

Investigation of hereditary cholesterol deficiency (CD) in Holstein Cattle at the state farms in Türkiye

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ABSTRACT

Türkiye is exposed to international animal and semen movements. For this reason, Türkiye is very affected by diseases and hereditary disorders. State Farms is a state organization that raises and distributes breeding cattle. This study was aimed at investigating the presence and distribution of mutant alleles causing cholesterol deficiency (CD) disorder in Holstein cattle on state farms. For this purpose, blood and sperm samples were collected from 466 Holstein cattle. The real-time PCR method was used for genotyping. A total of seven cattle were found to be heterozygous. The frequency of the mutant allele was determined to be 0.75%. The kinship of four cattle carrying the mutant allele with Mauglin Storm, the bull in which this disease was first detected, was determined. The presence of cattle carrying the mutant allele in Türkiye is quite low compared to other countries. The Apolipoprotein-B (APOB) mutant allele was found at low frequencies and detected for the first time at the State Farms in Türkiye. Therefore, it is necessary to develop control programs by screening other Holstein herds.

Introduction

In intensive production, obtaining healthy herds is one of the main goals. Hereditary diseases also negatively affect the health of herds. Hereditary diseases are defects that occur in the form of structural disorders, anomalies, and syndromes that are transmitted from generation to generation depending on genes. Therefore, knowing the genetic background of calves is very important in terms of breeding strategy. Today, animals with defective genes can be detected more easily.

Artificial insemination is extensively used in cattle breeding. Therefore, as long as a hereditary disease carrier bull is used in artificial insemination, it spreads the abnormal gene, causing its frequency to increase. It has been reported that 29% of 273 Holstein bulls whose semen was imported to Türkiye in 2015 carried at least one hereditary defect (10). One of these diseases is a hereditary disorder called cholesterol deficiency (CD), which was

detected in Holstein cattle by German researchers in 2015. Cholesterol deficiency is an autosomal recessive disease that results in death in calves. Affected animals show signs of hypocholesterolemia and hypolipidemia (13). It has been reported that the digestive system of calves shows normal activity, their stools vary between yellow and olive green, and the normal odor and stool density vary between soft (normal) and liquid (intermittent diarrhea) (18).

The source of the mutation is based on a bull named Mauglin Storm, born in 1991 (17, 23). The use of this bull's sons and grandchildren in artificial insemination has increased the spread of the disease (4). The major disadvantage of hereditary disorders arises when the mutation occurs in bulls that are far superior in terms of yields. It has been calculated that the economic loss due to the disease in Germany is 1.3 million euros per year (13).

It has been reported that the mutation that causes the disease in homozygous animals affected by the disease

occurs on the Apolipoprotein-B (APOB) gene (17, 22). CD is an autosomal recessive disease seen in homozygous mutant animals due to the APOB mutation, resulting in a 1299 bp insertion of the APOB gene on the 11th chromosome between the 24th and 25th nucleotides of the 5th exon in Holstein cattle (17).

Although it has been reported that this hereditary defect shows autosomal recessive inheritance, in an article (9), it was stated that some of the heterozygotes showed clinical signs, and the penetrance effect of the gene decreased in heterozygotes compared to homozygotes with full penetrance effect. Therefore, it was concluded that the disease is a metabolic disorder with incomplete dominant inheritance and incomplete penetrance in heterozygotes. In a study, it was determined that non-carriers had significantly higher serum cholesterol than carriers. It has been determined that CD carriers have shorter milking times and lower body weights than other control groups (3).

In Germany, the mutant gene frequency at which 80% of 234 homozygous calves died by the age of one was reported to be 8.7% (13). In Canada, the carrier frequency decreased from 17% in 2012 to 12% in 2016 (5). In a study conducted in Germany, 17.4% of 264 bulls were found to be carriers (17). The frequency of carriers in Holstein bulls born in Germany between 2012 and 2015 has been reported as 12.7% (22). In Poland, 9 out of 27 bulls with Mauglin STORM pedigree were found to be carriers (11). It has been reported that 10 of the 539 cows in Taiwan are carriers, and the frequency is 1.86% (16).

