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A novel method for reliable and rapid detection of BC, BLAD, CVM, and DUMPS in cattle

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ABSTRACT - This study aimed to optimize a reliable and rapid genotyping assay to detect the carriers of bovine citrullinemia (BC), bovine leukocyte adhesion deficiency (BLAD), complex vertebral malformation (CVM), and deficiency of uridine monophosphate synthase (DUMPS) in cattle populations. We developed real-time polymerase chain reaction (RT-PCR)-based assays to distinguish wild-type and defective alleles of BLAD, CVM, BC, and DUMPS. Twenty-four bulls in the International Center for Livestock Research and Training were genotyped. At the same time, genotyping was performed for DUMPS and BC using PCR-RFLP, and sequencing was performed for all diseases and compared with the RT-PCR kit we developed. None of the bulls carried mutant alleles of these hereditary autosomal recessive lethal defects. It takes only 2 h for the assay to be completed, including DNA extraction from the sample. These consequences indicate that RT-PCR is an easy, reliable, and rapid method for detecting BLAD, CVM, BC, and DUMPS carriers. Studies show a high frequency of mutant alleles of these genetic defects that cause genetic diseases, and this requires routine test systems to eradicate genetic diseases of economic importance.

Keywords: bovine citrullinemia, bovine leukocyte adhesion deficiency, complex vertebral malformation, deficiency of uridine monophosphate synthase, genetic disease in cattle, real-time PCR-based assay

1. Introduction

Genetic disorders in cattle are among the most critical issues in animal husbandry, and their effects on populations must be controlled. Known hereditary disorders in cattle are caused mainly by genes with autosomal recessive inheritance. The characteristic of autosomal recessive genes is that they cause a diseased phenotype only when both alleles are present. Therefore, defective genes may be inherited without identification (Agerholm, 2007).

Bovine leukocyte adhesion deficiency (BLAD), caused by a single base mutation in the *CD18* (ITGB2) gene (Windsor and Agerholm, 2009), is a disease characterized by persistent and progressive neutrophilia, increased susceptibility to infectious agents in the first two months of life, gingivitis, ulcerative and granulomatous stomatitis, enteritis, pneumonitis, periodontitis infection in soft tissues, and death at the age of 2-8 years (Nagahata, 2004; Agerholm, 2007; Meydan et al., 2010).

Uridine monophosphate synthase deficiency (DUMPS) occurs on bovine chromosome 1, with a single-point mutation characterized by the conversion of cytosine to thymine at codon 405 of exon 5 (Patel et al., 2006). Growth and development in homozygous recessive embryos cease approximately 40 days after fertilization, and embryonic mortality develops (Robinson et al., 1983; Schwenger et al., 1994; Citek et al., 2006).

Complex vertebral malformation (CVM) is caused by a single point mutation in nucleotide 559 of the *SLC35A3* (solute carrier family 35 member 3) gene located in bovine chromosome 3, characterized by the transversion from guanine to thymine (Thomsen et al., 2006).

Bovine citrullinemia (BC) disease causes argininosuccinate synthetase (ASS) enzyme deficiency, which converts citrulline to argininosuccinate in urea metabolism. This leads to citrulline accumulation, a more toxic product than ammonia, during urogenesis (Grupe et al., 1996; Patel et al., 2006). Accurate identification of animals is an essential part of controlling autosomal recessive inherited genetic diseases because animals carriers of the genetic disease may not show any clinical symptoms. Many genotyping methods have been developed to date, but most require several technical steps and need to be more suitable for automation or easy high-throughput genotyping (Tammen et al., 1996; Bendixen et al., 2002; Chu et al., 2008; Meydan et al., 2010).

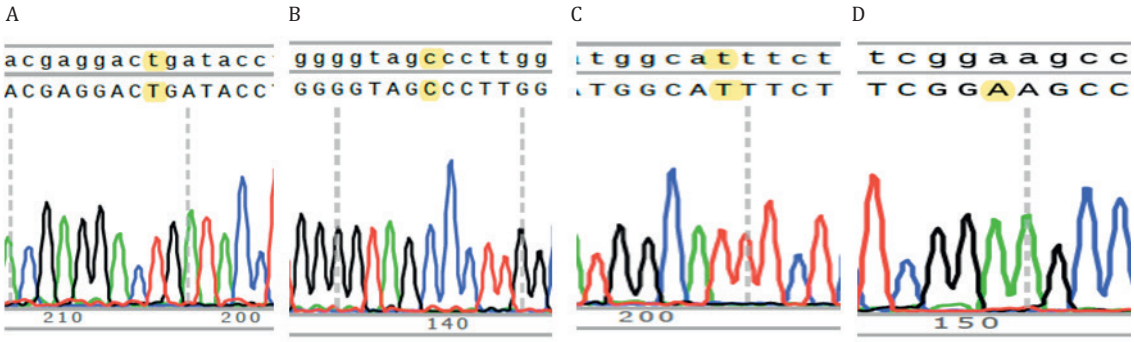
In this study, we developed a real-time polymerase chain reaction (RT-PCR)-based genotyping assay to detect heterozygous carriers of CVM, BLAD, BC, and DUMPS.

