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CHOLESTEROL DEFICIENCY MUTATION HCD DOES NOT IMPACT MILK PRODUCTIVITY AND BLOOD LEVELS OF CHOLESTEROL AND TRIGLYCERIDES IN RUSSIAN HOLSTEIN BLACK AND WHITE CATTLE

M.V. POZOVNIKOVA[1], T.E. LIHACHEVA[2], A.A. KUDINOV[1], V.B. LEJBOVA[1], N.V. DEMENTEVA[1]

[1]*All-Russian Research Institute for Farm Animal Genetics and Breeding — branch of Ernst Federal Science Center for Animal Husbandry*, 55A, Moskovskoe sh., St. Petersburg—Pushkin, 196625 Russia, e-mail marina.qpr@gmail.com (✉ corresponding author), kudinov_aa@list.ru, leib1406@yandex.ru, dementevan@mail.ru;
[2]*Saint-Petersburg State Agrarian University,* 2, Peterburgskoe sh., St. Petersburg—Pushkin, 196601 Russia, e-mail likhacheva.spb@gmail.com
ORCID:
Pozovnikova M.V. orcid.org/000-0002-8658-2026
Lihacheva T.E orcid.org/0000-0001-8456-5911
Kudinov A.A. orcid.org/0000-0002-7811-576X
Lejbova V.B. orcid.org/0000-0002-7017-9988
Dementeva N.V. orcid.org/0000-0003-0210-9344
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Abstract

The spread of lethal and semi-lethal mutations in cattle populations results in embryonic and postembryonic mortality of calves. The use of a limited number of sires creates the danger of wide spread of genetic abnormalities. Genetic markers identify carriers of a mutation in the absence of information about phenotypic manifestations of the disease. Cholesterol deficiency mutation (HCD, haplotype cholesterol deficiency), a recessive defect of Holstein cattle, is characterized by the death of calves in the first days or months of life. The extent of this genetic defect worldwide is currently very high, 6 to 17 %. In general, there is little information about the relationship of recessive mutations with dairy cattle productivity, and data on the effect of the HCD mutation, first described in 2015, on breeding traits are extremely limited. This paper is the first to report data on a genetic study of the APOB gene region on the BTA11 chromosome and milk production indices depending on the HCD status in a Russian dairy cow population. The obtained results indicate that in the studied population the HCD mutation does not reduce the pedigree value of animals in terms of milk production and milk quality (for fat and protein). The study was performed in a breeding farm of the Leningrad region in 2017. Random sample of Holstein black and white cattle include cows (n = 451) born in 2009-2015 and the calves (n = 7) with clinical signs of diarrhea and proven HCD carriers in pedigree (sires, sires of sires). Genotyping of animals was carried out by PCR using allele specific primers. The productivity of lactation 1 and 2 (milk, yield of milk fat and protein) was studied depending on the genotypes according to HCD. The ANOVA variance analysis and calculation of means were carried out with RStudio program on the basis of a single-effect model. Estimated breeding value of milk, fat and protein yields in kg was calculated using BLUP Animal Model. The concentration of triglycerides and cholesterol was determined with an automatic biochemical analyzer RX Daytona (Randox Laboratories, UK). According to the results of the study, 35 cows (7.76 %) of those tested are the HCD carriers. Among the calves, one calf was defined as a carrier and one heifer with homozygous HCD genotype for *APOB* gene had all symptoms of the disease. It is established that the HCD^+ cows are not inferior to their peers on milk productivity. The cows with the mutant allele of the *APOB* gene born in 2013 significantly exceeded healthy animals: during lactation 1 by 1219 kg ($p \le 0.01$) for milking, by 13.8 kg for milk fat yield, and by 19.9 kg for milk protein yield ($p \le 0.05$); during lactation 2 by 1392 kg ($p \le 0.001$) for milking, by 44 kg ($p \le 0.05$) for milk fat yield, and by 39.8 kg for milk protein yield ($p \le 0.01$). The average estimated breeding value (EBV) of HCD carriers is 6.8 % higher in milk yield, 8.1 % in fat and 4.8 % in protein compared to HCD^- animals. Monitoring of progeny of HCD carriers using Illumina Bovine IBDv3 (50k) did not reveal significant haploblocks in the *APOB* gene region, therefore, selection for increased milk productivity would not lead to a significant increase in the incidence of HCD carriers. Comparative analysis of biochemical indices in the first half