In China, 7 out of 138 bulls (5.07%) and only 1 out of 90 cows (1.11%) were identified as carriers. That is, the mutant allele frequency was determined to be 2.53% in bulls and 0.56% in cows. In the pedigree analysis, the carrier bulls and the cow were based on the cow named Braedale Baler Twine (HOCANF 6860888), the daughter of Mauglin Storm (15). The carrier rate of 1633 Holstein cows in China was determined to be 3.62% (59 cows) (12). The average frequency of mutant gene carriers was found to be 14.6% in 14 herds with 917 cows in Canada (24). In the study conducted in Russia, 35 of 451 cows were found to be carriers, and the carrier frequency was calculated at 7.76% (19). According to Pozovnikova et al. (20), 147 out of 1817 cows in Russia were carriers, and the carrier rate was 8.09%; 50 out of 171 cows whose fathers were carriers (29.23%); and 27 out of 160 cows and heifers whose fathers were carriers were reported to be carriers (16.87%). The carrier rate in cows of a local breed obtained with the contribution of Holstein in Russia was determined to be 4% (25). The carrier rate among Holsteins in Uruguay has been reported as 2.61% (21). In India, 1 out of 60 Holstein bulls was found to be carriers (frequency 1.67%) (14).

In this study, it was aimed to investigate the presence, distribution, and pedigree relationships of the mutant allele that causes an autosomal hereditary disease called "Cholesterol Deficiency (CD)" in Holstein cattle raised at State Farms.

Materials and Methods

A total of 466 Holstein cattle samples were taken from state farms, 25 of which were bull semen and 441 blood samples.

Genomic DNA was isolated using the "Zymo Research/D3025 Quick-gDNA™ Blood MiniPrep" DNA isolation kit. DNA samples were checked for their integrity on an agarose gel, and their amounts were measured using a spectrophotometer device (Thermo Multiscan GO). The amount and quality of the controlled DNA samples were diluted to 30-50 ng/μl and stored at -20 °C.

"Primer 200 nM (60 bases) Primer/P200HPLC" as primer

APOB.e3.WT.F_5'-CTGCAAAGCCACCTAGCCTA-3',
APOB.e3.WT.R_5'-GCCTCTTCTGTTTCTGGGGG-3',
and APOB.e3.Ins. R_5'-TCACGAGTGAATGCCT
CAC-3' primers from the insertion site were used.

"Bioline/BIO-92020, SensiFAST SYBR HI-ROX Kit" was used as the green master mix.

In real-time PCR, primers recommended by Schütz et al. (24), APOB.e3.WT.F_5'-CTGCAAAGCCACCTAGCCTA-3', APOB.e3.WT.R_5'-GCCTCTTCTGTTTCTGGGGG-3', and APOB.e3.Ins.R_5'-TCACGAGTGAATGCCTCAC-3' from the insertion site, were used. For each PCR reaction, 50 ng/μL of DNA, 1x SYBR Green Master Mix (Bioline, SensiMix SYBR Hi-ROX), and 0.2 μM of each primer were added and made up to 25 μL with ultra-distilled water. The initial denaturation of the Real-Time PCR device (ThermoFisher StepOne plus Real-Time PCR) will be 10 min at 95 °C, followed by 35 cycles of 15 sec at 95 °C, 20 sec at 57 °C, and 30 sec at 72 °C. Measurements were made at the end of each cycle. Then, high-resolution melting curve (HRM) analysis was performed between 65 and 95 °C. Mutant and normal alleles were determined according to the HRM analysis.

LongRange PCR Protocol: Long-range PCR was performed to confirm the heterozygous and homozygous samples in real-time PCR analysis. For this purpose, APOB.e3.WT.F_5'-CTGCAAAGCCACCTAGCCTA-3' and APOB.e3.WT.R_5'-GCCTCTTCTGTTTCTGGGGG-3' primers were used (24). A PCR mix (20 μL) consisting of 1xPCR buffer, 3.5 mM MgCl₂, 0.2 mM dNTPs, 0.3 pmol forward primer, 0.3 pmol reverse primer, 1 U Platinum Taq polymerase (Invitrogen, 10966034), and 20

ng/ μ L DNA was prepared. PCR instrument (ThermoFisher, Veriti) for 2 minutes: one cycle at 95 °C, 45 sec at 94 °C, 30 sec at 57 °C, and 1 min at 72 °C, 35 cycles, and a final stage at 72 °C for 10 minutes.

