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Methylation-specific PCR analysis in Col8A$_1$ promoter in Creole cattle carrier of rob(1;29)

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Abstract Robertsonian translocation (rob(1;29)) is the most frequent structural chromosomal abnormality in cattle. Heterozygous carriers have a normal phenotype but show a 3-5% decrease in fertility. Chromatin decondensation was evaluated similar to the inactive X chromosome when submitted to demethylating agent. Based on this result, and the concept that imprinted genes are essential in embryonic development, we decided to query genes located on BTA1 and BTA29 that could undergo genome imprinting. The collagen typeVIII-α$_1$ (Col8A$_1$) acted on extracellular matrix structural proteins. DNA bisulfite conversion and sequentiation methods were used to compare its differential methylation patterns. It was performed on eight Creole cattle DNA blood samples from normal and rob(1;29) carriers. An *in silico* screening for CpG islands in its promoter uncovered a single region of 454 bp prone to methylation. BiQ-Analizer software was used to show the selective conversion of unmethylated cytosines to uracils obtaining the following results: unmethylated CpGs: 0.000 (0 cases), methylated CpGs: 0.802 (77 cases) and CpGs not present: 0.198 (19 cases). No differences between samples were observed in this highly methylated region. This technique was successfully applied so it is a straightforward methodology that can be utilized to evaluate different tissue associated to specific gene expression.

Keywords: bisulfite, collagen, Creole cattle, cytosines, methylation

INTRODUCTION

Robertsonian translocation (rob(1;29)) is the most frequent structural chromosomal abnormality in cattle (Ducos et al. 2008). Heterozygous carriers generally have a normal phenotype but show a 3-5% decrease in fertility. This negative effect is mainly observed in fertility trait indexes such as, calving interval, non-return to service and culling rates due to an early embryonic loss (Bonnet-Garnier et al. 2006; Bonnet-Garnier et al. 2008). Ducos et al. (2008) reported cytogenetic screening of livestock population in Europe where a high percentage of cattle carriers of this Robertsonian translocation (rob(1;29)) was showed. A delay of development in embryos carrying this rearrangement and a low homozygous frequency in cattle populations is reported. These observations were based on early embryos and oocyte/spermatozoa quantification experiences where either female or male parents were carriers of rob(1;29) (Bonnet-Garnier et al. 2006; Bonnet-Garnier et al. 2008). Recent work, using clastogenic and DNA demethylating agent (aphidicolin and 5-azacytidine-C) on chromosomes of Creole cows carrying rob(1;29), revealed heterozygosity in fragile site expression rob(1q$_{13/21}$;29) and chromatin despiralization of rob(1;29) similar to the inactive X chromosome of mammals (Artigas et al. 2008a; Artigas et al. 2008b; Artigas et al. 2010). Based on these results we decided to query genes located on BTA1 and BTA29 that could undergo genome imprinting. This heritable modification of cytosine residues within CpG dinucleotides represents an important epigenetic mark that affects gene expression in diverse species (Vrana, 2007; Biliya and Bulla, 2010). Particularly on BTA1 we are focusing our studies on extracellular matrix structural proteins that could be associated to placental mammal-specific orthologous groups as collagen genes: collagen typeVI-α$_1$ (Col6A$_1$), collagen typeVI-

α_2 (Col6A_2), collagen typeVIII-α_1 (Col8A_1) and collagen typeVIII-α_2 (Col8A_2) (Elsik et al. 2009). We firstly selected these genes family as the Col9A_3 is showed maternal human imprinted (Luedi et al. 2007). It is also known that on BTA29 is the imprinted gene insulin like growth factor II (IGF_2) that plays a key role in mammalian growth (Schmutz et al. 1996; Vrana, 2007). In the present study we performed an *in silico* screening for CpG islands in the collagen typeVIII-α_1 (Col8A_1) promoter located on BTA1$q_{13/21}$, to uncover putative targets of methylation. DNA bisulfite conversion and sequentiation methods were used to compare differential methylation patterns in the bovine Col8A_1 promoter. Bisulfite-conversion based PCR methods are the most commonly used techniques for methylation mapping. In the sodium bisulfite methodology, unmethylated (U) cytosine residues are converted to uracil by oxidative deamination, which is subsequently recognized as thymine while the methylated (M) cytosine remains as 5-methylcytosine (5-mC). The target sequence can be then amplified with PCR primers that are specific for bisulfite modified DNA (Frommer et al. 1992).

The aim of this study was to optimize the DNA bisulfite conversion and methylation-specific PCR methods for the bovine Col8A_1 promoter in a tissue where this gene is not expressed. We focus our analysis of methylation patterns in blood samples of Creole cattle carriers of the rob(1;29) and cattle with normal karyotypes, as they will be our animals model to process other tissues as fibroblasts, semen and early embryos.

MATERIALS AND METHODS

Total of eight Creole cattle DNA samples were extracted from peripheral blood using the classical method of phenol-chloroform. All samples belong to the genetic reserve located at the National Park of San Miguel (Uruguay). We selected 5 cows with normal karyotype (ID: 60, 734, 808, 4530, 8692), 1 male (40) and 2 females (50, 767) carriers of rob(1;29). For the bisulfite conversion, 1 ng of DNA of each sample was processed according to the protocol referred to as Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA from solutions with low concentration of DNA (Qiagen, Alameda, CA, USA). The presence of CpG islands, overlapping the 5'-UTR in the bovine Col8A_1 (Gene ID: 538564), was examined using the program (Li and Dahiya, 2002). A target region of 880 bp in the promoter of the gene was used to detect putative CpG islands using the following parameters: CpG island size >100 and GC% >60.0. Primers for unmethylated (U) and methylated (M) DNA are shown in Table 1. PCR amplifications were performed as standard protocols. The annealing temperature was set at 53ºC. Amplicons were purified using EXOSAP-IT and sequenced to examine strand specific methylation. Sequences were aligned using BioEdit (Hall, 1999). BiQ Analizer software (Bock et al. 2005) was used to analyze the selective conversion of unmetilated cytosines to uracils by bisulfite treatment (Frommer et al. 1992).