of the dry period did not reveal significant differences in the blood cholesterol (3.04±0.31 mmol/l and 3.33±0.12 mmol/l, respectively) and triglycerides (0.197±0.01 mmol/l, and 0.170±0.01 mmol/l) between groups of latent HCD carriers and cows free from this mutation. Our study has shown that the use of HCD carriers does not reduce productivity in the dairy herd. However, monitoring for this genetic defect is necessary, as incorrect selection of animals can lead to the birth of a sick and non-viable offspring, which in turn will cause economic losses in the farms

Keywords: cattle, genotyping, HCD, haplotype cholesterol deficiency, lethal recessive mutation, apolipoproteine B, gene *APOB*, milk yielding, triglycerides, cholesterol

Artificial insemination and use of limited number of sires creates a risk of lethal recessive mutations in cattle [1]. Not many abnormalities may be visually observed. DNA screening with the use of high density SNP chips identifies mutations without phenotypic disease manifestations [2]. Such method is used to determine fertility haplotypes found in cattle and becoming the mortality factor at different stages of animal growth. Screening of domestic Holstein and Black Pied Holstein cattle breeds had shown that frequency of known mutations CVM, BLAD, DUMPS, BY, HCD, HH1, HH3, HH4, and HH5 reaches 10 % in cows and 4 % in sires [3]. Monitoring of hazardous recessive mutations in the cattle herd is mandatory and allows for timely exclusion of mutation carriers from the breeding, significantly reducing the economic losses. Thus, inspection of Holstein sires and timely culling of BLAD and CVM carriers in the Leningrad region decreased the frequency of such mutations up to 1-2 % [4-6].

Haplotype cholesterol deficiency (HCD) is a new recessive genetic defect in Holstein cattle. Identification of such haplotype associated with loss of calves in early postnatal period due to occurrence of therapeutically incurable idiopathic diarrhea was reported by S. Kipp et al. in 2015 at Interbull conference in Orlando (USA). Homozygous animals have disturbance of lipid metabolism and hypocholesteremia. Low blood cholesterol level was detected in heterozygous calves lacking clinical signs. Search of genome associations by 44747SNP scanning (Illumina BovineSNP50 BeadChip, version 2; 54Kv2; llumina, Inc., USA) in the affected calves had resulted in identification of homozygous region of 1.01 Mb on BTA11 (positions from 77274120 bp to 78290130 bp), which indicated autosomal monogenic inheritance of such disorder by recessive or codominant type. Significant SNP is located in position 72248536 bp at distance of nearly 5 Mb from the defective haplotype [7]. Search for genome mutations by genome-wide association studies (GWAS) revealed 22 SNPs in total (from 64367438 to 83585365 bp), which had reached validity threshold near the defective haplotype. Genome studies based on 54K SNP Chip genotypes allowed identification of casual region on chromosome BTA11. Based on analysis of sick animal pedigrees, well-known Canadian Holstein sire Maughlin Storm was found to be the carrier of such disorder [8, 9]. Further studies had shown that this mutation results from an insertion of 1299 bp in exon 5 of *APOB* gene (apolipoprotein B) on BTA11 with a shift of the reading frame in codon region for amino acid residue 135 in APOB. This leads to 97 % truncation of the protein [10].

