



Detection of the Brachyspina mutation in Uruguayan Holstein cows using real time PCR and melting curve analysis

María Teresa Federici Rodriguez^{1*}  Rody Artigas²  Sofia Guerra¹  Andrea Branda Sica¹ 
Noelia Vázquez²  Paula Nicolini³  Fernando Dutra Quintela⁴  Silvia Llambí² 

¹Instituto Nacional de Investigación Agropecuaria (INIA), Canelones, Uruguay. E-mail: mfederici@inia.org.uy. *Corresponding author.

²Facultad de Veterinaria, Universidad de la República (UDELAR), Montevideo, Uruguay.

³Instituto Superior de la Carne, Universidad de la República (UDELAR), Tacuarembó, Uruguay.

⁴División Laboratorios Veterinarios (DILAVE), Ministerio de Agricultura y Pesca (MGAP), Treinta y Tres, Uruguay.

ABSTRACT: *Brachyspina syndrome (BS) is a rare monogenic autosomal recessive hereditary disorder of the Holstein Friesian breed caused by a deletion of 3.3Kb in the Fanconi anemia complementation group I (FANCI) gene on BTA-21, which leads to a frame-shift and premature stop codon. Some of the consequences of BS are the reduction of the fertility rate and milk production. This study developed a simple, sensitive, rapid cost-effective assay method based on real time PCR and melting curve analysis for the detection of BS carrier animals. Sixty-eight normal homozygous and four heterozygous carrier genotypes were detected and confirmed through traditional PCR-electrophoresis analysis. We concluded that the assay we have developed proved to be a reliable, highly precise and low-cost tool, which could be used to monitor the presence of the BS mutation in uruguayan Holstein breed.*

Key words: *Bos taurus, Holstein, Brachyspina syndrome, real time PCR, melting curve analysis.*

Detecção da mutação da Brachyspina em vacas Holandês Uruguaias usando PCR em tempo real e análise da curva de fusão

RESUMO: *A síndrome de Brachyspina (BS) é um defeito hereditário monogênico autossômico recessivo raro da raça Holstein Friesian causado por uma exclusão de 3,3 KB no gene FANCI localizado no cromossomo bovino 21, o que leva a um deslocamento de quadro e um códon de parada prematuro. Uma consequência da BS é a eficiência de reprodução reduzida e a produção de leite. O objetivo deste estudo foi o desenvolvimento de um método simples, rápido e sensível, baseado em PCR em tempo real e análise da curva de fusão para identificar animais portadores de BS. Sessenta e oito genótipos homocigotos normais e quatro heterocigotos foram detectados e confirmados através da análise tradicional de PCR e electrophorese. Concluímos que o novo método é uma ferramenta confiável, altamente precisa e de baixo custo, que poderia ser usado para monitorar a presença da mutação BS na raça Holandês uruguaias.*

Palavras-chave: *Bos taurus, Holandês, síndrome de Brachyspina, PCR em tempo real, análise da curva de fusão.*

INTRODUCTION

Brachyspina syndrome (BS) (OMIA, 2020) is a rare monogenic autosomal recessive hereditary disorder of the Holstein Friesian breed (frequency of 0,06 in uruguayan cattle; ARTIGAS & FEDERICI, 2020). It is caused by a deletion of 3.3Kb in the Fanconi anemia complementation group I (*FANCI*) gene on BTA-21 which leads to a frame-shift and premature stop codon. It was first reported by AGELHOLM et al. (2006) in Denmark, followed by AGERHOLM & PEPERKAMP (2007); TESTONI et al. (2008) and ANGERHOLM et al. (2010) in other countries like Netherlands, Italy, and