2. Material and Methods

This study was carried out in accordance with the decision of the Experimental Animals Local Ethics Committee (dated 22.02.2012 and numbered 66) of the International Animal Husbandry Research and Education Center (Formerly the Central Animal Husbandry Research Institute).

The animal material of the study consists of 24 elite bulls (11 heads of Simmental, nine heads of Brown, and four heads of Holstein) from research institutes in Ankara, Turkey (geographically coordinated at 39.97007392551079 and 33.10986402546703). Blood samples were taken from the vena jugularis into EDTA tubes. DNA isolation from whole blood was performed using QIAamp 96 DNA™ commercial isolation kit in QIAcube HT automatic isolation device; the amount of DNA obtained was measured with NanoPhotometer™ (Implen) brand micro-volume spectrometer, and the DNA concentration of each sample was standardized to 25 ng/µL.

Three control DNA templates were synthesized to represent the distinct genotypes associated with each disease. Control DNA templates were not extracted from actual animals but were instead obtained synthetically through plasmid studies. For genotyping, a real-time-based method that analyzes amplification plots was employed. This approach utilizes probes specifically designed to generate amplification curves in their designated signal channels. Mutant-specific probes exclusively hybridize with mutant DNA sequences, producing a characteristic amplification curve in the HEX signal channel. Wild-type probes are tailored to bind solely with wild-type DNA sequences, resulting in a unique amplification curve in the FAM signal channel. Precise genotyping is performed by determining CT values specific to HEX and FAM amplification curves. Recessive homozygous genotype templates were generated by cloning synthetic gene templates and confirmed by sequencing (Figure 1).



A: bovine citrullinemia; B: bovine leukocyte adhesion deficiency; C: complex vertebral malformation; D: deficiency of uridine monophosphate synthase.

Figure 1 - Synthetic mutant-type homozygote genotyping sequencing analysis results.

Genotyping for DUMPS and BC were performed by PCR-RFLP, DNA sequencing, and RT-PCR methods; CVM and BLAD genotypes were determined by DNA sequencing and RT-PCR methods (Tables 1 and 2, Figure 2). The BC genotypes were determined in agarose gel electrophoresis with 2% ethidium bromide. The DUMPS genotypes were determined in MetaPhor agarose gel electrophoresis

Table 1 - PCR primers, PCR profiles, restriction enzymes, and PCR product lengths

Genetic defect	Primer	PCR profile	PCR product	Restriction enzyme
		94 °C 3 min / 35 cycles		
BLAD	F: 5'-GAATAGGCATCCTGCATCATATCCACCA-3'	94 °C 30 sec		
	R: 5'-CTTGGGGTTTCAGGGGAAGATGGAGTAG-3'	65 °C 30 sec	354 bp	-
		72 °C 30 sec		
		72 °C 10 min		
		94 °C 5 min / 35 cycles		
CVM	F: 5'-CAGATTCTCAAGAGCTTAATTCTA-3'	94 °C 60 sec		
	R: 5'-TATTTGCAACAACAAGCAGTT-3'	58 °C 60 sec	281bp	-
		72 °C 30 sec		
		72 °C 10 min		
		94 °C 5 min / 40 cycles		
DUMPS	F: 5'-GCAAATGGCTGAAGAACATTCTG-3'	94 °C 60 sec		
	R: 5'-GCTTCTAACTGAACTCCTCGAGT-3'	58 °C 60 sec	210 bp	Ava I
		72 °C 90 sec		
		72 °C 5 min		
		94 °C 3 min / 40 cycles		
BC	F: 5'-GGCCAGGGACCGTGTTCATTGAGGACATC-3'	94 °C 30 sec		
	R: 5'-TTCCTGGGACCCCGTGAGACACATACTTG-3'	57 °C 30 sec	204 bp	Ava II
		72 °C 30 sec		
		72 °C 10 min		

BC - bovine citrullinemia; BLAD - bovine leukocyte adhesion deficiency; CVM - complex vertebral malformation; DUMPS - deficiency of uridine monophosphate synthase; bp - base pair (Meydan et al., 2010; Schwenger et al., 1994; Grupe et al., 1996).

