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Original article

Bovine Lymphocyte Intestinal Retention Deficiency – detection of causal mutation among Holstein-Friesian bulls using the PCR-RFLP technique

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Abstract

The aim of this study was to develop a PCR-RFLP diagnostic test to detect the causal mutation for Bovine Lymphocyte Intestinal Retention Deficiency (BLIRD). A total of 217 bulls were tested, including 112 bulls being sons of a known BLIRD-carrier sire and 105 bulls used in insemination from 2020 - 2024 with no indication of BLIRD in their pedigree data. Genomic DNA was isolated from the commercial semen straw. The method developed for identifying the causative BLIRD mutation involved PCR amplification of a 412 base pair fragment of the ITGB7 gene and its digestion with the restriction enzyme Ssi I, which allowed for the clear and cost-effective genotyping of BLIRD. In the first group of 112 bulls, 60 BLIRD carriers and 2 BLIRD-affected bulls were identified. They were sons of internationally known top sires, Manfred (US2183007), Convincer (US2249055) and Justice (US22358313). In the second group of 105 bulls, 7 BLIRD carriers were found, giving a frequency of 6.67% among bulls born within the last five years. Further research should be conducted on a larger number of bulls, particularly those for which there is a risk of carrier status based on the bull's pedigree.

Keywords: Holstein-Friesian bulls, genetic defect, BLIRD, PCR, RFLP

Introduction

Within the last three decades, many genetic defects have been discovered and screened among breeding bulls to monitor and control their spread, especially in Holstein-Friesian dairy cattle (Gozdek et al. 2024). BLIRD (Bovine Leukocyte Intestinal Retention Deficiency) is one of the newest autosomal recessive genetic defects found in Holstein cattle (Besnard et al. 2023). It is caused by a single nucleotide substitution G>A within *ITGB7* gene (Integrin Beta Subunit 7) located on chromosome BTA5 in genomic position g: 26807079 (according to the *Bos taurus* genome map ARS-UCD 1.3). This mutation changes the codon GGC into AGC, which leads to the substitution of the amino acid Glycine to Arginine, and is registered under the identifier rs444441523 (www.ensembl.org). The *ITGB7* gene codes a protein of the same name, which belongs to a family of adhesion receptors that function in signaling from the extracellular matrix to the cell. Integrins are heterodimeric integral membrane proteins composed of an alpha chain and a beta chain. Specific ITGA4/ITGB7 (alpha-4/beta-7) is an adhesion molecule that mediates lymphocyte migration and homing to gut-associated lymphoid tissue and interacts with the cell surface adhesion molecules MADCAM1 which is normally expressed by the vascular endothelium of the gastrointestinal tract (Higgins et al. 2000). Although homozygous mutants did not show any specific gross lesions during necroscopy, they did reveal excessive increase of white blood cells and lymphocyte counts. Moreover, these animals showed a 27% reduction in average daily gain, delayed age of first insemination and premature culling (Besnard et al. 2023). The key ancestor of BLIRD is believed to be a bull, Emprise Bell Elton (US 1912270). O-Bee Manfred Justice (US 122358313), a grandson of Elton, is also a known carrier (according to the Nordic Cattle Genetic Evaluation website).

The aim of the study was to develop a PCR-based diagnostic test to detect causal mutation for BLIRD and to assess its occurrence in Polish Holstein-Friesian bulls.

Materials and Methods

Two hundred and seventeen Holstein-Friesian bulls were included in the study. They were kept in four Polish insemination and breeding centers and were actively used in the insemination of cows in Poland from 2000 - 2023. One hundred and twelve bulls (born from 2000 - 2009) have in their pedigree records a known BLIRD carrier. Another 105 bulls (born from 2020 - 2024) were randomly selected among the newest generation of bulls used in artificial insemination (AI) with no indication of BLIRD carrier in their pedigree.

Genomic DNA was isolated from the half volume of one commercial semen straw using the NucleoMag 200 Purification Kit or NucleoSpin Tissue Kit according to the manufacturer's instructions (Macherey-Nagel, Germany).

Since the material for DNA isolation was commercial semen straws produced by AI centers according to appropriate veterinary regulations, no Ethic Commission Approval was required.

All DNA samples come from the DNA Bull Repository (hosted by the University of Warmia and Mazury, Poland), which consists of bulls reared in Poland from 2000-2024 and which had already been tested for other genetic defects.

