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Research Article

Identification of the ERV Insertion of *APOB* Gene and Deletion of *TFB1M* Gene Associated with Lethal Haplotypes of Holstein Cattle Reared in Balıkesir Province, Türkiye

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Abstract: Cholesterol deficiency (CDH) and lethal haplotype 5 (HH5) are autosomal recessive genetic defects of Holstein cattle. HH5 and CDH are associated with embryonic mortality and calf loss, respectively, resulting in severe economic losses in the dairy cattle breeding. The aim of the present study was to investigate the presence of HH5 and CDH haplotypes and to determine the carrier and haplotype frequencies for these haplotypes in Holstein cattle reared in Balıkesir province of Türkiye. Polymerase chain reaction (PCR) was used to detect the deletion of the transcription factor B1 *(TFB1M)* gene and the retroviral insertion into the apolipoprotein B *(APOB)* gene, causing CDH and HH5, respectively. A total of 450 Holstein cows born between 2011 and 2018 were screened for causative mutations of both haplotypes. As a result, the carrier frequencies for CDH and HH5 were 4.67% and 7.56% and the haplotype frequencies were 0.023 and 0.038, respectively. 12.22% of the cows were identified to be carriers of at least one genetic defect. This study demonstrated the presence of CDH and HH5 haplotypes in Holstein cattle reared in Türkiye.

Keywords: *Holstein, Cholesterol deficiency, Lethal haplotype 5, Genetic defects*

Balıkesir'de Yetiştirilen Holştayn Irkı Sığırlarda Letal Haplotiplerle İlişkili *APOB* Geni ERV İnsersiyonu ve *TFB1M* Geni Delesyonunun Belirlenmesi

Öz: Kolesterol eksikliği (CDH) ve letal haplotip 5 (HH5) Holştayn ırkı sığırlarda görülen otozomal resesif genetik kusurlardır. HH5 ve CDH süt sığırı yetiştiriciliğinde önemli ekonomik kayıplara neden olan embriyonik ölüm ve buzağı kayıpları ile ilişkilidir. Bu çalışmanın amacı, Balıkesir'de yetiştirilen Holştayn ırkı sığırlarda HH5 ve CDH haplotiplerinin varlığını araştırmak, taşıyıcı ve haplotip frekanslarını belirlemektir. HH5 ve CDH'ye neden olan transkripsiyon faktörü B1 *(TFB1M)* geni delesyonu ile apolipoprotein B *(APOB)* geni retroviral insersiyonunu tespit etmek için polimeraz zincir reaksiyonu (PCR) kullanılmıştır. 2011-2018 yılları arasında doğan Holştayn ırkı toplam 450 inek haplotiplerin nedensel mutasyonları yönünden taranmıştır. Sonuçta, CDH ve HH5 için taşıyıcı frekansları sırasıyla %4.67 ve %7.56 ve haplotip frekansları 0.023 ve 0.038 olarak tespit edilmiştir. İneklerin %12.22'sinin en az bir genetik kusurun taşıyıcısı olduğu belirlenmiştir. Bu çalışma, Türkiye'de yetiştirilen Holştayn ırkı sığırlarda CDH ve HH5 haplotiplerinin varlığını ortaya çıkarmıştır.

Anahtar sözcükler: *Holştayn, Kolesterol eksikliği, Letal haplotip 5, Genetik kusurlar*

Introduction

In modern dairy cattle breeding, intensive selection and the widespread use of elite sires have led to significant genetic improvement in economically important traits, but also to increased inbreeding and reduced genetic diversity and survival traits worldwide. The accumulation of inbreeding has increased the frequency of lethal recessive alleles, resulting in a higher incidence of genetic defects in dairy cattle populations [1-3].

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The large-scale genomic data obtained as a result of the routine use of single nucleotide polymorphism (SNP) array genotyping in dairy cattle breeding programs over the last decade has enabled the identification of recessives. Lethal recessives can be discovered from haplotypes that, although common in the population, are never seen in the homozygous state in living animals [4-6]. After the discovery of the first three haplotypes (HH1, HH2, HH3) in the American Holstein in 2011 [4], 43 haplotypes (HH0-HH38, HHB, HHC, HHD, CDH) with reduced homozygosity have been identified in different Holstein populations so far [6]. Sixteen of these haplotypes, including CDH and HH5, have been listed in the Online Mendelian Inheritance in Animals (OMIA) database as likely causal variants for inherited traits and disorders in the Holstein breed [7].