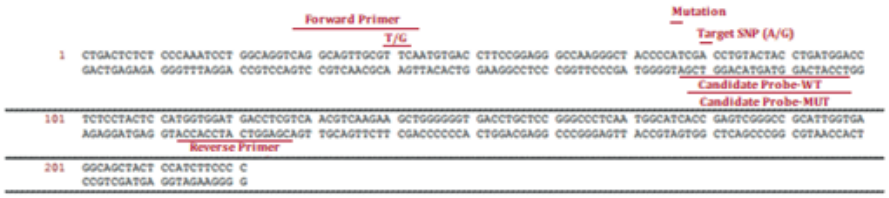
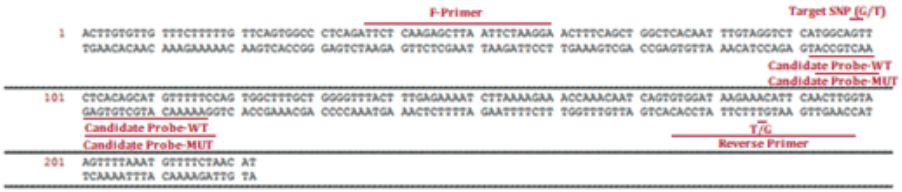
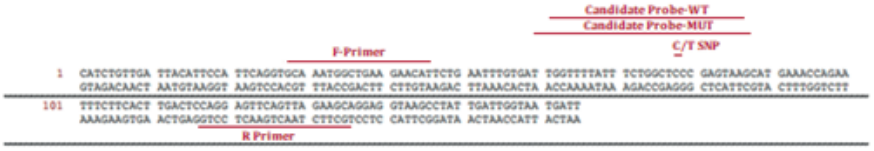
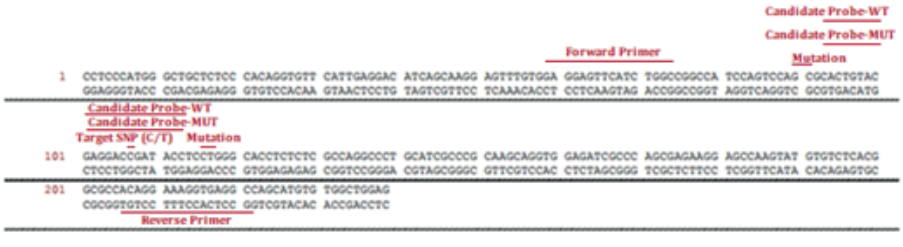
Table 2 - Primer and probe sequences in real-time PCR-based assays for BC, BLAD, CVM, and DUMPS

Genetic defect	Primer and probe	Oligo sequence (5′ to 3′)	Reporter (5′)
	ASS1-FWD	GAGGAGTTCATCTGGC	
BC	ASS1-REV	GCCTCACCTTTCCTGT	
	ASS1-Probe-WT	CACTGTACGAGGACCGATACC	FAM
	ASS1-Probe-MUT	CACTGTACGAGGACTGATACCT	HEX
	ITGB2-FWD	AGGCAGTTGCGTTC	
BLAD	ITGB2-REV	CGAGGTCATCCACCA	
	ITGB2-Probe-WT	TCCATCAGGTAGTACAGGTCGA	FAM
	ITGB2-Probe-MUT	CATCAGGTAGTACAGGCCGA	HEX
	SLC35A3-FWD	TTCTCAAGAGCTTAATTCTAAGG	
CVM	SLC35A3-REV	CAAGTTGAATGKTTCTTATCCA	
	SLC35A3-Probe-WT	AAAAACATGCTGTGAGAACTGCCAT	FAM
	SLC35A3-Probe-MUT	GAAAAACATGCTGTGAGAAATGCCA	HEX
	UMPS-FWD	GCAAATGGCTGAAGAACA	
DUMPS	UMPS-REV	GCTTCTAACTGAACTCCTGG	
	UMPS-Probe-WT	TGGTTTTATTTCTGGCTCCCGAGTA	FAM
	UMPS-Probe-MUT	TTGGTTTTATTTCTGGCTTCCGAGTAA	HEX

BC - bovine citrullinemia; BLAD - bovine leukocyte adhesion deficiency; CVM - complex vertebral malformation; DUMPS - deficiency of uridine monophosphate synthase.

with 4% ethidium bromide. In addition, CVM, BLAD, DUMPS, and BC samples were studied on ABI 3130 DNA Sequencer (Applied Biosystems) sequencing device using BigDye™ Terminator v3.1 Cycle kit, and genotyping results were analyzed with Sequencing Analysis 5.2 program.

Amplifications were performed in a RT-PCR system (QuantStudio 7 Flex™, Applied Biosystems) using TaqMan probes and specific primer pairs. Four sets of probes and primer pairs were designed based on the published sequences of the *CD18* (GenBank Accession Number Y12672), *SLC35A3* (GenBank Accession Number AY160683), *UMPS* (GenBank Accession Number JN039033), and *ASS* (GenBank Accession Number JN082727) genes. In each probe set, the first probe that perfectly matched the

Genetic defect	Genome
BLAD	
CVM	
DUMPS	
BC	

RT-PCR - real-time polymerase chain reaction; BC - bovine citrullinemia; BLAD - bovine leukocyte adhesion deficiency; CVM - complex vertebral malformation; DUMPS: deficiency of uridine monophosphate synthase; bp - base pair.

Figure 2 - View of the designed RT-PCR probes on the genome.