DNA Sequencing Analysis: Before DNA sequencing analysis, 4 μ l of PCR product, 0.5 μ l Exonuclease-1 (ThermoFisher, EN0581), and 1.0 μ l FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher, EF0652) were added to clean the amplified PCR products. The prepared mixture was kept in the PCR device for 15 minutes at 37 °C and 15 minutes at 85 °C. Then, for sequencing PCR, 12 μ l of 1x sequencing buffer, 1 μ l of BigDye solution, 5 μ l (1 pmol) of F/R primer, and 2 μ l of cleaned PCR product were added per sample. The PCR device was programmed for 2 minutes and 1 cycle at 96 °C, 10 seconds at 96 °C, 20 seconds at 54 °C, and 4 minutes and 30 cycles at 60 °C.

The products obtained as a result of the sequence PCR were cleaned according to the Ethanol-EDTA-Sodium acetate method. For this purpose, 1 μ l of EDTA (pH 8.0), 1 μ l of 3M sodium acetate (NaOAc, pH 5.07) and 50 μ l of Ethanol (98%) were added to each sample. The plate was covered with aluminum sealing and turned upside down 4-5 times and kept in the dark at room temperature for 15 minutes. Then it was centrifuged at 4000 rpm at +4 °C for 30 min. After centrifugation, the sealing on it was removed and inverted on a napkin and centrifuged at +4 °C for 1 minute at 700 rpm. Then, 70 μ l of 70% ethanol was added to the samples, covered with plate sealing, and centrifuged at 4000 rpm for 10 minutes at +4 °C. After centrifugation, the sealing on it was removed and inverted on a napkin and centrifuged at +4 °C for 1 minute at 700 rpm. The plate was then incubated in a dark place at room temperature for 60 min. After adding 15 μ l of Hi-Di Formamide to each sample, the plate was sealed and vortexed vigorously and centrifuged briefly at 1300 rpm. After centrifugation, the sealing was removed and the plate was closed with septa and loaded into the DNA sequence analysis device (ThermoFisher, ABI 3500 Genetic Analyzer). The sequences obtained as a result of the DNA sequence analysis were arranged with the Sequencher Version 5.4.6 (Gene Codes Co) package program.

PCR Analysis: Primers proposed by Schütz et al. (24) and used in real-time PCR were used in PCR analysis. For each PCR reaction, 50 ng/ μ L of DNA, 5xPhusion HF Buffer (Thermo, F530L), 0.2 μ M of each primer, and 1 U of Phusion Taq Polymerase were added and made up to 25 μ L with ultra-distilled water. In the gradient PCR analysis

to determine the T_m degree of the primers, the PCR device uses the initial denaturation at 98 °C for 1 min, followed by 40 cycles at 98 °C for 5 sec, 54–64 °C for 20 sec, and 72 °C for 20 second the last stage was programmed to last 10 minutes at 72 °C. The T_m temperature was determined to be 64 °C, and PCR of all samples was performed at this T_m temperature. After PCR, mutant and normal alleles were checked with 2% agarose gel electrophoresis.

Agarose Gel Electrophoresis: To prepare the agarose gel, 1 g of agarose and 50 ml of TAE (Tris-Acetate-EDTA) solution were mixed and heated in a microwave oven for 2 minutes. 1 μ l of RedSafe (INtRON, 21141) dye solution was added to the prepared 2% agarose gel. The gel was then poured slowly into the tray. The prepared gel was kept at room temperature for 30 minutes and at 4 °C for 30 minutes for polymerization. After polymerization, the agarose gel was placed in a tank containing 1xTAE solution. The tank was filled with 1xTAE solution to cover the gel. 8.5 μ l of the loading solution (1X Loading Dye) and 2.5 μ l of the PCR product mixture were added to each well. After loading, 7 cm/V was applied to the gel for 30 minutes. After the run was completed, the gel was removed from the tank and visualized using the gel imaging system. A DNA ladder (ThermoFisher, SM0323) was used to determine the size of the PCR product.