Cholesterol deficiency a newly identified recessive inherited genetic defect in Holstein (OMIA: 001965-9913), that causes calf mortality in the dairy cattle breeding. The causative mutation of CDH results from a 1.3 kb insertion of an endogenous retrovirus (ERV) into the fifth exon of the apolipoprotein B gene on chromosome 11 (BTA11:77.959 kb). The premature stop codon in protein synthesis as a result of the insertion causes the amino acid number of the protein to remain below 140 [7-10]. The premature truncation of the protein results in an inability secretion of chylomicrons, and leads to malabsorption of dietary fat and fat-soluble vitamins in the intestine, and is assumed to impair cholesterol metabolism and transport in the circulation and liver [10,11]. The disease-associated haplotype descended from the Canadian Holstein sire Maughlin Storm (ID HOLCANM000005457798) born in 1991, suggesting autosomal recessive inheritance [12,13]. Clinical signs of the disease in homozygous calves include chronic diarrhea, insufficient development, and severe hypocholesterolemia. Affected calves do not respond to symptomatic treatment and usually die within the first 6 months of their life [10,14,15]. Heterozygous carriers (calves, bulls and nonlactating females) are clinically healthy but show reduced cholesterol and lipoprotein concentrations [11,16,17].

HH5 is an autosomal recessive inherited genetic defect in Holstein cattle (OMIA: 001941-9913), caused by a deletion affecting in the mitochondrial *TFB1M* gene, that affects mitochondrial protein translation and causes embryonic lethality before day 60 of gestation [2,7,10]. The deletion of 138 kb (93.233 kb to 93.371 kb on BTA 9) contains the entire *TFB1M* gene and was traced back to Thornlea Texal Supreme, born in 1957. It has been reported that the frequency of HH5 carriers in European and North American Holstein cattle is approximately 4-5% [10]. Recently, Häfliger et al.[6], found the strongest association to date between heifers non-return rate after 56 days and HH5.

HH5 and CDH are associated with embryonic loss and calf mortality, resulting in severe economic losses [18,19]. Balıkesir is one of the leading provinces in Türkiye for cattle numbers and is an important center for milk production. Therefore, the objectives of the present study were to investigate the presence of CDH and HH5 haplotypes and to determine the carrier and mutant allele frequencies for these haplotypes in Holstein cattle reared in Balıkesir province of Türkiye.

Material and Methods

Ethical Approval

This study was approved by the local ethics committee on animal experiments of Balıkesir University (protocol number: 2019/12-8).

Sample Collection and DNA Isolation

A total of 450 blood samples were collected randomly from Holstein cows, born between 2011 and 2018 and raised in five dairy farms in Balıkesir province of Türkiye. Blood samples were collected from the tail vein into a sterile tube containing K3 EDTA anticoagulant and stored at -20°C. Genomic DNA was isolated from whole blood using the PureLink genomic DNA mini kit (Invitrogen, CA, USA) according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR)

To identify CDH and HH5 carriers in Holstein cows two PCRs were performed for each sample using a common forward primer and two reverse primers for mutant and wild-type alleles of the *APOB* gene [20] and the *TFB1M* gene [10], respectively. Information on the genetic defects and the nucleotide sequences of the primers used for PCR analysis are shown in the *Table 1*. All PCR amplification reactions were performed in a Biometra TAdvanced thermal cycler (Analytik Jena, Germany).

PCR amplification for the *APOB* gene was performed in a total volume of 25 µL reaction mixture containing 50 ng of genomic DNA, 6 pmol of each primer (Oligomer Biotechnology, Ankara, Türkiye), 12.5 µL of 2X Taq DNA polymerase master mix (Ampliqon Inc., Odense, Denmark), and 9.05 µL of nuclease-free water (Ambion Inc., Austin, TX, USA). Thermal cycler conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec annealing at 62°C for 30 sec, extension at 72°C for 30 sec, and a final extension step at 72°C for 7 min.