Other authors found an insertion of reduced endogenic retrovirus ERV2-1 in LTR (Long Terminal Repeats) on BTA11 in exon 5 of *APOB* gene, which resulted in stop-codon not far from the insertion. This preterm stop-codon in open *APOB* gene reading frame caused truncation of protein length by 140 amino acids. It was established that such preterm reduction results in inability to remove chylomicrons from the intestinal cells, which causes cholesterol malabsorption [11]. Apolipoprotein B (APOB) is necessary for synthesis of chylomicrons and lipoproteins of very low density in the intestines and liver. Apolipoproteins are protein components of lipoproteins, usually amphiphilic, which spe-

cifically bind lipids to form lipoprotein particle [12, 13].

High carriage percentage of HCD in Holstein herd was found in different countries: 5.07 % in China (sires, n =138) [14], 17.4 % in Germany (sires, n = 264) [10], 17 and 12 % in Canada for cows of 2012 and 2016 years of birth, respectively [15]. Based on pedigree analysis of 584 sires used for breeding in Russia, it was found that 10.3 % males (60 sires) were latent carriers of mutant allele of *APOB* gene [16]. Herewith, origin of sires varied (Canada, America, and Austria). Based on genotyping of 41 sires, whose fathers were HCD carriers, 17 animals (39 %) were latent carriers of the mutation. Timely screening of the populations for genetic defects in cattle and correct selection of animals could reduce economic losses. According to J.B. Cole et al. [17], economic losses from embryonic and post-embryonic deaths in USA due to lethal mutations comprised nearly 11 million US dollars annually.

In this paper, we had for the first time performed genetic assessment of the region of *APOB* gene and had compared diary output of cows in one of the Russian populations depending on the HCD status. Our findings show that HCD mutation does not reduce pedigree value of animals by milk yield and milk quality in terms of fat and protein.

Our purpose was to assess spread of HCD mutation in a population of Russian Black Pied Holstein cows as associated with milk yield and lipid metabolism indicators.

Techniques. Black Pied Holstein cows (*Bos taurus taurus*) of one of pedigree farming units in the Leningrad Region (2017, n = 451) were randomly sampled, animals were born in 2009-2015 years. Sampled calf (n = 7) had clinical diarrhea signs and HCD carriers in their pedigree (fathers, father's fathers).

DNA was extracted by phenol method [18] from blood taken from tail vein.

The primers used for PCR genotyping were 5′-GGTGACCATCCTCTCTCTGC-3′ (the universal forward primer), 5′-AGTGGAACCCAGCTCCATTA-3′ (the revers primer for amplification of 249 bp fragment) to identify wild allele, and 5′-CACCTTCCGCTATTCGAGAG-3′ (the forward primer for 436 bp fragment) to identify mutant allele of *APOB* gene (indel-polymorphism) [19]. PCR protocol was as follows: 1 min at 95 °C (initial denaturation); 30 s at 94 °C, 30 s at 60 °C, 30 s at 72 °C (35 cycles); 10 min at 72 °C (amplifier Thermal Cycler T1000, Bio-Rad Laboratories, Inc., USA). Reaction mixture contained 67 mM Tris-HCl (pH 8.6), 1.5 mM $MgCl_2$, 16.6 mM NaOH, 0.125 mM each of dATP, dGTP, dCTP, and dTTP, 0.5 μm of each primer, 50-100 μg of matrix DNA and 2.5 U of Taq DNA polymesare (Sibenzyme LLC, Russia). Resulting DNA fragments were separated by horizontal electrophoresis at 10 V/cm in 1× TBE buffer with ethidium bromide (0.1 μg/ml) in 2.0 % agarose gel (Agarosa LE 2, Helicon, Russia). Amplicon sizes were determined with molecular weight marker ThermoScientific Gene Ruler Ultra Low Range DNA Ladder (Fermentas, Lithuania). A video system Gel Imager 2 (Helicon, Russia) was used for gel documentation and data processing.

Linkage disequilibrium (LD) was analyzed in a sample of daughters of HCD sires form Leningrad Region. Genotyping (BovineSNP50 BeadChip v. 3, Illumina, Inc., USA) was conducted as per the manufacturer's protocol. Genotype studies were limited by region of *APOB* gene on BTA11 chromosome, including 33 SNPs of nearly 2000 kbp in length. LD (R^2) was calculated with PLINK 1.9 software [20].