Canada, respectively. Many other studies detected carriers for the allele of the disease despite not reporting clinical cases (VANRADEN, 2011; FANG, 2013; SAHANA, 2013; RÚSC & KAMINSKI, 2015; LI, 2016; ARTIGAS & FEDERICI, 2020). Brachyspina syndrome is caused by a mutation in the *FANCI* gene (Fanconi Anemia complementation group I) that is located on the bovine chromosome BTA 21. *FANCI* encodes for a monoubiquitin protein with a fundamental role in DNA repair processes (FANG, 2013). The deleterious allele is produced by a 3.3 Kb deletion spanning exons 25 to 27 of the gene and the splicing of exon 24 with 28, which produces a non-functional protein. The disease produces mostly

embryonic mortality (CHARLIER, 2012) and usually the birth of dead calves can be observed with marked malformations as growth retardation, inferior brachygnathism, caudal displacement of the ears, shortening of the spine and disproportionately long limbs (AGERHOLM, 2006; AGERHOLM, 2010; TESTONI, 2008).

Diagnostic tests for BS are based in traditional end point PCR followed by gel electrophoresis (CHARLIER, 2012). With the advances in molecular genomics, microarray platforms including many genetic disease tests became commercially available for cattle; however, they are still too expensive for monitoring just one mutation. Conversely, detection through PCR and electrophoresis analysis is laborious and time consuming. Also, it requires the amplification of the intact 3.3 kb. fragment which could be difficult to amplify in the case of low-quality DNA samples, obtained from hair samples or quick DNA extraction procedures which are usually used for the screening of a large number of samples. For that reason, we detected BS deletion using a more sensitive and reproducible technique: real time PCR followed by melting curve analysis.

Real time PCR has previously been reported to detect other mutations that cause genetic disorders in Holstein cattle by using high resolution melting (HRM) analysis: the bovine leukocyte adhesion deficiency (BLAD) and the complex vertebral malformation (CVM), both caused by SNP (single nucleotide polymorphism) (GABÓR, 2012; ILIE, 2014).

The objective of this study was to optimize a rapid, economic and sensitive one step assay to detect the BS recessive mutation through real time PCR followed by melting curve analysis and validate it using end-point PCR and electrophoresis in a reference population of Holstein breed cows. Moreover, we discussed advantages and disadvantages of the novel assay compared to other genotyping platforms.

MATERIALS AND METHODS

Samples used in this study were collected from a reference population of uruguayan Holstein breed stored in the Genomic DNA Bank stored both at the Unit of Biotechnology of INIA Las Brujas and the Genetic Section of Veterinary Faculty (UdelaR). Blood collection, DNA extraction and evaluation were performed through collaborations between the following institutions: Instituto Nacional de Investigación Agropecuaria (INIA), Dirección de

Laboratorios de Genética Veterinaria (DILAVE-Treinta y Tres) and Universidad de la República (UdelaR). DNA extraction method used was a modified protocol of GREEN & SAMBROOCK (2012) and DNA quantity and quality were evaluated using the spectrophotometer NanoDrop® ND-1000. This study included 72 high quality DNA samples with Absorbance ratio 260/280 between 1.7 and 2.0 DNA.

To optimize this novel methodology, we used DNA from eight normal samples (homozygous AA) and four BS carrier animals (heterozygous Aa) previously genotyped through end point PCR and electrophoresis (ARTIGAS & FEDERICI, 2020). Real time PCR amplifications were conducted using the *Rotor Gene Q* (Corbett Research) instrument in a 25 uL-reaction volume containing 12.5 uL of the Master Mix (*Type-it*® HRM PCR Kit QIAGEN, Hilden, Germany), 50 ng of genomic DNA, 0.3 uM of BS primers (CHARLIER, 2012) and another pair of primers which amplify an internal amplification control (IAC) (MIRK, 1995) (Table 1). The optimized PCR amplification cycle consisted on an initial denaturation step at 95 °C for 5 m, 40 cycles of 95 °C for 30 s, 55 °C for 1 m and 72 °C for 2.5 m; followed by a final extension of 72 °C for 5 m. The melt curve step was conducted by ramping the temperature from 72 to 95 °C with a hold of 90 s on the 1st step and 5 s on next steps. Fluorescence data was collected in the green channel. In figure 1 we showed the different combination of both primer concentration tested and annealing temperature tested. The melting curve was viewed by plotting the negative derivative of fluorescence (-Rn) versus temperature using Rotor Gene Q Software 1.7.75. After protocol optimization, the 72 DNA samples were genotyped.