Statistical Analysis: Gene frequencies were determined by the gene counting method. The frequency of gene A was found by dividing the sum of twice the number of homozygous (AA) individuals and the number of heterozygous individuals by twice the total number of individuals. The frequency of the B gene was found by doing a similar procedure for the B gene.

Gene Frequency = (Number of Homozygous Individuals x 2) + Number of Heterozygous Individuals / Total Number of Individuals x 2.

Whether the genotype frequencies fit the Hardy-Weinberg equation was checked with the chi-square test.

Results

Real-Time PCR: As a result of the real-time PCR analysis, the amplification graph of the samples is given in Figure 1. As a result of the HRM analysis performed at the end of the real-time PCR analysis, it was observed that in some samples, it gave a single peak at 85.8 °C, and in some samples, two peaks at 85.7 and 92.6 °C (Figure 2 and Figure 3). Samples with a single peak (Figure 2) were evaluated as not carrying the mutant allele (homozygous), while samples with two peaks (Figure 3) were considered to be carrying the mutant allele (heterozygous).



Figure 1. Real-Time PCR amplification plot of the samples.

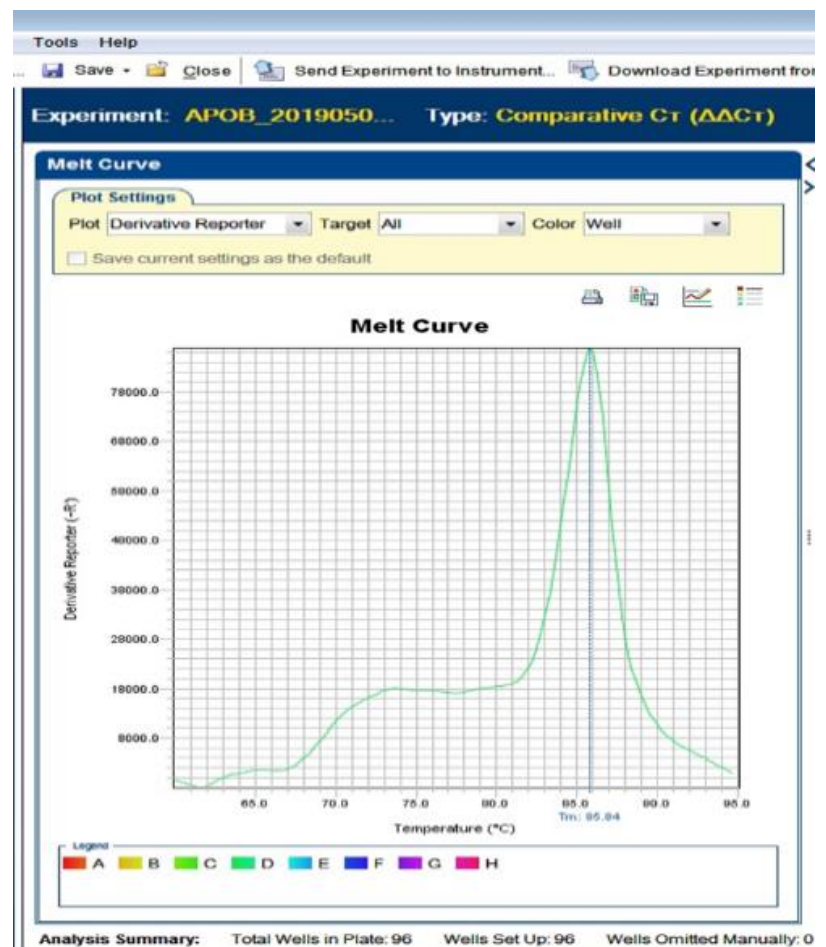


Figure 2. HRM (High Resolution Melting) curve analysis results of the samples (Homozygous).