Mutation recorded under identifier rs444441523 within the *ITGB7* gene was identified by Polymerase Chain Reaction (PCR) followed by RFLP (Restriction Fragment Length Polymorphism) relying on digestion of the PCR amplicon by a specific restriction enzyme capable of recognizing a polymorphic site of interest. A pair of primers (forward: 5'AGTAGAGCTGGTGGGGG TAG3' and reverse: 5'AAATCCAGGGTTCCACCA GC3') were designed by Primer3 software (Untergasser et al. 2012) and used to amplify a 412 bp fragment of the *ITGB7* gene. The PCR thermal profile consisted of a pre-denaturation step (of 95°C for 3 min) followed by 35 cycles of: 94°C for 30 s, 63°C for 30 s and 72°C for 30 s and a final extension at 72°C for 5 min. PCR was performed in a reaction mix containing 70-80 ng of genomic DNA, 0.18 µL (25 pM) of each primer, 1.0 µL PCR Buffer (20x, Eurx), 1.5 µL dNTPs mix (2.0 mM each), 1 unit of Tfl DNA Polymerase (Eurx), 3.0 µL of $MgCl_2$ (25 mM) and deionized water added to reach a volume of 21 µL. Eight microliters of specific PCR product were digested with 0.8 units of FastDigest Ssi I (Aci I) restriction enzyme (Thermo Scientific) during 30 min of incubation at 37°C. Restriction fragments were electrophoresed in standard 2.5 % agarose gel stained with ethidium bromide and observed using a Fluor-S™ MultiImager (Bio-Rad). Positive control, i.e., DNA from a known BLIRD carrier was used as a reference sample. Amplicons obtained from the DNA of BLIRD GG, AG and AA bulls were cut out from the agarose gel, purified using a Gel-Out kit (A&A Biotechnology, Gdańsk, Poland) and sequenced using an Applied Biosystems sequencer in Genomed Ltd (Poland). The forward and reverse strands were analyzed using BioEdit v. 7.2.0 software.

Fig. 1. Genomic and sequence context of Single Nucleotide Polymorphism rs444441523 causing Bovine Lymphocyte Intestinal Retention Deficiency (BLIRD).
A. Fragment of chromosome BTA5 showing location of the ITGB7 gene and the neighboring genes (retrieved from www.ensembl.org).
B. Location of BLIRD causal mutation within the 8th exon the ITGB7 gene (retrieved from www.ensembl.org).
C. Genomic sequence containing BLIRD causal mutation R (A>G) (in red) amplified using PCR primers (in green).

Results

An overview of the experiment is shown in Fig. 1. A fragment of exon 8th of the *ITGB7* gene containing BLIRD causal mutation was successfully amplified by the use of specific PCR primers. An example of genotyping *ITGB7* missense mutation A>G is shown in Fig. 2. Within the entire amplicon (412 bp), for Adenine in the polymorphic site (A allele, mutant), Ssi I enzyme (recognizing sequence CCGC) was not able to recognize this site but found another non-polymorphic site and cut a 412 bp amplicon into two smaller fragments: 310 and 102 bp. For the G allele (wild), an additional recognition site for enzyme Ssi I appeared exactly in the polymorphic site, which was observed by the additional cutting of a 310 bp fragment into two smaller fragments: 251 and 59 bp. For heterozygous (AG) individuals, four fragments occurred: 310, 251, 102 and 59 bp. In other words, the Ssi I enzyme cut the amplicon always once (in a non-polymorphic site) when the animal was affected (AA genotype) or twice when the animal was non-affected (GG genotype). The amplicon from a BLIRD carrier (AG genotype) was cut once by Ssi I (from one parent) and twice (from another parent), showing four restriction fragments. Sequencing of three bulls genotyped as BLIRD free (GG), carrier (AG) and affected (AA) confirmed the reliability of genotyping with the use of Ssi I enzyme (Fig. 3).

In the first group of 112 bulls (having a known BLIRD carrier in their pedigree) and born from 2000-2009, 60 BLIRD carriers and 2 bulls affected by BLIRD were identified. They were sons of three sires: Manfred (US2183007), Convincer (US2249055) and Justice (US22358313). In the second group of 105 bulls, which have in their available pedigree no data of known BLIRD carriers, 7 BLIRD carriers were found, giving a frequency of 6.67% among bulls born from 2020-2024.