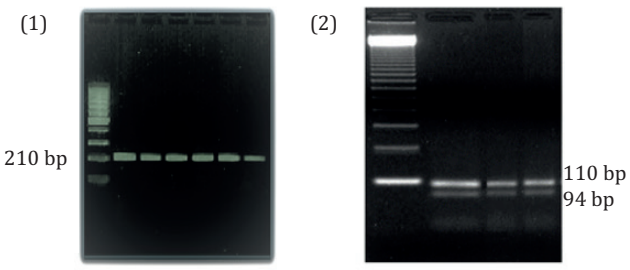
wild-type sequence variant was 5'-labeled with 6-carboxy-fluorescein (FAM); the second probe that matched the mutant sequence variant was 5'- labeled with hexachloro-fluorescein (HEX), and both probes included a non-fluorescent quencher (Black Hole Quencher-1; BHQ1) (Table 2).

Four independent RT-PCR reactions were performed for each sample to determine the CD18, UMPS, ASS, and SLC35A3 loci genotypes. The 20 µL reaction mixture was prepared as 50 mM KCl, 50 mM TRIS-HCl, 300 nM dNTP, 2 mM MgCl2, and 2 units of DNA polymerase, 200 nM primer, and 100 nM fluorescent probe. The PCR conditions were 95 °C for 4 min, followed by 35 cycles of 95 °C for 20 s, 56.5 °C for 30 s, and 72 °C for 30 s. All experiments were conducted in triplicate.

3. Results

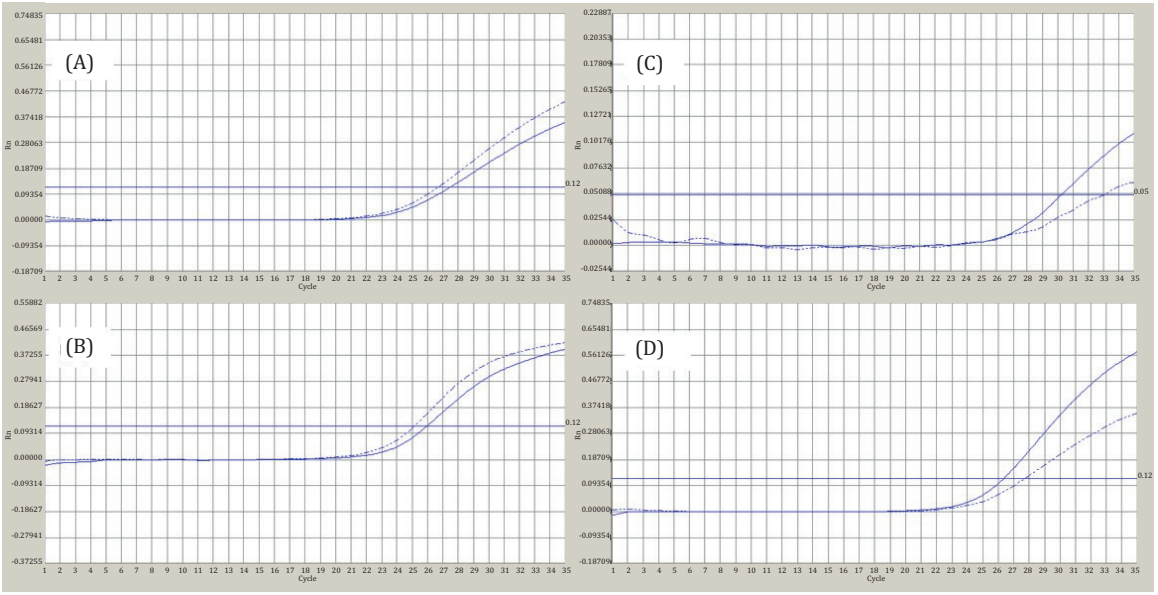
One of the traditional methods frequently used in genotyping studies for BC and DUMPS diseases is the PCR-RFLP method. The PCR products obtained from the amplification for the PCR-RFLP process were incubated with AvaII and AvaI restriction enzymes (Table 1). Afterward, BC fragments were visualized in agarose gel electrophoresis with 2% ethidium bromide, and DUMPS fragments were visualized in MetaPhor agarose gel electrophoresis with 4% ethidium bromide. Wild type BC allele gave two fragments at 110 and 94 bp, and wild type DUMPS allele gave a single fragment with a length of 210 bp (Figure 3), and no mutant allele was found. Allelic mutations can be performed by analyzing the real-time amplification plots by the real-time-based method, which is based on the designed probes only generating the amplification curve in the target signal channel. According to this theory, mutant probes only hybridize with the mutant targets and generate a typical amplification curve from the HEX signal channel; meanwhile, the wild-type probes hybridize with the wild-type targets and generate a typical amplification curve from the FAM signal channel. Thus, genotyping can be performed accurately by comparing the amplification curves (Figures 4-6).