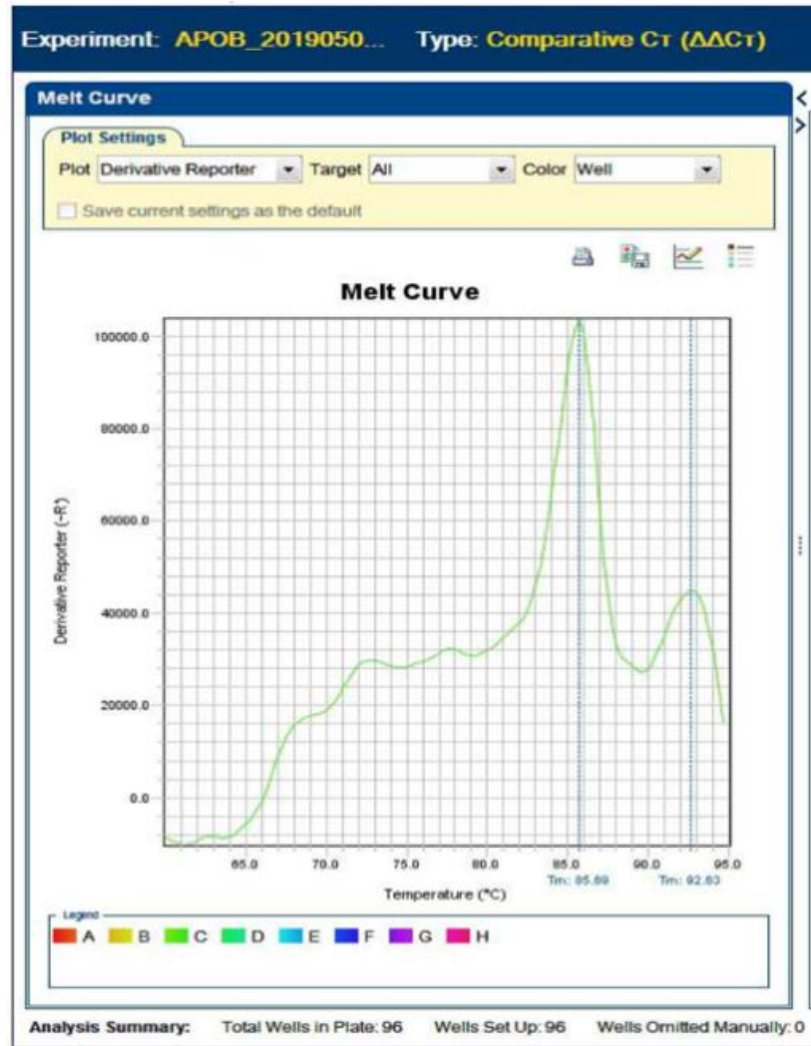


Figure 3. HRM (High Resolution Melting curve) analysis results of the samples (Heterozygous).

Sequence Analysis: DNA sequencing analysis was performed using APOB.e3.WT.F and APOB.e3.WT.R (long-range PCR) to confirm the samples evaluated as heterozygous and homozygous in real-time PCR. As a result of the sequencing analysis, no difference was observed in homozygous and heterozygous samples, and DNA sequences of 206 bp (base pair) length were obtained in all. In DNA sequencing analysis, the bases in the insertion region of the 1287 bp-long mutant allele were not found. In the agarose gel, the PCR products were determined to be 206 bp in length, and the band with bases in the insertion region could not be visualized. In other words, homozygous and heterozygous individuals could not be distinguished from each other.

PCR: In the analysis, it was determined that the heterozygous samples had two bands with a length of 206 and 132 bp.

Genotype and Allele Frequencies: Distributions of heterozygous and homozygous genotypes observed in

agarose gel electrophoresis, expected genotype frequencies, and chi-square analyses are given in Table 1, and allele frequencies are given in Table 2. Of the 466 samples analyzed, 25 of which were bulls, 459 were homozygous for the wild allele. Seven cows were identified as heterozygous for the mutant allele. All 25 bulls sampled from Sultansuyu Farm were found to be homozygous for the wild allele (Table 1). It was determined that 7 cows were carriers: 3 in Çukurova Farm, 3 in Ceylanpınar Farm, and 1 in Kocas Farm. It has been demonstrated by X^2 analysis that the difference between observed and expected genotype frequencies in farms and in total is not significant (Table 1). The population appears to be in equilibrium in terms of the alleles studied. Mutant allele frequency varies between 0.00 and 1.97%, according to the farms (Table 2). When all the farms were evaluated together, the mutant gene frequency was calculated at 0.75%. The frequency of the mutant gene is quite low.

