C)** Melting curves of C+ amplicons (2-3), forward primers (4-5), and reverse primers (6-7).

In XIST exon 4, cDNAs were amplified using the specific primers XIST4 forward and reverse, with an expected amplicon of 96 base pairs in size. However, the melting temperature of samples that used cDNA synthesized with the XIST4 reverse primer proved to be slightly higher than that of the positive controls and samples that used cDNA synthesized with the forward primer (Figure 4).

In exon 6, the samples that used cDNAs synthesized with the specific primers XIST6 forward and reverse were amplified, with an expected amplicon of 159 base pairs, except for sample 6, which showed nonspecific amplification (Figure 4).

As was observed for the female fetal cotyledon sample, the amplicons obtained from the male samples were also sequenced and analyzed using the BLASTN tool. The sequencing of XIST1 amplicons (exon 1) from the positive control and XIST1 reverse samples showed 99% identity with the sequences of the bovine XIST transcripts for the X2 and X3 variants (Figure S5). For the XIST1.1 reverse amplicons, also in exon 1, samples showed 100% identity with the sequence of the bovine XIST transcript compared to the X2 variant (Figure S6). Thus, the results showed that there was only detection of sense transcripts (referring to cDNA synthesized with reverse primers) in exon 1 of the XIST gene in male fetal cotyledons.

Amplicons produced by the positive control and XIST4 forward primers showed 99% identity with the sequences of the bovine XIST transcripts for the three variants (X1, X2, and X3) (Figure S7). However, the sequencing results of the samples referring to the cDNA synthesized with the reverse primer did not align with any XIST sequence deposited in GenBank. Despite this, an identity of 93% was obtained with an Ovis canadensis sequence, similar to the samples that used the XIST4 reverse primer in female fetal cotyledons. These results indicate that while sense transcripts were detected (referring to cDNA synthesized with the forward primer), no antisense transcripts (from cDNA synthesized with the reverse primer) were detected in exon 4 of the XIST gene in male fetal cotyledons (Figure S7).

Amplicons from exon 6 (XIST6) referring to the positive control and XIST6 forward primers were sequenced and showed an identity of 100% with the sequences of the bovine XIST transcripts for the three variants (X1, X2, and X3). However, the result of the amplicon sequencing using cDNA synthesized with the reverse primer (line 7 on the agarose gel, Figure 4), did not align with any XIST sequence deposited in GenBank, confirming the melting temperature result, which was different from those of the XIST locus-specific amplicons (Figure S8). However, they found homology with two other regions of the *Sus scrofa* and *Felis catus* species. Thus, the results indicate that sense transcripts were detected (referring to cDNA synthesized with the forward primer), but no detection of specific antisense transcripts (from cDNA synthesized with the reverse primer) in exon 6 of the XIST gene in fetal cotyledons of male (Figure S8).

In general, the amplicon sequencing results from female fetal cotyledons showed that there was sense transcription throughout the entire XIST locus (exons 1, 4, and 6) and antisense transcription only at the beginning of the gene in exon 1 (XIST1 and XIST1.1). On the other hand, the sequencing of amplicons from male fetal cotyledons showed only sense transcription along the XIST locus (exons 1, 4, and 6) (Figure 1B and 1C).

DISCUSSION

XIST is the main gene that regulates XCI. Thus, the results presented in this work, characterizing the transcriptional profile along the XIST locus in the bovine placenta, are important to improve our understanding of XCI and the influence of breeding techniques on the inactivation of X in cattle. XIST is essential for the initiation and maintenance of inactivation (Gribnau and Grootegoed, 2012). When XCI does not occur correctly, the development of the embryo is affected, preventing it from developing normally (Barakat et al., 2010).

Serious consequences for the development and growth of the embryo and placenta occur when there is a loss of imprinted gene expression (Mann et al., 2004). We know that XCI in mice is imprinted (Okamoto et al., 2004) and that genomic imprinting is not stable in placental tissues, which detected biallelic expression in several genes and suggested that epigenetic disruptions occur in some specific tissues during preimplantation development in vitro (Mann et al., 2004). In cattle, it has been shown by analysis of MAOA gene expression that XCI in bovine placentas is imprinted, with the paternal X is preferentially inactivated (Xue et al., 2002). Considering that XCI is an essential process for normal embryonic development, understanding XCI in cattle can also offer the possibility of identifying epigenetic markers associated with embryonic quality.

To characterize the expression profile along the XIST locus, we used the SS-RT-PCR method, which can distinguish sense and antisense transcripts sharing the same region of the genome, but transcribed in opposite directions.

Antisense transcripts usually have a part superimposed on the RNA sense, may or may not encode proteins, and generally regulate sense RNAs that encode proteins (Faghihi and Wahlestedt, 2009). As is the case with TSIX in mice, which is an antisense transcript and negative modulator of XIST, which overlaps throughout the lncRNA XIST repressing it (Lee et al., 1999, Lee and Lu, 1999, Stavropoulos et al., 2001). Another study showed the presence of a new antisense transcript of XIST in mice known as XistAR, which is expressed on the inactive X chromosome and induces the expression of XIST in addition to being emerging in exon 1 of XIST (Sarkar et al., 2015).