To validate the results obtained by the real time PCR protocol developed, traditional PCR-electrophoresis technique was used to genotype the same 72 samples. End point PCR reactions were performed in a final volume of 25 uL following FANG et al. (2013) PCR conditions: 100 ng of genomic DNA, 10 uM of each primer, 2.5 uL of PCR 10X buffer (Mg²⁺ Plus), 4 ul of dNTP 5 mM and 1.5 U of Taq polymerase. The cycling program included an initial denaturation of 94 °C for 5 min, followed by 35 cycles of 94 °C 30 sec, 58 °C 1 min and 72 °C 2.5 min, with a final extension of 72 °C for 10 min. After that, we performed an electrophoresis in 1.5 % agarose with Good View™, using a 1000 and 100 bp molecular weight marker (Invitrogen). Amplicons from both real time PCR products (duplex PCR) and end point PCR were visualized using SC805-BIOTOP and BioSens Gel Imaging System V2.0.

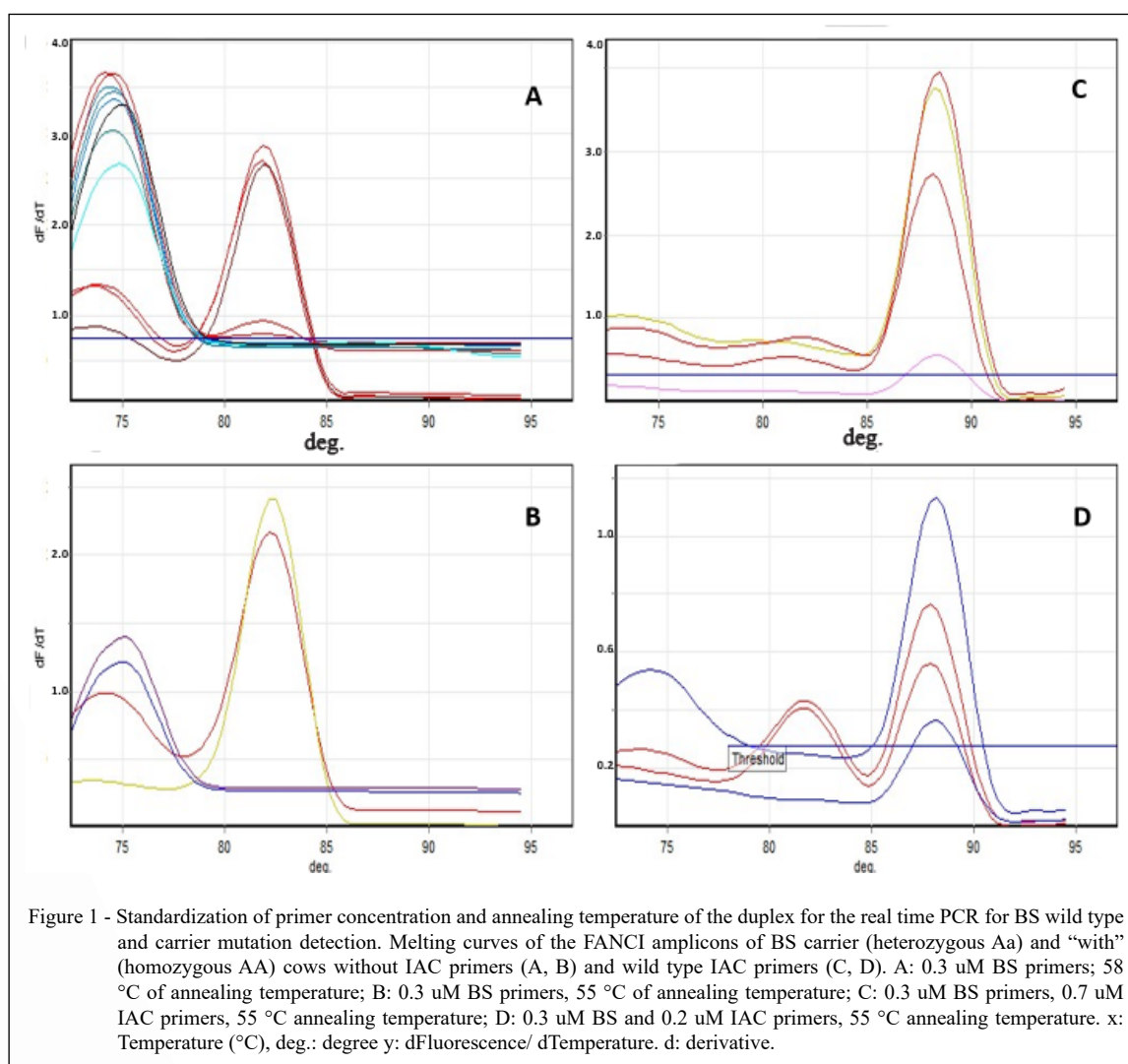
Table 1 - PCR primers, (BS, IAC): F: forward, R: reverse sequence, melting temperature (T_m) and amplification product size in base pairs (bp.)