PCR amplification for the *TFB1M* gene was performed in a final volume of 25 µL, containing 50 ng of genomic DNA, 5 pmol of each primer (Oligomer Biotechnology, Ankara, Türkiye), 12.5 µL of 2X Taq DNA polymerase master mix (Ampliqon Inc., Odense, Denmark), and 9.30

Table 1. Information on the genetic defects and the nucleotide sequences of the primers used for the PCR analysis

Locus[a]	Chr[b]	Gene	Mutation and Type of Variant[c]	Primer (Sequence 5′-3′[d]	Chromosomal Positions of Primers[c]	Amplicon Length	Tm[e] (°C)
HH5	9	*TFB1M*	g.93223651 to 93370998del; deletion	CF: AGATATGCTAAAGTTTACCTAGAAGAA	BTA9: 93371172 - 93371146	442 bp (Wild) 256 bp (Mutant)	57.5
				WT-R: CTGAAGCTCCATTCTGAGTCAT	BTA9: 93370731 - 93370752		
				Del-R: TGCTCTATGAATTTTGTGAATGGT	BTA9: 93232580 - 93232603		
CDH	11	*APOB*	g.77958995ins1.3kb; insertion	CF: TGCAAAGCCACCTAGCCTAT	BTA11:77958901 - 77958920	366 bp (Mutant) 171 bp (Wild)	62
				Ins-R: CACTCCTAATTGCCCAGGAA	within the insertion		
				WT-R: AGATGATGCCCCTCTTGATG	BTA11:77959071 - 77959052		

[a] *HH5, Holstein haplotype 5* [10]; *CDH, Cholesterol deficiency haplotype* [9,20]; [b]*Chromosome;* [c] *Genomic positions refer to the Bos taurus UMD 3.1 genome assemble;* [d] *CF, common forward primer; WT-R, wild-type reverse primer; mutant-type reverse primers: Del-R, deletion specific reverse primer, Ins-R, insertion specific reverse primer;* [e] *Tm: melting temperature*

µL of nuclease-free water (Ambion Inc., Austin, TX, USA) under the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57.5°C for 30 sec, extension at 72°C for 30 sec, and a final extension step at 72°C for 7 min.

A sample, provided by Professor Stanislaw Kamiński, known to be a carrier of CDH was used to control the amplification of the mutant allele in the PCR optimization. The first samples identified as CDH or HCD carriers were used for control of the mutant allele in PCR reactions (as a positive control). The PCR products of *APOB* and *TFB1M* genes were electrophoresed on 1.5% agarose gel stained with GelRed (Biotium, Hayward, CA, USA) in 0.5X TBE (Tris-borate-EDTA) buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and visualized under ultraviolet light.

Data Analysis

Carrier and haplotype frequencies of CDH and HH5 were estimated by direct counting. The distribution of carriers by date of birth was considered in periods of 2 years. Tables and graphs were generated using Microsoft Excel for Microsoft 365 (Microsoft Corporation, Redmond, WA).

Results

A total of 450 cows were analyzed by PCR for the detection of causative mutations for CDH and HH5. The PCR products of the *APOB* and *TFB1M* genes were analyzed by 1.5% agarose gel electrophoresis and visualized under ultraviolet light *(Fig. 1-A,B)*. As a result of electrophoresis, two DNA bands specific for both mutant and wild-type alleles were expected to be present in carrier animals, and only one DNA band specific for the wild-type allele in normal animals. Heterozygous carriers of CDH had two DNA fragments of 171 and 366 bp, whereas normal animals (wild-type homozygotes) had only one DNA fragment of 171 bp *(Fig. 1-A)*. HH5 carriers had two DNA fragments of 256 and 442 bp, whereas normal animals had only one DNA fragment of 442 bp *(Fig. 1-B)*.

CDH analysis revealed that of the 450 cows were genotyped, 429 were identified as normal and 21 were identified as carriers for CDH. As a result of the HH5 analysis of 450 cows, 416 were identified as normal and 34 were identified as carriers of HH5. As expected, no homozygous mutants were found for either haplotype. Carrier frequencies were found to be 4.67% and 7.56% for CDH and HH5, respectively, and the haplotype frequencies were calculated to be 0.023 and 0.038, respectively. While no cows carrying both haplotypes were found, 12.22% of the population was observed to carry at least one of the genetic defects.

It was observed that the CDH carrier frequency was highest (12.94%) in cattle born in 2011-2012 and followed a gradually decreasing trend in the following years, first decreasing to the range of 3.41-3.45% in cattle born between 2013 and 2016, and then decreasing to the lowest level (1.86%) in cattle born in 2017-2018 *(Fig. 2-A)*. The HH5 carrier frequency was observed as 2.35% in cattle born in 2011-2012, increased to 9.09% in cattle born in 2013-2014, reached the highest level (18.97%) in cattle born in 2015-2016 and then decreased to the lowest level (1.24%) in cattle born in 2017-2018 *(Fig. 2-B)*.