Table 1. Primers for methylation-specific PCR in the Col8α_1 promoter.

Primer	Product size	GC%	Sequence
Left M primer	191	44.00	ATTCGATAAATTGTTTTATAACGG
Right M primer	191	54.17	CAAAAAACCCTTCTTATACTCGTC
Left U primer	192	53.21	AGTTTGATAAATTGTTTTATAATGG
Right U primer	192	58.62	CCAAAAAACCCTTCTTATACTCATC

RESULTS AND DISCUSSION

All samples were successfully converted using the sodium bisulfite methodology. The query for putative target methylation regions on the promoter of the Col8A_1 gene revealed only one 454 bp CpG island.

The eight sequence traces were aligned with sequence NM_001101176, *Bos taurus* collagen, typeVIII-α_1 (Col8A_1), position from 2068 to 2255 (Table 2). The alignment of the Col8α_1 sequence revealed 37 converted cytosines (75,51%), 10 methylated cytosines (20,41%) that correspond to CpG islands, and 2 unspecific alignment (4,08%). Two positions (2165 and 2186 from NM_001101176) were unspecific

since some individuals showed unconverted cytosines, but others showed two peaks corresponding to a converted cytosine and an unconverted cytosine. The BiQ Analizer methylation analysis showed the following results: unmethylated CpGs: 0.000 (0 cases), methylated CpGs: 0.802 (77 cases) and CpGs not present: 0.198 (19 cases). Methylation results are represented in a Lollipop-style chart (Figure 1). All CpG islands in the promoter region of the gen Col8α_1 were methylated in the eight bovine samples. No differences, between the cows with normal karyotype and rob(1;29) carrier samples, were observed in this highly methylated region. Further studies are required to query the methylation patterns in the collagen gene family on BTA1. The bisulfite conversion technique was successfully applied in this study; it is a simple and straightforward methodology that can be utilized to evaluate target genome imprinting in domestic animals. Now, it could be incorporate to other tissue associated to specific gene expression, like embryos and fibroblast to know genetic cause of embryo mortality and to apply in reproduction program as animal biotechnologies.

Table 2. Position of methylated and unmethylated cytosines in the eight Creole cattle sequence traces with respect to sequence NM_001101176.

Types of Cytosines	NM_001101176 positions
Unmethylated cytosines (converted by MSP into uracils)	2075, 2079, 2082, 2084, 2093, 2097, 2102, 2105, 2108, 2109, 2112, 2120, 2123, 2126, 2128, 2129, 2138, 2139, 2140, 2147, 2150, 2153, 2162, 2163,2169, 2171, 2174, 2183, 2197, 2199, 2210, 2217, 2218, 2228, 2243, 2252, 2255
Methylated cytosines	2072, 2090, 2110, 2116, 2132, 2213, 2219, 2230, 2234, 2237

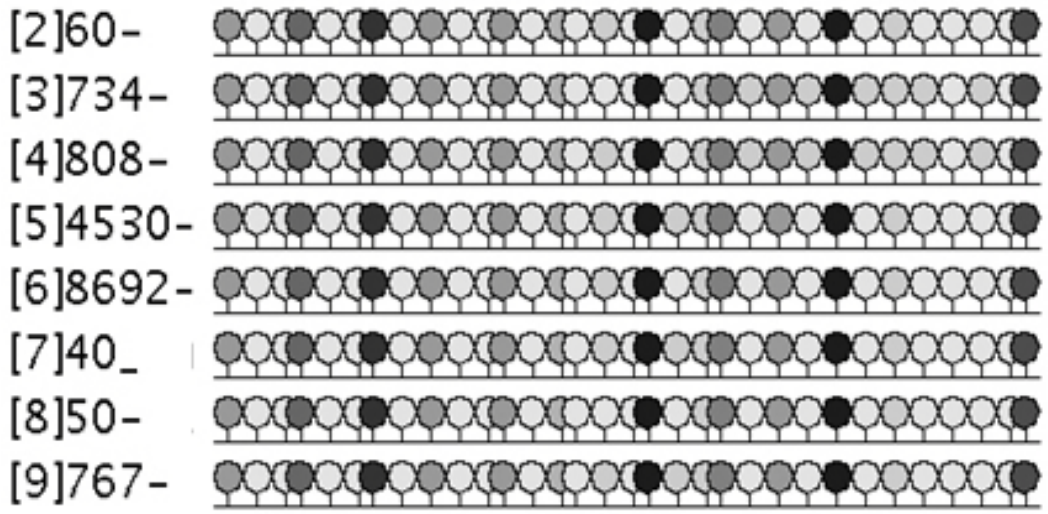

Fig. 1 Lollipop-style chart showing methylation patterns for five samples with normal karyotype (ID: 2-6) and three rob(1;29) carriers (ID:7-9). Filled (black) circles correspond to methylated Cs, unfilled (white) circles correspond to unmethylated Cs.

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