Cholesterol and triglyceride blood levels were determined in HCD^+ animals (n = 3) and in conventionally healthy animals (HCD^-, n = 14) of lactation 2 to lactation 4 in the first half of interlactation period. Blood for biochemical studies was collected from the tail vein with vacuum system Vacuette (Greiner

Bio-One, Austria) 2 hours after feed supply (from 10.00am to 11.00am). In 30-40 min afterwards, vials with blood were centrifuged for 20 min at 3000 rpm. Concentration of triglycerides and cholesterol was measured (a biochemical analyzer RX Daytona, Randox Laboratories, Great Britain) with the use of Cormay reagents (Poland).

Records of dairy yield in cows born in 2012-2014 years were taken from the pedigree cards (form 2MOL). Yield during lactation 1 and lactation 2, milk fat yield, and milk protein yield were accounted for.

Correlation between the genotypes of cows and analyzed traits was identified by statistical significance of differences between the mean values. Found value t_d was compared to *t*-Student table [21]. Dispersion analysis ANOVA and calculation of the means was conducted with RStudio software [22] based on the following model with one fixed effect: $y_{ij} = \mu + HCD_j + e_{ij}$, where y_{ij} is estimated breeding value (EBV) in cow *i* by production traits, μ is mean, HCD_j is fixed haplotype effect, e_{ij} is unknown residual. Data was statistically processed with Microsoft Excel and AtteStat (http://www.studmed.ru/programma-attestat-1205_1778bebd8f9.html) software. Mean (*M*) and standard error of mean (±SEM) are presented.

Estimated breeding value (EBV) for milk yield, fat and protein production was calculated by BLUP Animal Model based on phenotypic data of 2016 [23]. Haplotype effect was shown as 0 (HCD^-) and 1 (carrier HCD^+).

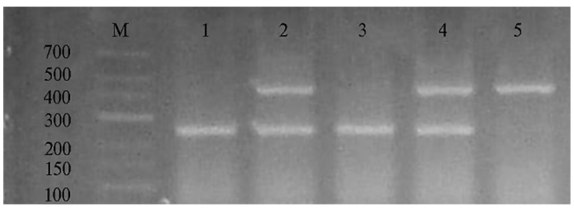

Fig. 1. Electrophoregram of PCR products for indel polymorphism in *APOB* gene in Black Pied Holstein cows: M — molecule weigh marker; 1, 3 — healthy animals (249 bp fragment), 2, 4 — heterozygous carriers (fragments of 249 bp and 436 bp), 5 — homozygous animal (436 bp fragment) (Leningrad Region, 2017).

Results. Figure 1 shows typical electrophoregram of PCR products for indel polymorphism in *APOB* gene.

Totally, we have tested 55.7 % of breeding herd suspected for mutant HCD allele of *APOB* gene. Based on study results, 35 cows (7.76 %) and one female calf were heterozygous carriers of HCD mutation.

Homozygous HCD genotype for *APOB* gene was found only in one heifer with sharp retardation in growth and development, debilitation, and incurable diarrhea (Fig. 2). The calf's weigh at birth was 39 kg, at 2-month age was 49 kg, and at 3-months age was 49 kg; average daily weight gain from the birth to 2 months of age was 166 g. Genotyping showed the mother cow to be HCD-positive, and the father also was the mutant allele carrier. As per parental pedigrees, Maughlin Storm 5457798 sire was among the mother's predecessors, whereas Breadale Goldwyn 10705608 sire was among the father's predecessors. These sires and their descendants are already used for many years in artificial insemination programs in Russia and are found to be latent HCD carriers.