Discussion

The present study demonstrated the presence of defective alleles for HH5 and CDH in the Turkish Holstein population. HH5 carriers in the population were observed

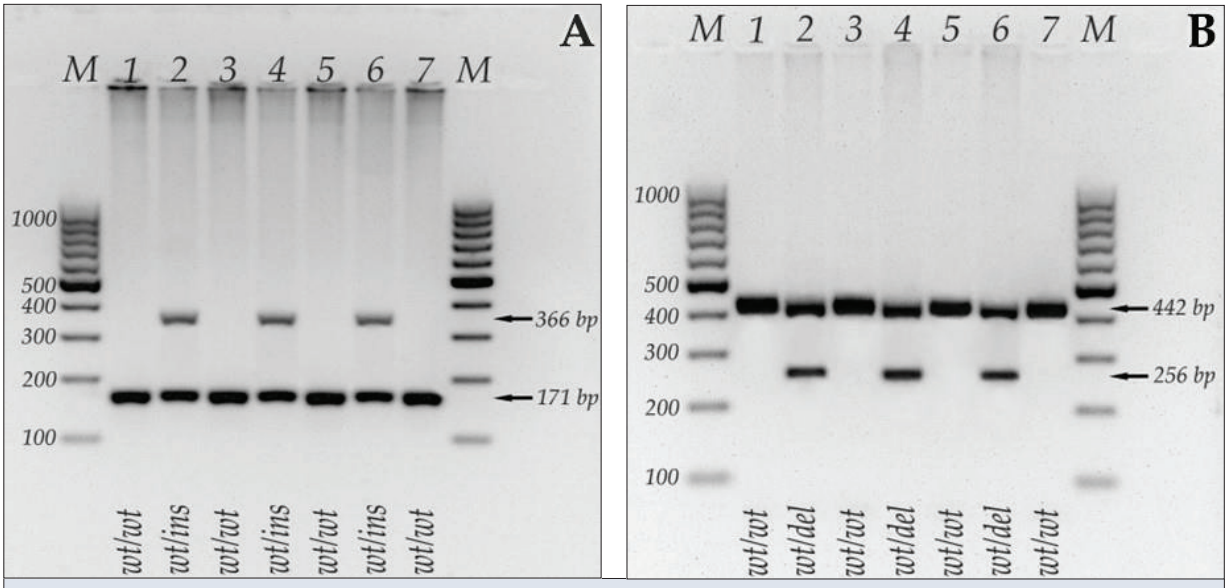


Fig 1. Agarose gel electrophoresis of the diagnostic PCR products for CDH and HH5, respectively. A- Lane M,100 bp DNA ladder; lane 1, 3, 5, 7 normal animals with one DNA fragment of 171 bp; lane 2, 4, 6 CDH carriers with two DNA fragments of 171 and 366 bp. B- Lane M,100 bp DNA ladder; lane 1, 3, 5, 7 normal animals with one DNA fragment of 442 bp; lane 2, 4, 6 HH5 carriers with two DNA fragments of 442 and 256 bp

to be more than CDH carriers. Studies investigating the prevalence of HH5 and CDH carriers in different countries have shown a wide variation. The frequency of CDH carriers in Türkiye (4.67%) was lower than previously reported for Holsteins in Germany (8.7% and 12.7%) [10,14], Kazakhstan (11%) [21] and Russia (5.66% and 7.76%) [22,23]. However, it was higher than that reported in Uruguay (2.61%) [2] and China (3.62%) [3]. The HH5 carrier frequency (7.56%) was found to be higher than previously reported for Holsteins in Germany (5.5%) [10], Russia (2.23%) [22], Uruguay (0.26%) [2] and China (4.30%) [3]. The results indicate that the prevalence of HH5 carriers in the Turkish Holstein population is higher than those of reported in other countries, while the prevalence of CDH carriers is lower than those of reported in most of the other countries. This may be related to the prevalence of the HH5 and CDH carriers among the sires that were allowed to import semen into Türkiye at that time. Consistent with the results of the present study, Inal and Cam [24], reported that among the 273 Holstein sires whose semen was allowed to be imported into Türkiye in 2015, there were 22 HH5 and 15 CDH carriers. The study, which was identified HH5 and CDH as the two most common haplotypes, respectively, by examining the records in the sire catalogs, also indicated that information on the genetic structure of about half of the sires could not be obtained [24].