Our results on the evaluation of exons 1, 4, and 6 of XIST showed that sense transcription occurred throughout the XIST locus, while antisense transcription only occurred in exon 1 in the fetal cotyledon of female (Figures 1B and 1C). Sequencing showed 99% to 100% homology with the lncRNA XIST of *Bos taurus*, which suggested that the transcripts expressed in the sense direction represent the XIST RNA. As it was transcribed in exons 1, 4, and 6, we believe that it is the same RNA expressed throughout the locus. However, it is possible that sense transcripts emerging in exon 1, in addition to representing XIST, can also represent the shorter lncRNA RepA, as in mice, considering that RepA transcribes in the same direction as XIST and is critical for the initiation of XCI. (Zhao et al., 2008).

In the scientific literature, research on antisense transcription at the XIST locus in cattle is scarce. For this reason, information from other organs, tissues, or other mammalian species can be used to gain a better understanding of XCI in cattle. One study compared XCI in mice, cattle, and human species, and found that, among 11 genes identified in murine species, only XIST, TSIX, and JPX were found in the XCI region of cattle (Chureau et al., 2002). However, no homology was observed between the region promoter TSIX in

cattle and humans compared to TSIX in mice (Chureau et al., 2002). This raises important questions regarding the regulatory role of this gene in the XCI of other mammals (Migeon, 2003).

The antisense transcript that we detected here may be the XistAR counterpart of mice, or other RNA(s) that have not yet been characterized. A study in our laboratory reported the existence of several transcripts in cattle that overlap the regions that have homology to the TSIX and XistAR genes of mice and humans (Mendonça et al., 2019). However, unlike in mice, TSIX is completely superimposed on the XIST gene (Shevchenko et al., 2011). In cattle, the antisense transcript overlaps only in a part of XIST (Horvath et al., 2011). These results suggest that this antisense transcript detected in exon 1 is not from TSIX. However, we were unable to detect antisense transcription in exons 4 and 6.

The detection of a sense transcript, most likely XIST, in the fetal cotyledon of female is in agreement with the literature, which demonstrates that XIST is expressed in the cells and tissues of females, fulfilling its role in XCI in mammals (Brockdorff et al., 1991; Kaneda et al., 2017). However, we suggest that the antisense transcript that we identified could be the counterpart of XistAR in mice, because it is present only in exon 1. This antisense transcript induces the expression of XIST (Sarkar et al.; 2015), which contributes to XCI, since the fetal placenta of females was analyzed. Moreover, our interpretation is in accordance with the results of an *in silico* analysis that detected the homology of six mouse transcripts with XistAR, which overlapped with exon 1 of bovine XIST (Mendonça et al., 2019).

In male fetal cotyledons, contrary to what was expected, the expression of sense transcripts, most likely the XIST RNA, was observed throughout the XIST locus (exons 1, 4, and 6). However, we did not identify antisense transcription. Despite evaluating different tissues, a previous study also identified antisense transcription in exon 1 in male gonads and somatic tissues (Farazmand et al., 2004). Similarly, Mendonça et al. (2019) identified antisense transcription in testicular tissue in XIST exons 1, 2, and 6 (Mendonça et al., 2019). Mann et al. (2004), showed that in mice, there was aberrant expression of XIST in male placentas and suggested that the TSIX antisense transcript was either inactivated or that transcription was not initiated (Mann et al., 2004).

Our findings disagree with those of Kaneda et al. (2017), who reported that XIST is not expressed in male cells (Kaneda et al., 2017). However, our results are in accordance with those of Mendonça et al. (2019), who detected sense transcription in the bovine testicular tissue and assumed that XIST may be expressed in a particular way in each tissue (Mendonça et al., 2019).

Because of the complexity of XCI and the limited knowledge of this event in cattle, the characterization and study of the gene expression profile of transcripts involved in this process are extremely important for livestock production. Our results show that there is sense transcript along the entire XIST locus in female and male bovine fetal cotyledon and presence of antisense transcripts only at the beginning of the XIST locus in female fetal cotyledon, contributing to the literature regarding the understanding of XCI in bovine. Furthermore, they reinforce the importance of evaluating with caution results of gene expression studies using cDNA synthesized with Oligo(dT) or random primers, since there may be transcription in both strands of DNA thus overestimating the transcript levels studied. The fact that we used the SS-RT-PCR method resulted in significant results in relation to gene expression, as we were able to distinguish sense transcription from

antisense, which other studies have normally not been able to do. With this, our results show the relevance of taking into account the possibility of antisense transcript expression when proposing to perform gene expression studies, especially at ncRNA locus, where transcription of both DNA strands is not rare. The more detailed information we have from the XCI process, the better the understanding of this process. Consequently, this will allow us to alleviate the problems that occur in epigenetic reprogramming, mainly with the use of ARTs.

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AUTHORS' CONTRIBUTIONS

P.S.C., A.F.L.R., and L.N.V. performed the bioinformatics analyses. P.S.C., L.N.V., T.C.F.S., A.R.C., and M.M.F. performed the genomic analyses. P.S.C and M.M.F. designed the experiment, interpreted the results, and wrote the manuscript. All authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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