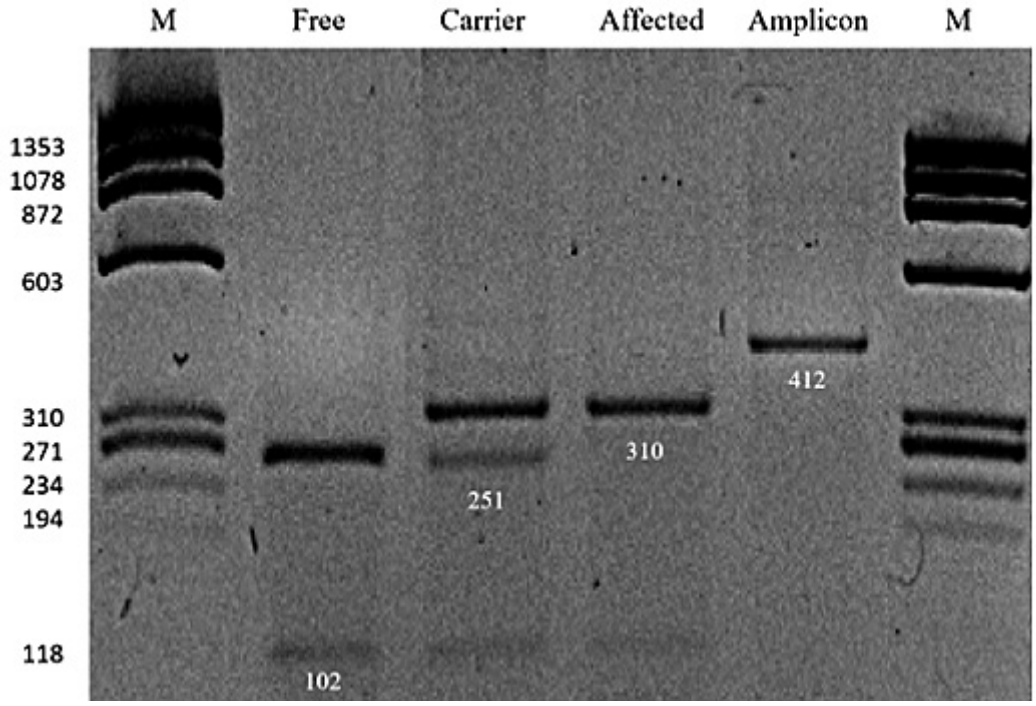

Fig. 2. Example of electrophoresis of 412 bp amplicons of the ITGB7 gene containing a polymorphic site A>G causing BLIRD. From the left: lines 1 and 6 – M (DNA size marker PhiX174/Hae III). Line 2 - bull non-affected by BLIRD with the GG genotype (251, 102 and 59 bp); line 3 – bull being BLIRD carriers with the AG genotype (310, 251, 102 and 59 bp); line 4 – bull affected by BLIRD (AA genotype) showing two restriction fragments: 310 and 102 bp; line 5 - Amplicon 412 bp uncut by Ssi I). The smallest restriction fragment of 59 bp (for free and carrier animals) as well as a fragment of 118 bp (in the M line) are not visible because they diffused from the gel.

Two bulls Valjean (FR2941260084) and AltaIota (US061898306) (born in 2004 and 2005, respectively) were identified as BLIRD affected. The analysis of DNA Bull Repository revealed 148 new bulls as sons of BLIRD carriers and BLIRD-affected sires (born from 2011-2014). Among them, 44 and 45 sons of the bull AltaIota and the bull Man-O-Man were found, respectively.

Discussion

This study describes a simple and inexpensive method of detecting the causal mutation of BLIRD. The method relies on PCR amplification of the 412 bp fragment of the *ITGB7* gene followed by its digestion by restriction enzyme Ssi I, which cuts (or not) the polymorphic site capable of differentiating between animals either affected, non-affected or carriers of BLIRD.

Since the Ssi I enzyme cuts in other non-polymorphic sites within the 412 bp amplicon, the method has an internal control of digestion (which checks whether the enzyme was added to the sample or whether the enzyme was sufficiently active for full digestion of the amplicon). The overall genotyping, including DNA isolation, PCR amplification and restriction enzyme digestion, takes about six hours. The restriction fragments are of sufficient size to be easily resolved in standard agarose gel. The low price of the Ssi I enzyme and the requirement of only basic lab equipment (PCR thermocycler, electrophoresis submarine, agarose gel and UV transilluminator) makes the method inexpensive and available for the average genetic laboratory.

