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A Novel Multiplex Polymerase Chain Reaction Method for the Identification of Brachyspina Syndrome Carriers in Chinese Holstein Cattle

Li Y[1,2], Zhai L[1], Fang L[1], Zhang S[1], Liu L[2], Zhu Y[2], Xue J[2], Xiaoqing L[2], Qiao L[2] and Sun D[1*]

[1]Department of Animal Genetics and Breeding, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China

[2]Beijing Dairy Cattle Center, Beijing 100192, China

[*]**Corresponding author**: Dongxiao Sun, College of Animal Science and Technology, China Agricultural University, Yuanmingyuan West Road, Beijing 100193, China, Tel: +86 10 62734653; E-mail: sundx@cau.edu.cn

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Abstract

Brachyspina syndrome (BS) is a monogenic autosomal recessive hereditary defect in Holstein dairy cattle. It is caused by a 3.3-kb deletion in the Fanconi anemia complementation group I (FANCI) gene on BTA21, which removes exons 25 to 27 and leads to a frame-shift and premature stop codon in exon 28. A consequence of BS is reduced reproduction efficiency and milk production in the dams due to loss of homozygous fetuses. In this study, we successfully developed a novel single-tube multiplex PCR assay to identify the recessive allele of BS with the bovine mitochondrial ATP synthase F0 subunit 8 (ATP8) genes as an internal positive control. We used this technique to detect BS alleles in a total of 63 Holstein bulls and 500 Chinese Holstein cows born between 2013 and 2015. Among them, 42 carriers were found (two sires and 40 cows). Combined with our previous study that assayed 206 Holstein bulls and 136 cows (born between 1996 and 2012) and identified 10 bull carriers and three cow carriers, the overall observed carrier frequency is 4.5% in bulls (269 assayed), and 6.8% in cows (636 assayed). Our method provides not only a reliable, economic, and practical diagnostic technique for BS carrier detection, but also promotes BS detection and monitoring system for the genetic improvement project of Chinese Holstein cattle.

Keywords: Brachyspina syndrome; Genetic defect; Chinese Holstein; Multiplex-PCR

Introduction

Brachyspina syndrome (BS) is a recessive, monogenic disorder in Holstein cattle that causes either early-term abortion (most common) or stillborn calves (rare) when a fetus is homozygous recessive for the lethal allele [1,2]. Affected fetus delivered near term display lesions such as severely reduced body weight, a significantly shortened spine (brachyspina), long and slender limbs, inferior brachygnathism, and malformation of the internal organs. The first case of BS was reported in Denmark in 2006 [1], and then more cases were subsequently reported in the Netherlands [3], Italy [4], Germany [5] and Canada [6]. Similar to other Holstein cattle genetic defects (such as complex vertebral malformation and bovine leukocyte adhesion deficiency) that have been spread internationally through disproportionate insemination using frozen semen mainly from one elite ancestor, most of the reported BS cases can be traced back to just two individuals: an elite USA Holstein bull, named Sweet Haven Tradition [3-5], and a Canadian ancestor named Round Oak Rag Apple Elevation [6]. Two sons of Sweet Haven Tradition, Bis-May Tradition Cleitus and Roth rock Tradition Leadman are primarily responsible for spreading the recessive lethal allele of BS.

The genetic mechanism of BS is a 3329 bp deletion in the bovine Fanconi anemia complementation-group 1 (FANCI) gene on BTA21, which removes exons 25 to 27 (of 37 total) and causes a frame shift and premature stop codon in exon 28 [2]. Therefore, a PCR method that amplifies a long product was developed to detect the lethal mutation [2]. Considering that the genetic materials (mainly frozen semen) of Sweet Haven Tradition and his offspring have been imported to China over the past decades, our previous study assayed 206 Holstein bulls (born from 1996 to 2012) and 136 Holstein cows in the Beijing region using the long PCR method. As expected, BS carriers were found (10 carrier bulls and three carrier cows) [7]. However, the long PCR is generally time consuming (3–4 hours) and dependent on a specific LA-Taq enzyme. Thus, in the present study, we developed a novel single-tube multiplex PCR method for identifying the recessive lethal BS allele, and examined an additional 63 Holstein bulls (born from 2013 to 2015) and 500 Chinese Holstein cows to provide information for the genetic improvement project of Chinese Holstein cattle.