The CT values for the FAM probe across all diseases are presented in the graphic prepared from the bulls (Figure 7). It was observed that CT values varied between diseases and their standard deviations were quite low, which refers to the correct working states of the probes. For the test results of elite bulls, FAM CT values expressing wild type in BLAD genotyping were found between 24.33 and 23.25, and the mean value was 23.65±0.24. In CVM genotyping, FAM CT values expressing wild type were between 23.65 and 22.47, and the mean value was 23.07±0.35. In DUMPS genotyping, FAM CT values expressing wild type were found between 22.42 and 21.27, and the mean value was 21.92±0.31 in BC genotyping, FAM CT values expressing wild type were found between 27.41 and 26.07, and the mean value was 26.81±0.36. In genotyping, HEX amplification expressing the mutant allele could not be detected, and the reliability of the method was confirmed in this respect. During our experiments utilizing the synthetic mutant homozygous allele, we observed distinct HEX-labelled CT values. For BLAD, the values ranged between 17.60 and 19.2; for BC, the values were between 23.7 and 25.8; for DUMPS, the values were between 16.6 to 19.5; and for CVM, the observed range was between 24.2 and 26.4.



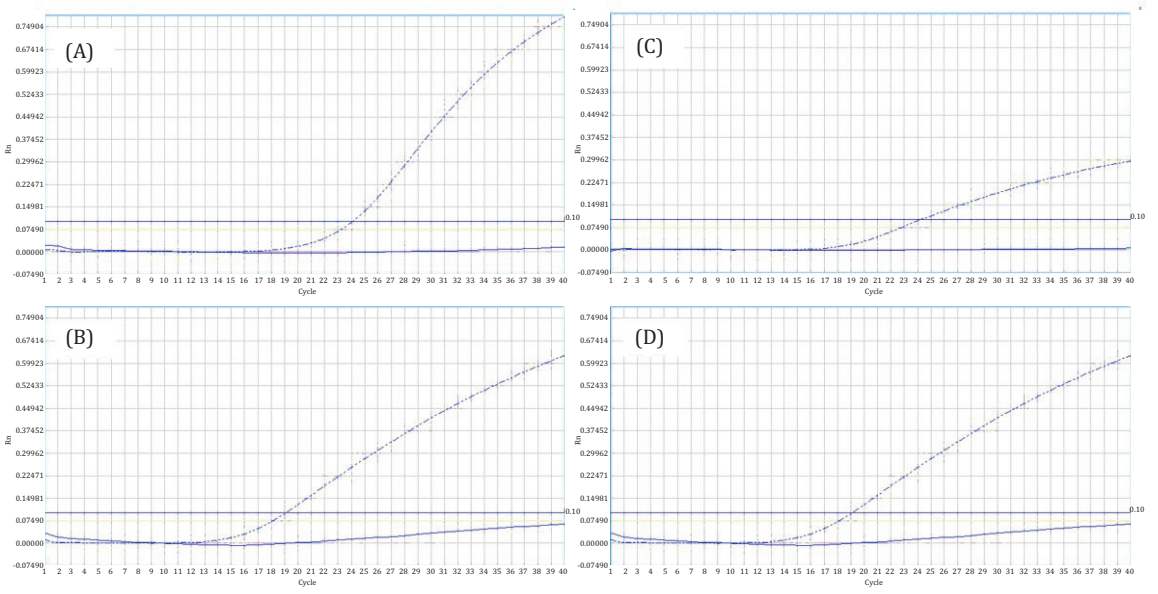
1: Wild-type DUMPS genotype; 2: Wild-type BC genotype.

Figure 3 - Demonstration of deficiency of uridine monophosphate synthase (DUMPS) and bovine citrullinemia (BC) genotypes on agarose gel.



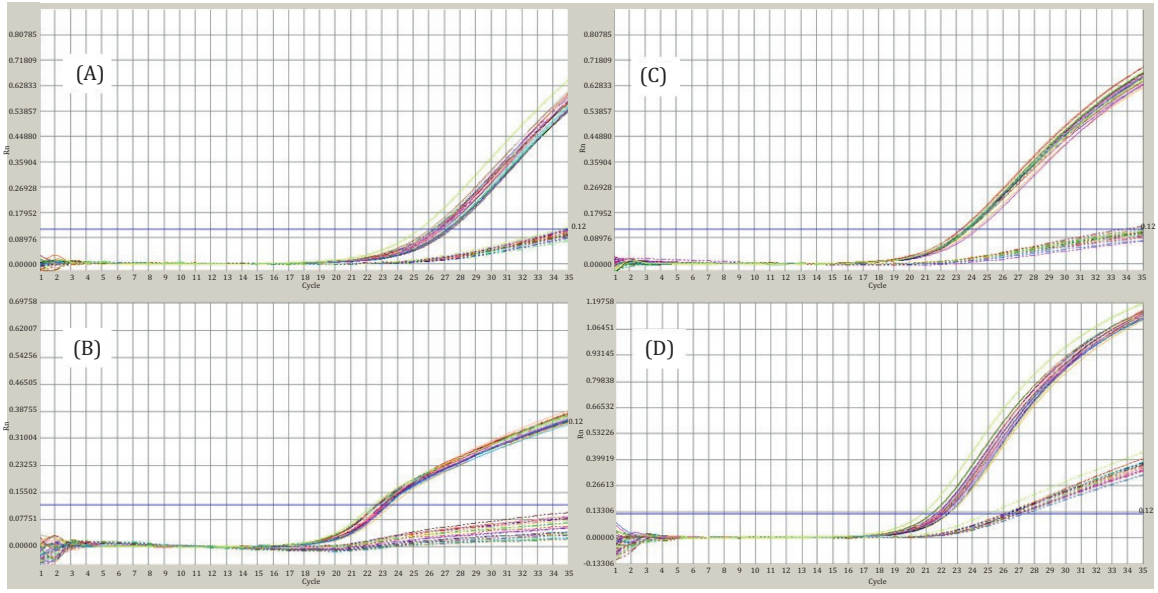
FAM-labelled probe is complementary to wild-type allele and HEX-labelled probe is complementary to mutant-type allele.
The solid line represents FAM, the dashed line represents HEX.