Primer	Primer sequence 5' - 3'	Primer (T _m)	Product sizes (pb)
BY- F	5'GCTCAAGTAGTTAGTTGCTCCACTG 3'	58	3.3kb/409 bp
BY- R	5'ATAAATAAATAAAGCAGGATGCTGCTGAAA 3'	55	3.3kb/409bp
IAC- F	5'AGGCAGTTGCGTTCAACGTG 3'	54	159pb
IAC-2	5'CCGACTCGGTGATGCCATTGA 3'	56	159pb

RESULTS AND DISCUSSION

Independent PCR reactions for each pair of primers were firstly performed in order to avoid unspecific amplification fragments and determine the

temperature range of each fluorescence peak. Due to de low amplification efficiency of the mutated allele compared to the high amplification efficiency of the IAC fragment, the concentration of both primer sets was also adjusted (Figure 1). We optimized the



best conditions in order to obtain similar amplicon amplification efficiency in the same PCR reaction. The combination of annealing temperature and primer concentration that yielded the best result was ultimately selected.

An IAC is a non-target product incorporated into an assay to prevent false negative results in the event of a PCR failure. A positive IAC peak and missing target amplicon peaks in one assay would indicate that the target products were not successfully amplified. A PCR accompanied with a negative IAC and missing target products usually indicates that the reaction was inhibited as a result of malfunctioned PCR running program, or the presence of inhibitory materials (LIU, 2018). We choose a small fragment on the gene CD 18* using the primers described by MIRK (1995), which were previously used to detect BLAD (Bovine Leucocyte Adhesion Deficiency), another recessive genetic disease present in uruguayan Holstein Friesian breed (BRANDA SICA, 2016). Due to its small size (150 bp) the endogenous control fragment is efficiently amplified. When IAC primers are used in the PCR reaction, the fluorescence of BS amplicon peak decreased due to IAC competition for the PCR reaction components of the mix (Figure 1: C, D). For that reason, we decreased concentration of IAC primers in PCR reaction from 0.7 μ M (FEDERICI & BRANDA SICA, 2018) to 0.2 μ M, in order to obtain two easily distinguishable peaks. The BS amplicon peak was not distinguishable when the IAC amplicon peak was very efficiently amplified (Figure 1, C). A peak for “primer dimer” of about 73.5- 75 °C were observed in both homozygous and heterozygous genotypes (Figure 1, A, B). We can observe in Table 1 that melting temperature of BS primers differed: 58 °C (forward primer) and 55 °C (reverse primer). By using a temperature of 55 °C instead of 58 °C, the peak of “primer dimer” diminished, and probably favored a better annealing of the BS reverse primer in the FANCI gene increasing amplification efficiency of BS amplicon. (Figure 1 A, B). Finally, normalization allows elimination of differences due to the fluorescence background and increment the ability of the software to detect small differences of the melting profile.