Materials and Methods

Animals and DNA extraction

A total of 63 Holstein bulls born between 2013 and 2015 in the Beijing Dairy Cattle Center, a leading Holstein bull station in China, and 500 randomly selected Chinese Holstein cows from the Beijing Sanyuanlvhe Dairy Center were screened for BS. Frozen semen and blood samples were obtained from bulls and cows, respectively. Blood genomic DNA was extracted by using a TIANamp Blood DNA Kit (Tiangen Biotech, Beijing, China) following the manufacturer's instructions. Semen genomic DNA was isolated with a high-salt method as follows. First, each semen straw was emptied into a 1.5-mL tube containing 1 mL of saturated NaCl solution and then tubes were centrifuged for 5 min at 5000×g at room temperature. Second, the supernatant was removed and the sperm was suspended in 1 mL of saturated NaCl solution. The samples were then centrifuged for 5 min at 5000×g and the supernatant was removed. Third, the pellet was subsequently lysed by adding 450 μL of extraction buffer (10 mM Tris pH 8.0, 10 mM EDTA pH 8.0, 1% SDS, and 100 mM NaCl) and warmed to 55°C. Finally, 50 μL of 0.5 M dithiothreitol and 10 μL of proteinase K solution (20 mg/mL) were added to the samples, which were incubated at 55°C for 10 hours [8]. The DNA extracted from semen was treated the same as that extracted from blood in downstream steps.

PCR primer design and optimization of multiplex PCR assay

A single-tube multiplex-PCR assay, containing two pairs of primers, was developed. One pair of primers covering the 3329-bp deletion fragment of the FANCI gene (Gen Bank accession number:

AC_000178.1) as described by Charlier et al. [2] BSF-5′-GCTCAAGTAGTTAGTTGCTCCACTG-3′; BSR-5′ ATAAATAAATAAAGCAGGATGCTGAAA-3′. The other pair of internal primers, mtF/R, was designed as a positive control using primer3 software based on the mitochondrial ATP synthase F0 subunit 8 (ATP8) gene (Gen Bank accession number: HQ184045.1): 5′-TAAGTTAGAGATTGAGAGCC -3′/5′-GATAAGGGTTACGAGAGGGA -3′ with an expected PCR product size of 269 bp.

The multiplex-PCR was set up in a final volume of 25 μL containing 100 ng of genomic DNA template, 0.6 μM each of the above primers, 2.5 μL 10×PCR Buffer, 0.75 mM MgCl2, 2 μL of 2.5 mM deoxyribonucleotide triphosphate mixture, and 1.5 units of Taq polymerase (TaKaRa Biotechnology, Dalian, China). Initial denaturation was achieved at 94°C for 5 min, followed by 35 cycles of 30 sec at 94°C, annealing for 30 sec at 60°C, and extension for 30 sec at 72°C, followed by a final extension for 7 min at 72°C. The PCR products were analyzed on a 2.0% agarose (Biowest, Nuaillé, France) gel with a final concentration of 0.5 μg/mL of ethidium bromide (Tiangen Biotech, Beijing, China).

Sequencing

With the above BSF/BSR primers, the FANCI gene was amplified from all of the identified BS carriers. PCR products were purified by TIAN quick Midi Purification Kits (Tiangen Biotech) and then sequenced by an ABI PRISM377 DNA analyzer (Applied Bio systems, Foster City, CA, USA). Sequences were compared with the wild type sequence of the FANCI gene (AC_000178.1) using DNAMAN software.

Results

A single-tube multiplex PCR assay containing two sets of PCR primers was developed, one of which amplifies the bovine FANCI gene for BS-associated allele detection, while the other amplified the bovine mitochondrial ATP8 gene as an internal positive control. The ATP8 gene, encoding 66 amino acids, is expressed in normal animal cells, and is used to determine the amplification efficiency of the multiplex PCR. After successful amplification, samples derived from normal animals produced only one DNA fragment of 269 bp because the long fragment covering the 3329-bp deletion was not amplified. In contrast, BS carriers yielded two DNA fragments of 409 bp and 269 bp corresponding to the FANCI and ATP8 genes, respectively (Figure 1).

Using this method on samples obtained from a total of 63 Holstein bulls and 500 Chinese Holstein cows, BS alleles were examined in two bulls and 40 cows. Combined with our previous screen that identified 10 bull carriers and three cow carriers in a total of 206 Holstein bulls and 136 Chinese Holstein cows (born from 1996 to 2012) [7], the overall frequency of carriers was 4.5% in bulls and 6.8% in cows. Thus, the allelic frequency of the recessively lethal allele of BS in the population was 3.0% (Table 1). As expected, no mutant homozygote was found because the homozygous recessive genotype is lethal. To further confirm the accuracy and reliability of the novel multiplex PCR method, the 409-bp fragment from each of the 55 identified BS carriers was sequenced. By comparing the mutant sequence with the wild-type sequence of the FANCI gene in NCBI (AC_000178.1), we confirmed the absence of 3329 bp (BTA21:21184870-21188198) in BS carrier alleles (Figure 2), indicating the accuracy and reliability of our method.