Table 1. Distribution of genotypes for the APOB allele by farms (N=Number).

Farms	AA		AB		BB		X ²			
	Obs.	Exp.	Obs.	Exp.	Obs.	Exp.				
	N	%	N	%	N	%				
Cukurova (Adana)	76	96.21	76.04	3	3.79	2.92	-	0.00	0.03	NS
Ceylanpinar (Urfa)	73	96.06	73.03	3	3.94	2.93	-	0.00	0.03	NS
Kocas (Aksaray)	95	98.96	95.00	1	1.04	0.99	-	0.00	0.01	NS
Polatli (Ankara)	108	100.0	108.00	-	0.00	-	-	0.00	0.00	NS
Turkgeldi (Tekirdag)	82	100.0	82.00	-	0.00	-	-	0.00	0.00	NS
Sultansuyu* (Malatya)	25	100.0	25.00	-	0.00	-	-	0.00	0.00	NS
TOTAL	459	98.50	459.03	7	1.50	6.94	-	0.00	0.07	NS

AA: Wild type homozygote, AB: Heterozygote carrier, BB: Mutant homozygote; NS: Non-significant
Obs.: Observed, Exp.: Expected, *: Bulls.

Table 2. Allele frequencies of the APOB gene by farms.

Farms	A (%)	B (%)
Cukurova (Adana)	98.11	1.89
Ceylanpinar (Urfa)	98.03	1.97
Kocas (Aksaray)	99.48	0.52
Polatli (Ankara)	100.00	0.00
Turkgeldi (Tekirdag)	100.00	0.00
Sultansuyu (Malatya) - Bulls	100.00	0.00
General	99.25	0.75

A: Wild allele, B: Mutant allele.

Discussion and Conclusion

Real-Time PCR, Sequence Analysis and PCR: Menzi et al. (17) showed that Taq polymerase used prefers the shorter wild type allele (249 bp) in animals with mutant alleles in their long-range PCR analysis to show the insertion in the 5th exon of the APOB gene. Therefore, they reported that they could not amplify the PCR product of the mutant allele with a length of 1548 bp. Based on this analysis to detect carriers; they suggested PCR by using a primer from the insertion site. Therefore, Menzi et al. (17), PCR of all samples again using Phusion Taq polymerase with APOB.e3.WT.F, APOB.e3.WT.R, and APOB.e3.Ins.R used in real-time PCR. They were made and visualized by running on a 2% agarose gel. It was determined that the majority of the samples detected in the heterozygous structure in the Real-Time PCR application were homozygous. They stated that this was due to the fact that the primers in Real-Time PCR were affected by the T_m grade used, and the affinity of Taq polymerase was different depending on the change in the MgCl₂ concentration in the master mix used.

Genotype and Allele Frequencies: All 25 bulls in the study were homozygous for the wild allele. That is, bulls do not inherit the mutant allele that causes the disease. When the pedigrees of the fathers of the bulls were examined, no kinship ties with Mauglin Storm were

found. In a study examining bull pedigrees of imported semen in Türkiye, the carrier rate was reported as 5.5% (10). In this case, it can be said that it is possible that the mutant gene causing CD disease has spread throughout the country.

For this hereditary defect, it is seen that carrier frequencies have increased over the years in countries such as Germany and Canada, where control programs have not yet started (6, 7, 13, 17, 24). This situation shows that there is a need for eradication programs to reduce the frequency of the mutant gene. The frequency of the gene will increase if there is no intervention for eradication. The fact that the frequency of mutant genes in some countries such as China and Taiwan is lower than in some countries such as Germany and Canada can be attributed to the low import of semen whose pedigree is based on Storm.