Once the assay was standardized and the range of melting temperature for each peak was identified, low efficiencies of amplification could be detected using this method. The analysis of the dissociation curves of the amplification products revealed a clear differentiation between the samples of carrier heterozygous and those of

normal homozygous cows (AA) (Figure 2). Normal homozygous animals showed, as expected, a single temperature peak at 87.5- 88 °C, corresponding to the IAC fragment, while carrier heterozygous cows (Aa) presented a second peak in the temperature range of 81 to 82 °C, which represents the BS deleterious allele (Figure 2). Using this novel assay, we genotyped 72 DNA samples of cows of the reference population in which we detected four BS heterozygous carrier cows and 68 homozygous normal cows.

We confirmed the identity of the amplicon peaks through electrophoresis analysis of real time PCR and traditional end point PCR amplified fragments (Figure 3), compared to previously reported fragment sizes (CHARLIER, 2012; FANG, 2013). Real time PCR products of the heterozygous cows presented two amplification fragments (409 and 159 bp.) corresponding to the deleterious allele for BS and the IAC amplicon control, respectively. Conversely, real time PCR of normal homozygous AA cows presented only the endogenous control fragment of 159 pb. The traditional end point PCR amplicons presented two fragments (3738 bp., and the 409 bp) in the heterozygous cow (Figure 3, lane 12), and only one fragment (3738 bp) in the normal homozygous cow (Figure 3, lane 13), as previously described (CHARLIER, 2012; FANG, 2013).

Comparing both real time melting curves and traditional end point PCR amplification fragments through gel electrophoresis analysis, we concluded that BS allele fragment of 409 bp is represented by the peak of 87.5- 88 °C, while endogenous control gene PCR product of 150 bp is represented by the peak of 81- 82 °C. The last-mentioned fragment was detected in both heterozygous and homozygous animals while the former PCR fragment was only detected in heterozygous animals. Moreover, if we compare heterozygous carrier cows using both traditional end point PCR and melt curve analysis of real time PCR products, the fragment of 3738 bp is absent using real time PCR. It is not suitable to amplify through real time PCR due to the big size, as real time PCR products are recommended to be smaller than 400 pb. (WITTER et al., 2009). Instead, IAC fragment would amplify in all samples, as an internal reaction control. As we did not have any sample of cows with Brachyspina syndrome, samples presenting the BS allele of 409 bp. would be carrier cows. Heterozygous BS carriers would not be distinguished from recessive BS homozygous cows because the fragment of 409 bp would appear in both genotypes. Therefore, the technique presented here would not