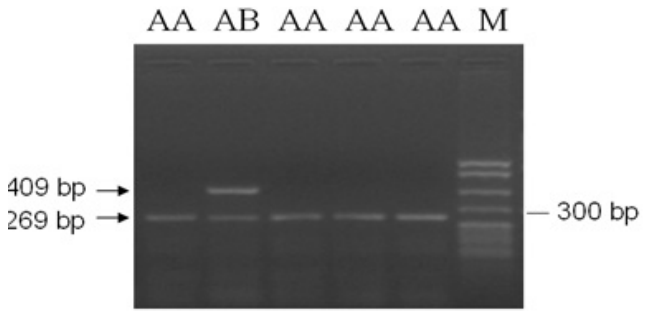

Figure 1: Multiplex PCR products of the FANCI gene and ATP8 gene in Chinese Holstein cattle. Lane M: 50-bp DNA ladder; lane AA: normal animals with one DNA fragment of 269 bp corresponding to the ATP8 gene; lane AB: BS carrier with both a 409-bp DNA fragment for the FANCI gene and a 269-bp DNA fragment for the ATP8 gene.

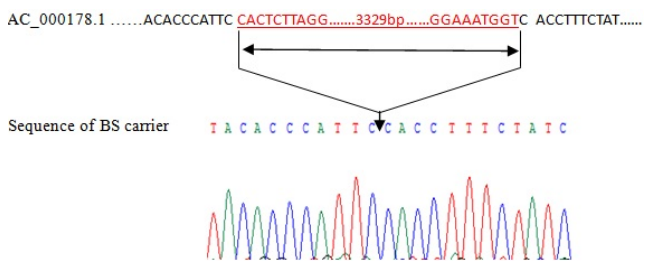

Figure 2: Sequence comparison of the FANCI gene between BS carriers and normal individuals, confirming a 3329-bp deletion was present in BS carriers.

Animals	No. individuals			Genotypic frequency	
	AA	AB	Total	AA	AB
Holstein bulls, this study	61	2	63	0.97	0.03
Holstein cows. this study	460	40	500	0.92	0.08
Holstein bulls, previous study	196	10	206	0.95	0.05
Holstein cows, previous study	133	3	136	0.98	0.02
Total	850	55	905	0.94	0.06

Table 1: Genotypic frequencies of BS carriers in Chinese Holstein cattle. Note: Normal animals were named "AA" genotypes, while BS carriers yielded 2 DNA fragments, i.e., 409 bp and 269 bp were named "AB" genotypes.

Additionally, with pedigree analysis, all 12 of the bull carriers were traced back to a common ancestor: the US Holstein sire Sweet Haven Tradition. No additional remote ancestors were found.

Discussion

The multiplex PCR method requires only a rapid amplification for results, which can be completed in 1.5 hours following DNA extraction. The previous long PCR method, in contrast, requires a minimum of 3–4 hours following DNA extraction [2,7]. The key issues for developing a multiplex PCR assay are primer design and optimization of amplification conditions. When designing primers, we adhered to the following principles: compatibility between primer pairs; amplification efficiency; Tm similarity of all primers; and an optimal difference between amplified fragment lengths to permit resolution of products on a 2% agarose gel. For optimization of PCR conditions, we first optimized a pair of primers for standard PCR and then determined the approximate proportion of PCR reaction system according to each product's respective amplification efficiency. Second, we determined annealing temperature and the optimal reaction system by multiplex PCR optimization. More importantly, we added a pair of internal primers, mtF/R, to the PCR reaction system to confirm amplification of each sample. Mitochondrial DNA is structurally stable and is present in multiple copies in cells, so it is an efficient template for PCR amplification. ATP synthase is widely distributed in the inner mitochondrial membrane, where it is involved in the mitochondrial respiratory chain complex oxidative phosphorylation, synthesizing ATP to supply the body's energy. The ATP8 gene encodes ATP synthase F0 subunit 8, an essential complex in normal cells. Our previous experiment showed that the ATP8 gene was always successfully amplified with high efficiency in genomic DNA samples from Holsteins. Thus, it was considered as an internal control for the multiplex PCR method for BS. The results demonstrate that the method described above is rapid, economic, and efficient for detecting BS carriers in large scale studies. However, real-time PCR assay is actually more sensitive and rapid than conventional PCR, and can easily be adapted by the dairy cattle farmer's community where animal health is a major concern for them. We will further develop a specific real-time PCR assay for BS diagnostic and apply it to examine more samples in the future.

The presence of BS carriers will inevitably result in economic losses in the management of dairy cattle herds [9]. All sires used for artificial insemination could be screened, efficiently allowing a breeding program to eliminate the BS allele from the Chinese Holstein population. Many genetic companies in the USA, such as World Wide Sires, Accelerated Genetics and Select Sires, have been aggressively screening both current and potential sires, to identify all possible carriers. Starting from August 2011, all US Holstein bulls that are carriers are identified with a "BY" and non-carriers will be listed as "TY."

Our current and previous data [7] confirm that the recessive lethal allele of BS exists in the Chinese Holstein population at an allele frequency of 3.6%, demonstrating the need for BS detection and monitoring in Chinese bull stations to gradually decrease the frequency of the harmful allele of BS in the Chinese Holstein population and reduce the losses to dairy farmers.

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