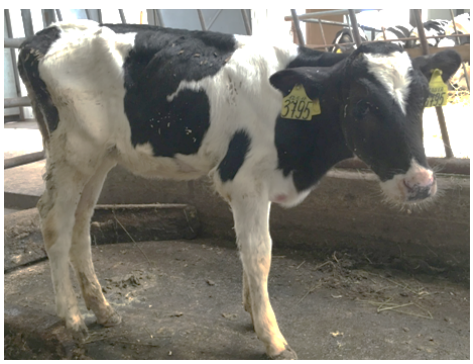

Fig. 2. Black Pied Holstein calf homozygous for mutant allele of *APOB* gene with typical clinical signs of cholesterol deficit syndrome (retardation in growth and development, debilitation, diarrhea) (Leningrad Region, 2017).

HCD^+ calves were as good as their peers and sometimes exceeded them by milk production values (Table 1). Thus, latent carriers of *APOB* gene mutant allele born in 2013 validly left be-

hind healthy animals. i.e. during the first lactation by 1219 kg milk yield ($p < 0.01$), by 13.8 kg milk fat yield, and by 19.9 kg milk protein yield ($p < 0.05$); during the second lactation by 1392 kg ($p < 0.001$), 44 kg ($p < 0.05$), and 39.8 kg ($p < 0.01$), respectively. No valid differences were found between the groups of calves born in 2012 and 2014.

1. Milk production in Black Pied Holstein cows according to genotypes for indel polymorphism in *APOB* gene (haplotype cholesterol deficit HCD) (*M*±SEM, Leningrad Region, 2017)

Year of birth	Status for HCD	Number of animals	Milk yield, kg	Fat, kg	Protein, kg
		Lactation 1			
2012	HCD^+	3	8750±440	342.6±28.6	281.7±20.4
	HCD^-	22	8894±256	333.7±10.1	280.8±7.8
2013	HCD^+	8	9471±261[a]	353.8±7.8	292.0±8.5[c]
	HCD^-	73	8252±142[b]	340.0±5.9	272.1±4.5[d]
2014	HCD^+	10	8780±378	346.5±10.0	268.9±9.1
	HCD^-	101	8646±126	347.0±5.4	271.0±4.1
		Lactation 2			
2012	HCD^+	3	10339±787	390.6±76.9	330.8±43.8
	HCD^-	22	9596±385	371.9±16.4	300.1±11.3
2013	HCD^+	6	10872±346[e]	428.7±20.1[g]	339.7±12.2[i]
	HCD^-	49	9480±233[f]	384.7±9.0[h]	299.9±6.9[j]

N o t e. Statistically significantly differences between HCD^+ and HCD^- are marked with letters: a, b at $p < 0.01$; c, d at $p < 0.05$; e, f at $p < 0.001$; g, h at $p < 0.05$; i, j at $p < 0.01$.

2. Dispersion analysis of breeding value estimates of Black Pied Holstein cows heterozygous for haplotype cholesterol deficit (HCD) (Leningrad Region, 2017)

Production trait	HCD effect	p-value
Milk	4.076	0.0442
Fat	6.617	0.0105
Protein	1.905	0.1680

Average EBV for milk, fat, and protein production for HCD carriers were 1100.8; 42.5 and 28.3 kg, respectively, with 1030.5; 39.3 and 27.1 kg for HCD^- animals. That is, EBV was higher in the first group for milk production by 6.8 %, for fat yield by 8.1 %, and for protein yield by 4.8 %. Dispersion analysis (Table 2) illustrates stable positive effect of HCD carriage on productive performance.

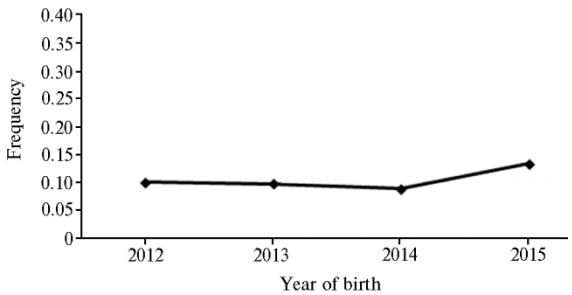

Fig. 3. Frequency of cholesterol deficit mutation (HCD) carriers among Black Pied Holstein calves of different years of birth (Leningrad Region, 2017).