Among all tested bulls, two affected bulls (recessive homozygotes) were identified. Because their breeding value was evaluated by using the traditional method (based on their daughters), they lived at least 5-6 years (the time necessary for obtaining milk performance data from a sire's daughters). This means that the clinical effects specific to BLIRD were not detrimental enough to eliminate these bulls from insemination centers. According to publicly available data on the breeding value of these two bulls, they had large numbers of daughters, i.e. 6,276 and 78,634, for Valjean and AltaIota, respectively. Since they have the AA genotype, all of their daughters inherited the A allele and became new BLIRD carriers. These numbers refer to progeny registered in the national system for genetic evaluation (SYMLEK), which includes ca. 38% of dairy cows in Poland. These data suggest that more daughters of these two BLIRD homozygous bulls were born. Both bulls were also the sires of sons, but their number is difficult to determine. BLIRD, as a new

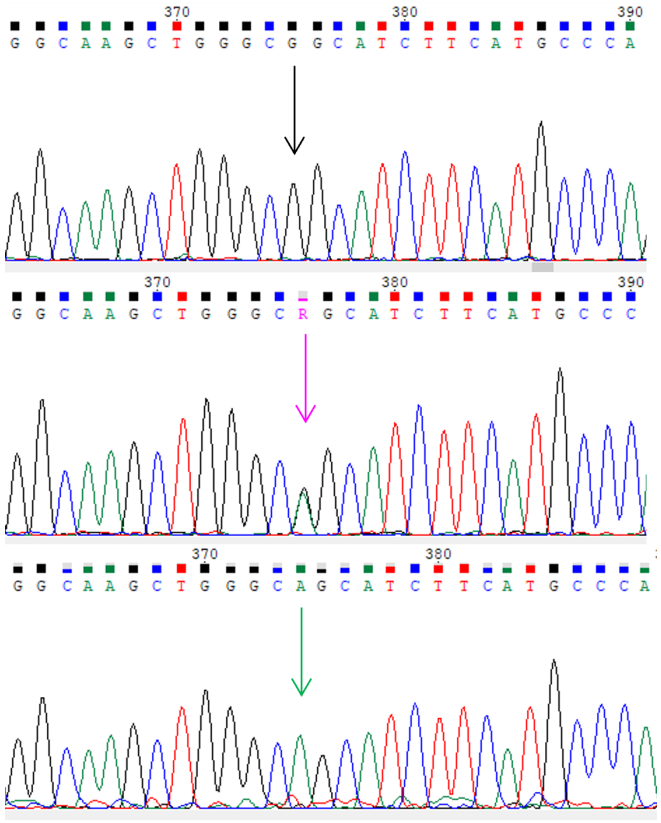

Fig. 3. Fragment of a 412 bp amplicon sequence obtained from bulls genotyped using the PCR-RFLP method as BLIRD free (upper), carrier (middle) and affected (lower). Only forward strands are shown. R indicates a polymorphic site (G or A).

genetic defect in Holstein-Friesian cattle, is monitored by insemination centers in different countries (personal communication), but there are only two scientific reports assessing the frequency of *ITGB7* alleles or genotypes. In Swiss Holstein cattle (Leunerberger et al. 2024), the *ITGB7* G allele has a frequency of 2.1%. In Australian Holstein cattle, BLIRD carrier frequency has been estimated at 12.24% and recessive homozygotes at 0.36% (van den Berg et al. 2024). Preliminary screening of Holstein cattle in France and Germany showed a prevalence of 0.3% and 0.1% of homozygous BLIRD calves, respectively (according to the Syntetics website).

It can be concluded that in the Polish Holstein-Friesian cow population, BLIRD causal mutation was widespread and will be transmitted to future generations. A trace of this trend was observed in this study by detecting 7 carriers among a sample of 105 bulls reared in the last 5 years. Therefore, genotyping bulls for BLIRD should be included in routine screening of young bulls, and the results should be registered in pedigree files in a very similar way as was done in the case of earlier genetic defects such as BLAD (Czarnik et al. 2007), CVM and *Brachyspina* (Kamiński 2023).

Conclusion

The developed PCR-RFLP method is a simple, cost-effective technique for to identifying the BLIRD genotype in Holstein-Friesian cattle in population-wide screening programs, especially among young bulls. Priority should be put on analyzing pedigree data to identify genetic lines in which BLIRD carriers have already been found or where there is a risk of their occurrence in current offspring.

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