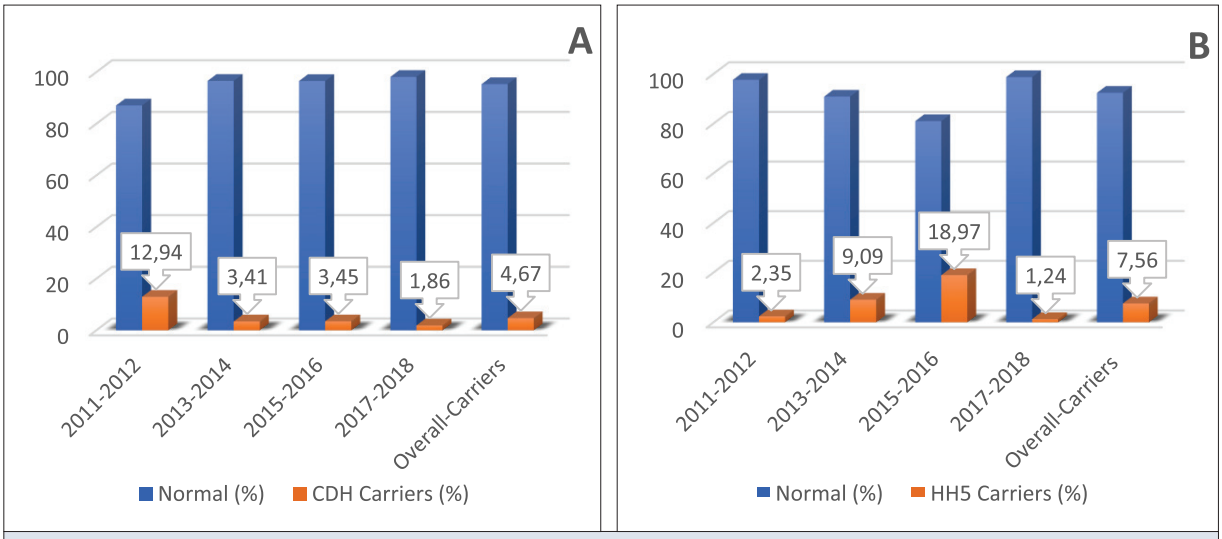


Fig 2. Distribution of carriers by year of birth. A- CDH carriers, B- HH5 carriers

Although the distribution of CDH and HH5 carriers in cattle born between 2011 and 2016 followed a different trend, the lowest carrier frequencies for both haplotypes were observed in cattle born in 2017-2018 *(Fig. 2-A,B)*. It can be said that the distribution of carriers according to birth dates between 2011 and 2018 is related to the prevalence of haplotypes both in the sires used for artificial insemination (AI) in Balıkesir from 2010 to the first months of 2018, and in the breeding cattle available in Balıkesir during the same period. During this period, there were no legal restrictions on the use of CDH or HH5 carrier sires for AI in Türkiye. Since the second half of 2018, the import of semen from HH5 carrier sires has been prohibited [25]. However, there are still no legal restrictions on the import of semen from CDH-carrier sires [26].

Recently, it has been suggested that CDH affects not only *APOB* mutant homozygotes, but also some heterozygous carriers by similar clinical signs due to incomplete penetrance. It has been reported that there is a significant difference between total cholesterol and triglyceride concentrations of clinically CDH-affected and non-affected *APOB* heterozygotes [27]. In this context, the potential impact of CDH on the dairy industry may be higher than estimated. It is important to raise awareness of this newly discovered haplotype among Turkish veterinarians to identify potential carriers and prevent the uncontrolled spread of the mutant allele of the *APOB* gene. DNA testing should be widely used for the identification of carriers and for the definitive diagnosis of affected calves.

In conclusion, this study demonstrated the presence of CDH and HH5 haplotypes in the Turkish Holstein population. It revealed the prevalence of the haplotypes in cows born between 2011 and 2018 in Balıkesir province. To prevent calf losses and reduce the future frequency of the haplotypes, carrier-to-carrier matings, which are expected to result in 25% calf losses and 50% carrier calves, should be avoided. The use of semen from carrier bulls in AI should be prevented to avoid the spread of the mutant allele throughout the country. Further studies should be conducted to identify haplotype carriers and investigate their prevalence in Holstein herds.

Availability of Data and Materials

The data used and analyzed in this study are available from the corresponding author (M. Gürses) on reasonable request.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contribution

MG designed and conducted the study. MG and ND collected the blood samples, carried out the extraction of genomic DNA, PCR analysis, and gel electrophoresis. MG drafted the manuscript. All authors have approved the final version of the manuscript.

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