Figure 4 - Real-time polymerase chain reaction amplification plot of heterozygote (carrier) type of (A) bovine citrullinemia, (B) bovine leukocyte adhesion deficiency, (C) complex vertebral malformation, and (D) deficiency of uridine monophosphate synthase.



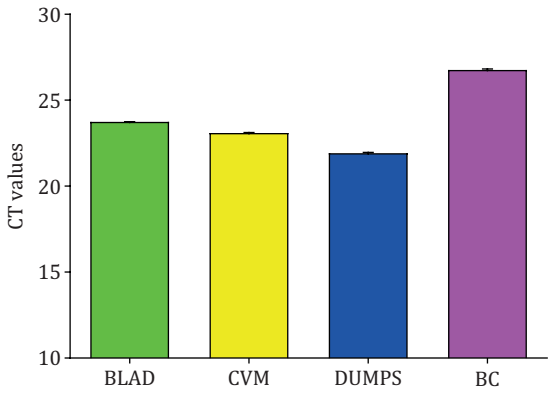
FAM-labelled probe is complementary to wild-type allele and HEX-labelled probe is complementary to mutant-type allele.
The solid line represents FAM, the dashed line represents HEX.

Figure 5 - Real-time polymerase chain reaction amplification plot of the mutant homozygote type of (A) bovine citrullinemia, (B) bovine leukocyte adhesion deficiency, (C) complex vertebral malformation, and (D) deficiency of uridine monophosphate synthase.



FAM-labelled probe is complementary to wild-type allele and HEX-labelled probe is complementary to mutant-type allele.
The solid line represents FAM, the dashed line represents HEX.

Figure 6 - Real-time polymerase chain reaction amplification plot of the wild-type homozygote of (A) bovine citrullinemia, (B) bovine leukocyte adhesion deficiency, (C) complex vertebral malformation, and (D) deficiency of uridine monophosphate synthase.



The figure represents the results of the 24 elite bulls tested.

Figure 7 - Real-time polymerase chain reaction detected FAM-labelled probe CT value of the wild-type homozygote of bovine citrullinemia (BC), bovine leukocyte adhesion deficiency (BLAD), complex vertebral malformation (CVM), and deficiency of uridine monophosphate synthase (DUMPS).

We optimized and applied RT-PCR to identify cattle carriers of CVM, BLAD, BC, and DUMPS. This technique produced a highly effective and sensitive tool for detecting CVM, BLAD, BC, and DUMPS heterozygote carriers from homozygous control genotypes. For this purpose, 24 elite bulls hosted in the institute were analyzed with the technique we developed, and none of the bulls were found to be a carrier of these diseases. The accuracy of RT-PCR genotyping results was evaluated by direct sequencing, using the BigDye™ Terminator v3.1 Cycle kit in the ABI 3130 DNA Sequencer (Applied Biosystems), and mutant-type genotyping results were analyzed with Sequencing Analysis 5.2 (Figure 1). The results showed that there was no inconsistency between the two test methods. These results showed that

RT-PCR is a reliable analysis for the genotyping of CVM, BLAD, BC, and DUMPS loci. Of the 24 elite bulls analyzed using our developed technique, none were found to be carriers of CVM, BLAD, BC, or DUMPS.

4. Discussion

Modern cattle breeding increasingly includes programs based on the international semen trade from high-genetic value elite bulls. With the widespread use of advanced breeding technologies, including artificial insemination and embryo transfer, individual bulls may produce thousands of offspring in many countries (Agerholm, 2007). It is clear that such widespread use of individual bulls may cause unwanted genes to spread within a breed.