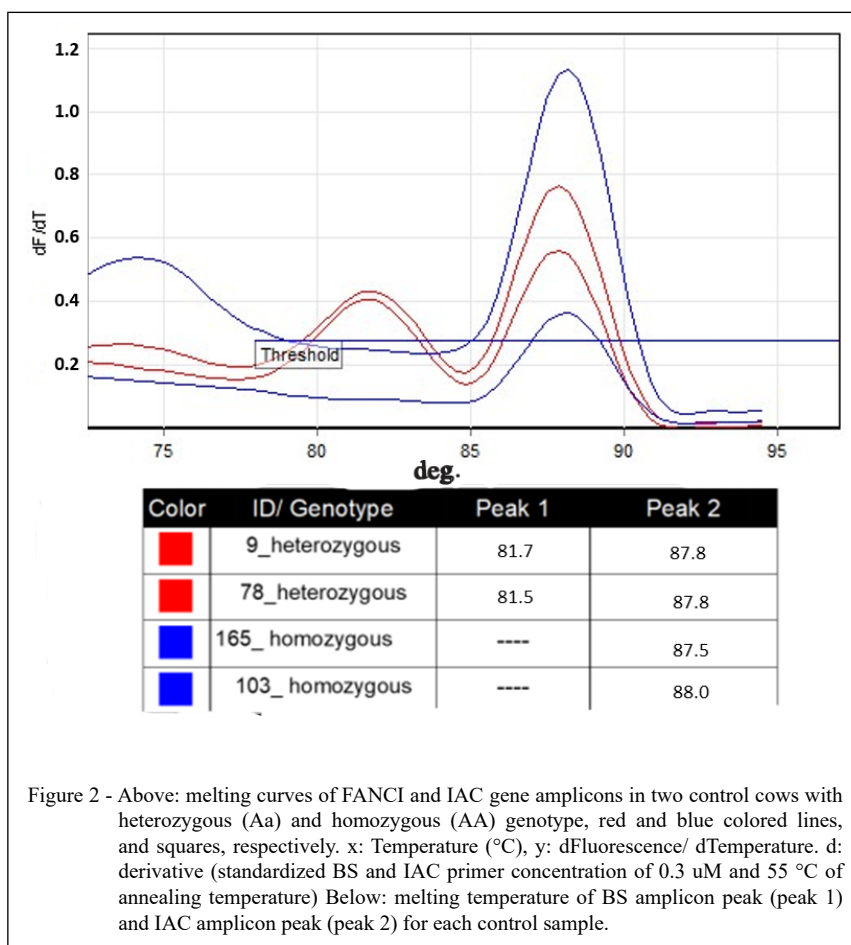
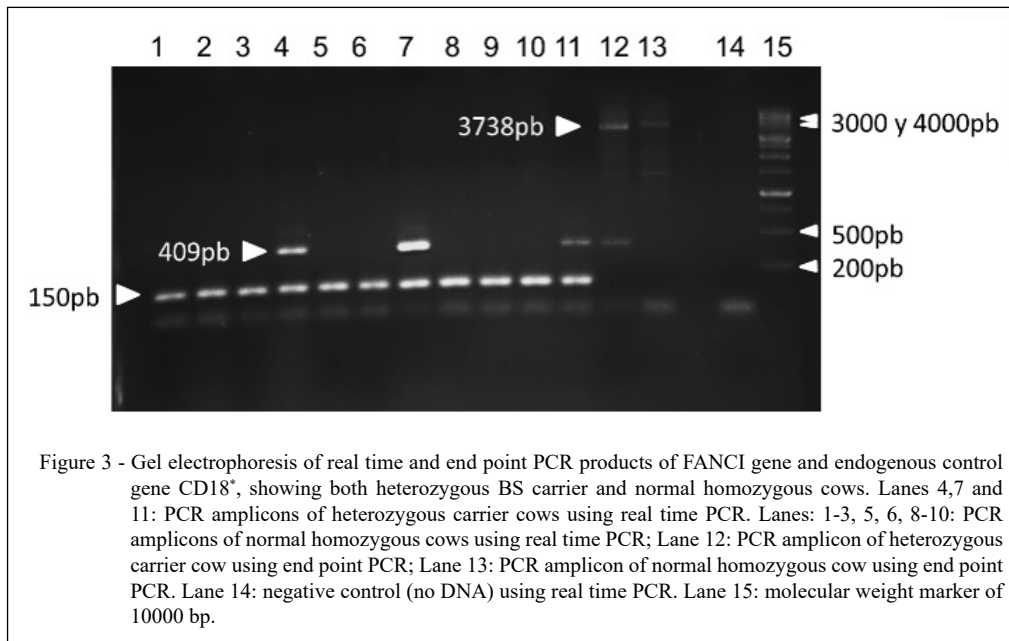


Figure 2 - Above: melting curves of FANCI and IAC gene amplicons in two control cows with heterozygous (Aa) and homozygous (AA) genotype, red and blue colored lines, and squares, respectively. x: Temperature (°C), y: dFluorescence/ dTemperature. d: derivative (standardized BS and IAC primer concentration of 0.3 uM and 55 °C of annealing temperature) Below: melting temperature of BS amplicon peak (peak 1) and IAC amplicon peak (peak 2) for each control sample.

be used for diagnosis tests of clinical cases. Instead, it would be very useful for population studies.