In our study, it was determined that 7 out of 466 cattle were carriers, and the carrier frequency was 1.5% (Table 1 and Table 2). Gürses and Dere (8) reported that the rate of CD carriers was 4.67% in 450 cows in the Balıkesir region of Türkiye. Although the carrier frequency of CD disease is high on private farms in the Balıkesir region, it is quite low on state farms. This can be seen as an advantage. Therefore, it would be easier to control or prevent the spread of the ABOP gene. For the control of the disease, the pedigrees of the bulls should be paid attention to, and if they are not specified in their

pedigrees, genetic tests should be made in the laboratory to ensure that they are used.

The Presence, Distribution, and Pedigree Relationships of The Mutant Allele: After the mutation in the APOB gene, which causes cholesterol deficiency, was detected in Türkiye, it is important to associate these animals with Mauglin STORM. The inbreeding status of seven carrier cows with Mauglin STORM was investigated (Table 1). It was determined that the fathers of TR63671712 and TR63678971 ear tag carrier 2 cows in Ceylanpınar Farm were ELITE born in 2004 with HODEUM000662151690 ear tags. He is the grandson of Mauglin Storm. The pedigree information of the other carrier TR16775699 cow with ear tags could not be reached. Thus, it has been proven that two cows are related to Mauglin Storm (1, 2).

The carrier of the cow's father with ear tag number TR68677555 born in 2014 in KOCAS farm is a bull named GUNNAR with 2006 birth date and HODEUM001500838492 ear tag (Table 1). The bull named GUNNAR was revealed to be the son of Mauglin STORM's grandson, Braedale GOLDWYN, who is known to be a carrier. Therefore, it has been proven that this cow is also related to Mauglin Storm. It was determined that the father of the cow with ear tag number 1245066 from the 3 carrier cows in Cukurova Farm was also Gunnar. However, it could not be determined from the available pedigree information of the other two carrier cows from where they got the mutant gene (1, 2).

It was understood that 4 of the 7 carriers in question were related to Mauglin STORM; the other 3 could not be associated with STORM according to the available information (Table 1). These findings indicated that carrier bulls were also carried through semen import. The mutant gene was found as a result of the importation of the semen of his sons and grandchildren, who are known to be carriers of Mauglin STORM in Türkiye. As it is known, for success in dairy cattle breeding, it is necessary to obtain the highest yield and to maintain this situation for years. For this, it is important to choose the bulls and cows correctly. Hereditary diseases are more easily identified with the development of technology, and the pedigrees of bulls include information on whether a bull carries a hereditary disease as well as its productivity characteristics. Considering that proven bulls pass on their hereditary characteristics to their offspring, the use of semen from these bulls and the importation of their semen can continue to be passed on to future generations through heredity, along with traits such as milk yield, fertility, and meat yield, as a result of the use of these proven bulls in artificial insemination in many countries. Mauglin Storm is the beginning of the mutation; the mutant gene spread throughout countries with his children, grandchildren, and daughters. Carrier bulls have been found in many

countries, such as Germany, the United States, the Netherlands, Canada, Belgium, England, Denmark, Luxembourg, and France. Mauglin Storm passes on both its good properties and cholesterol deficiency to its offspring. With the import of semen, this disease does not remain in a limited number of countries but spreads to more. Thanks to genetic tests, it can be determined whether the bulls are carriers of hereditary diseases, and information about the bull can be obtained when the bull catalogs and pedigrees of the bulls are examined. After the molecular detection of the disease, bulls with this disease have also become distinguishable, and in the pedigrees of the bulls of many countries, the bulls carrying the disease are shown with the CD code and those determined not to be carrying the disease are shown with the TC code. Türkiye also imports semen and female breeding cattle. For this reason, it is necessary to know whether bulls and breeding females carry hereditary diseases.

It was concluded that ABOP carrier cows were detected on state farms in Türkiye. For this reason, it is necessary to carry out controls for hereditary diseases in imported live animals and semen. The fact that clinical symptoms were observed in some of the heterozygous individuals suggests that the effect of the disease may be greater. For this reason, efforts should be made to completely eradicate the mutant gene by reducing its frequency. Cholesterol and triglyceride levels should be monitored and evaluated in individuals determined to be carriers. It is important to identify breeder cows, especially bulls, in terms of mutant genes.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

The contributions of the authors are equal.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was carried out after the animal experiment was approved by Ankara University Local Ethics Committee (Decision number: 2017-10-88).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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