Genetic disorders in cattle are among the most critical issues in animal husbandry, and their effects on populations need to be controlled. Known hereditary disorders in cattle are caused mainly by genes with autosomal recessive inheritance. The characteristic of autosomal recessive genes is that they cause a diseased phenotype only when both alleles are present. Therefore, defective genes may be inherited without identification (Agerholm, 2007; Meydan et al., 2010).

Bovine leukocyte adhesion deficiency was initially identified as a bovine granulocytopathy syndrome. Subsequent studies have shown that it is caused by a single base mutation in the *CD18* (ITGB2) gene (Windsor and Agerholm, 2009). It was first clinically diagnosed by Hagemoser et al. (1983). The disease prevents neutrophil leukocytes from reaching the site of infection through the endothelial layer. Despite the high number of neutrophils, inflammatory response fails due to the alteration of neutrophil function. The disease is characterized by persistent and progressive neutrophilia, increased susceptibility to infectious agents in the first two months of life, infection in soft tissues such as gingivitis, ulcerative and granulomatous stomatitis, enteritis, pneumonitis, periodontitis, and death at the age of 2-8 months. When sick calves are born, they are usually phenotypically normal, but high fever, chronic diarrhea, and other symptoms appear within a few weeks. Cattle with BLAD usually die before the age of one year before they can be diagnosed. Some cows can survive over two years, but their breeding and milk yields are meager (Nagahata, 2004; Agerholm, 2007; Meydan et al., 2010; Avanus and Altınel, 2017). Nagahata et al. (1987) classified the disease as autosomal recessive, and Kehrli et al. (1990) described the molecular basis. It is characterized by reduced expression of the heterodimeric β2 integrin adhesion molecule on the leukocyte. This situation causes multiple defects in the leukocyte, and insufficient mucosal immunity develops with defective leukocyte adhesion. This disease is caused by a point mutation in the 383rd nucleotide of the *CD18* gene found in bovine chromosome 1, which leads to the conversion of adenine to guanine (Kehrli et al., 1990; Shuster et al., 1992).

In mammalian cells, the uridine monophosphate synthase (UMPS) enzyme catalyzes the conversion of orotic acid to uridine monophosphate. The pyrimidine nucleotides form the structure of DNA and RNA. The uridine monophosphate synthase deficiency occurs on bovine chromosome 1, with a single-point mutation characterized by the conversion of cytosine to thymine at codon 405 of exon 5 (Patel et al., 2006). Growth and development in homozygous recessive embryos cease approximately 40 days after fertilization, and embryonic mortality develops. Abortion or embryo absorption usually occurs 40 days after pregnancy, and the disease causes recurrent reproductive problems. The UMPS activity in the spleen, muscle, liver, kidney, and mammary gland is seen to have decreased to half of the average value, but they seem phenotypically normal. DUMPS carriers of milk and orotic acid levels increased in the urine can be detected (Robinson et al., 1983; Schwenger et al., 1994; Citek et al., 2006).

Complex vertebral malformation was described by Agerholm et al. (2001) in the Danish Holstein population. The ancestor of the undesirable mutant allele, the American Holstein genus Penstate Ivanhoe Star (US1441440), and his son Carlin-M Ivanhoe Bell (US1667366) have been shown to cause the spread of the disease worldwide by artificial insemination. The disease is caused by a single point mutation in nucleotide 559 of the *SLC35A3* gene located in bovine chromosome 3, characterized by the transversion from guanine to thymine. This mutation leads to the transversion of valine to phenylalanine at position 180. This critical aminoacid change causes disturbances in nucleotide energy transfer and results in vertebral malformations (Thomsen et al., 2006). The disease causes perinatal

death due to abortion or vertebral anomalies by 80% during embryonic development. Surviving calves have low birth weight, scoliosis, cervical and thoracic vertebral anomalies, malformations of the carpal and dorsal joints, and cardiac anomalies (Agerholm et al., 2004).

Bovine citrullinemia disease was first described in the Australian Holstein population by Healy et al. (1991) following the importation of American Holstein bull sperm, Linmack Kriss King, into the Australian Holstein community. The disease causes ASS enzyme deficiency, which converts citrulline to argininosuccinate in urea metabolism. This leads to the accumulation of citrulline, a more toxic product than ammonia, during urogenesis. Blood, eye fluid, cerebrospinal fluid, and brain tissue accumulate high citrulline levels in patients. The transversion of cytosine causes bovine citrullinemia to thymine at codon 86 of exon 5 of the gene encoding the ASS enzyme of bovine chromosome 11 (Padeeri et al., 1999). Calves affected by BC seem normal after birth. However, depression starts from day two after birth, and feed intake stops, and from day 3, symptoms such as turning around and not being able to carry the head are observed. The disease progresses rapidly between days 3 and 5, and blindness develops. Homozygous calves die within the first seven days of their lives (Robinson et al., 1993; Grupe et al., 1996; Patel et al., 2006).