Among the tested animals of different years of birth, frequency of HCD mutations was nearly 10 % (Fig. 3) with a trend towards growth in offspring born in 2015, which highlights the need to constantly control the herd in pedigree farming units to reduce the number of HCD carriers.

We did not find any valid differences between HCD^+ and HCD^- cows in the blood cholesterol level (3.04±0.31 and 3.33±0.12 μmol/l, respectively) and blood triglycerides (0.197±0.01 and 0.170±0.01 μmol/l). For both groups, these metabolites were within the reference concentrations, i.e. 1.5-4.5 μmol/l for cholesterol and 0.05-0.3 μmol/l for triglycerides [24]. Milk yield for 305 days after the last completed lactation did not vary between groups and was 10302±791 and 10191±453 kg, respectively.

For better assessment of the influence of selection for milk production on the spread of HCD carriers, we have analyzed the linkage disequilibrium (LD) between SNP in *APOB* gene regions at distance of nearly 1000 bp from the gene in both directions. Average distance between SNPs on chip in the studied

region was nearly 55 kbp. Estimates had shown absence of haploblocks in this region of genomes of the HCD^+ sire descendants in the Holstein herd population of Leningrad region. Mean LD value was low ($R^2 = 0.077 \pm 0.008$).

It should be noted that according to pedigree records, Holstein sires of different origin (Netherlands, USA, Canada, and Russia) were used for breeding at the studied farms. Some of them have a confirmed status of latent HCD carriers. Literature sources contain insufficient information about the link between the recessive mutations and productive performance of milk-type cattle. In fact, however, the sires carrying recessive mutations often serve as enhancers, and wide spreading of the genetic defects is promoted by the fact that such defects are usually linked with economic trait genes [25]. Thus, analysis of pedigree records of sons and nephews of Skokie Sensation Ned, the stirps of DUMPS disorder, had shown that heterozygotes have significantly higher genetic potential of milk yield [26]. S. Saleem et al. [27] have noted that qualitative values of Holstein sire sperm remain unchanged despite the HCD status. In previous studies we have found that HCD status does not render significant effect on several reproductive parameters in cows (e.g. the age of the first insemination, first calving, and number of inseminations until conception, duration of service-period and intercalving period) [28].

A number of research papers report [29, 30] that sires and calves, the latent HCD carriers, have lower blood concentration of triglycerides and cholesterol compared to animals lacking mutant allele of *APOB* gene. However, blood concentration of cholesterol (as well as triglyceride concentration) is influenced by such factors as nutrition, physiological state of animals, and diseases of different etiology. Therefore, HCD defect could be diagnosed only by molecular testing. In our research, we did not find valid deviations from the normal values in blood biochemical indicators of HCD carriers, which could be due both to small sample size, as well as to genetically determined compensatory mechanisms in several animals [11, 31].

Tracking of selection and genetic characteristics in breed and determination of the genealogical affiliation of sire are required to control genetic defects [32, 33].

It may be interesting to note that HCD carriers do not reduce productive capacity in cattle herd. Moreover, average estimated breeding value in the studied population for milk, fat, and protein production in HCD carriers was higher than in the intact animals. Dispersion analysis had revealed stable positive effect of HCD carriage on productive performance. Such predominance lacks clear explanation, but could be possibly due to location of *APOB* gene in the genome area responsible for high milk production. At the same time, analysis of the linkage disequilibrium between SNP in this region shows lack of haploblocks in the of Holstein population of the Leningrad region. Accordingly, selection for increased productive capacity does not result in significant growth in frequency of HCD mutation carriers.

Therefore, we did not reveal any differences in blood concentration of cholesterol and triglycerides during the first phase of interlactation period in the studied population of Black Pied Holstein cows depending on the status for recessive HCD (haplotype cholesterol deficit) mutation. Use of cows which are the HCD carriers does not reduce productive performance in the herd. Nevertheless, monitoring of the populations for HCD carriage is necessary since use of heterozygous sires may result in sick and non-viable offspring, and, consequently, may cause economic losses to farming units.

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