The main advantages of real time PCR are that it is performed in a close tube system that uses minimal labor, has rapid turnaround time and only requires a PCR reaction, a DNA dye and a melting instrumentation (WITTEWER, 2009). In particular, the method described here to detect carrier animals of BS syndrome is a very efficient and sensitive tool, as we could efficiently discriminate heterozygous (carrier) from homozygous control genotypes (Figures 1 and 2). The great advantage of this method is the shortened time of analysis compared to traditional end point PCR and gel electrophoresis analysis (3 hours vs 4 hours, respectively), which is a time consuming and laborious technique. Moreover, the detection of individual genotypes in the same tube used for PCR amplification without the need of separation steps, avoids PCR product contamination and improve the sensitivity of the assay. Once adjusted and precisely identified each PCR product melting temperature, this method can detect very small fluorescence

peaks due to its high sensibility to small quantities of DNA. Similar genotyping methods like real time PCR-HRM have the disadvantage of being highly sensitive to contamination carryover from the DNA purification procedures. For this reason, DNA quality required should be high and homogenous among samples compared and the same DNA extraction method is recommended to be used for all the samples (GABÓR, 2012). High quality DNA is also required for melting curve analysis of real time PCR products; however, results are more reproducible as we can identify different intensity peaks for each fragment. Then, different DNA extraction methods would not affect results and samples could be compared. If the fragment identity of a peak is not clear using melting curve analysis, we can always check the identity of the peak through gel electrophoresis or DNA sequencing. Also, using traditional end point PCR some samples would not amplify the 3.3 kb fragment because it could be degraded. Instead, using the method we proposed, we do not need to detect this fragment, so DNA quality would not represent such a problem.



Compared to microchip-based genotyping platforms like GGP- LD 50K Dairy Cattle panel service (*GeneSeek*, California, USA), real time PCR and melting curve analysis is a better option for BS genotyping. Microarray panels are still expensive to use as a routine diagnostic technique: the cost per sample of high throughput platforms like microarray SNP panels is about 40 U\$ (BRANDA SICA, FEDERICI, 2018). Conversely, the cost per sample using end point PCR and gel electrophoresis analysis is around 4.0 U\$ (quoted with national market products, being agarose the highest cost). In contrast, the estimated cost per sample of the whole process of real time PCR and melting curve analysis is about 1.68 U\$, depending on the kit used. Our methodology would be the cheapest option because are not interested in all SNPs provided in the microarray but only in BS mutation. Before developing this technique, the detection of BS mutation could only be assessed through end point PCR and gel electrophoresis or microarray genotyping platforms; both more expensive.

Our novel technique was specifically adjusted in a Rotor Gene Q (Corbett Research) real time PCR; however, it could be adapted to any laboratory with a real time PCR machine. The 2-primer set system designed for genotype discrimination could be used through adjusting PCR conditions.

Due to its sensitivity, rapidity, and cost-effectiveness, we suggested that the novel assay

described here could be the most suitable option for the screening of large number of samples for the detection of BS heterozygous carriers in Holstein cow populations.

CONCLUSION

We have developed a rapid, sensitive and reliable one step- method based on real time PCR and melting curve analysis for the screening of BS in Holstein cows, which was validated with previously described, optimized end point PCR-electrophoresis analysis. We also showed that this novel method provides significant savings in cost and time compared to other genotyping platforms, which turns it a suitable option to implement as a routine molecular diagnostic tool for the screening of BS allele in dairy livestock. Brachyspina syndrome allele early detection would reduce reproductive losses through genetically directed breeding mating strategies in uruguayan Holstein cattle. To our knowledge, this is the first report of the use of real time PCR and melting curve analysis for the detection of BS Holstein carrier cows.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

No ethical approval was required for this study), INIA, CEUA, protocol # 20199.

DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

AUTHORS' CONTRIBUTIONS

1. Study conception and design, 2. Data acquisition, 3. Data analysis, 4. Results discussion, 5. Writing of the manuscript, 6. Approval of the final version of the manuscript.

M.T. Federici: 1, 2, 3, 4, 5, 6. R. Artigas: 1, 2, 3, 4, 5, 6. S. Guerra: 2, 3, 4, 6. A. Branda: 5, 6. N. Vazquez: 5, 6. P. Nicolini: 5, 6. F. Dutra: 6. S. Llambí: 1, 6.

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