Accurate identification of animals is an essential part of controlling autosomal recessive inherited genetic diseases because animals carriers of genetic diseases may not show any clinical symptoms. Identifying the molecular basis of genetic diseases enables rapid screening of populations for the removal of carrier animals from the population, thereby reducing the number of offspring affected by the disease and preventing economic losses. The development of molecular genetics has enabled the identification of heterozygous carrier animals in terms of genetic diseases by genomic analysis effectively and rapidly. Various studies have been conducted to eliminate sick and carrier animals from the Holstein cattle population worldwide. The highest prevalence of BLAD carriers was reported as 21.5% in Denmark (Jorgensen et al., 1993); for Turkey, this rate was 4% (Meydan et al., 2010). The highest prevalence of DUMPS carriers was 1.2% in the USA (Shanks and Robinson, 1990) and 32.5% for CVM in Japan (Nagahata et al., 2002). Turkey's highest reported BLAD and CVM carriers, a prevalence of 2.2% (Meydan et al., 2010) and 6.6% (Avanus and Altınel, 2017), respectively, has been reported. Regarding BC, heterozygous allele frequencies, which are quite high, with 1.55% in the Shandong province of China (Wang et al., 2009), have been reported to be relatively high in India and are 1.67% (Robinson et al., 1993). The highest allele frequency was found in Australia in 50% of Australian Holstein Friesian cows and 30% of males in artificial insemination centers because most Australian dairy cattle breeds are derived from Linmack Kriss King (LMKK) (Healy et al., 1991; Kotikalapudi et al., 2014).

Many genotyping methods (Shuster et al., 1992; Tammen et al., 1996; Bendixen et al., 2002; Chu et al., 2008; Meydan et al., 2010; Zhang et al., 2012) have been developed to date, but most require several technical steps and need to be more suitable for automation or easy high-throughput genotyping. Several molecular methods, such as PCR-RFLP (Meydan et al., 2010; Korkmaz-Ağaoğlu et al., 2015), PCR-PIRA (Kanae et al., 2005), PCR-HRM (Federici et al., 2018), and AS-PCR (Bendixen et al., 2002) sequencing, are used for the diagnosis of BLAD, CVM, DUMPS, and BC.

Since these approaches require essential equipment, they can be relatively cost-effective and easy to use. However, all involve several technical steps and are time-consuming; for example, at least 10 h are needed to obtain results after DNA extraction. Real-time PCR contains a probe labeled with the reporter dye. The quencher dye cleaved with Taq DNA polymerase during DNA amplification, allowing the reporter dye to fluoresce and accumulate. Amplification of the probe-specific product causes the probe to detach, increasing reporter fluorescence so that using different reporter dyes, division of allele-specific probes can be detected in a single RT-PCR assay (Livak, 1999). This method has proven to be a quick, infallible, accurate, and highly efficient technique for SNP analysis (Johnson et al., 2004).

In the current study, we developed a RT-PCR-based genotyping assay to detect heterozygous carriers for CVM, BLAD, BC, and DUMPS. With the RT-PCR-based analysis method for BLAD, CVM, DUMPS, and BC diagnostics developed by this study, only one amplification step is needed to obtain the results. The analysis takes approximately 2 h from DNA extraction. The developed method does not require post-PCR treatment, which reduces the risk of subsequent contamination. These advantages make

this RT-PCR-based method more useful for high-efficiency sample processing than other methods in diagnosing BLAD, CVM, DUMPS, and BC in cattle.

Although the results of the studies show that the frequency of heterozygous genetic disease alleles in the population is shallow, mutant alleles will spread to their offspring if no follow-up is made to eliminate this feature. Healthy cows mated with heterozygous cows will produce 50% homozygous and 50% heterozygous offspring; the offspring potential is 25% normal, 50% heterozygous, and 25% lethal when heterozygous cows mate with heterozygotes.

5. Conclusions

The real-time PCR-based method is ready for simple, reliable, highly efficient, and rapid genotyping for BLAD, CVM, BC, and DUMPS carrier detection in the cattle population. The reported high frequency of CVM, BC, DUMPS, and BLAD alleles worldwide demonstrates the necessity of applying a routine test system using our new method. By avoiding heterozygous bulls for these diseases in the cattle population, the number of carriers of hereditary fatal diseases caused by these recessive genes can be reduced by an intensive selection program. Therefore, a fast, reliable, and high-throughput diagnostic method would be included in breeding programs.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: Semen, Z. **Data curation:** Semen, Z. **Formal analysis:** Semen, Z. **Funding acquisition:** Semen, Z. **Investigation:** Semen, Z. and Karakas, V. **Methodology:** Semen, Z. **Project administration:** Semen, Z. and Karakas, V. **Software:** Semen, Z. **Supervision:** Semen, Z. **Validation:** Semen, Z. **Visualization:** Semen, Z. and Guvenc-Bayram, G. **Writing – original draft:** Semen, Z. and Guvenc-Bayram, G. **Writing – review & editing:** Semen, Z. and Guvenc-Bayram, G.

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