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Identification of monomorphic and polymorphic genes associated with recessive fertility defects in Holstein cows reared in Kazakhstan

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ABSTRACT

Haplotypes of candidate genes namely: apoptotic protease activating factor 1 (*APAF1 p.Q579X* or *HH1*), glycinamide ribonucleotide formyltransferase (*GART* or *HH4*), structural maintenance of chromosomes 2 (*SMC2* or *HH3*), and haplotype cholesterol deficiency (*HCD*) genes associated with recessive fertility defects (loss of fertility) were investigated in imported Canadian Holstein cows reared at "Medeu Commerce" LLP breeding farm in Kazakhstan. The genotypic profiling of the *APAF1/HH1*, *GART/HH4* fertility haplotype carriers was carried out by PCR-RFLP methods using BstC8I and Tru9I and MseI, while the genotypic profiling of the *SMC2/HH3*, and *HCD* fertility haplotype carriers was carried out using our own primer designed by internal primer marker methods. The study revealed that the PCR-RFLP diagnostic markers *APAF1/HH1* and *GART/HH4* for recessive fertility defects were monomorphic in the Canadian Holstein cows investigated. However, the diagnostic markers *SMC2/HH3* and *HCD* fertility haplotype carriers (our own design diagnostic markers) were polymorphic, with frequencies of 3% and 11%, respectively, in the investigated Canadian Holstein cows. The study concluded that genetic monitoring of recessive fertility defects enables the timely identification of carriers of harmful lethal mutations, and control of the fertility haplotype elimination process.

Key words: loss of fertility; mutations; SNP; genetic defects; haplotypes; PCR-RFLP; *APAF1*; *HH1*; *SMC2*; *HH3*; *GART*; *HH4*; *HCD*; *APOB*; Holstein cow

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Introduction

Decreased fertility in cows is a major problem in dairy farming and is a consequence of the inbreeding observed in intensively selected and specialized cattle breeds (GEREŠ et al., 2009; TROJAČANEC et al., 2012; FRITZ et al., 2013; SZENCI et al., 2018; FOLNOŽIĆ et al., 2019; ĐURIČIĆ et al., 2020; KOVACS et al., 2020; STOJANOV et al. 2020). Animal reproduction is a complex trait that depends on many factors, such as male and female fertility, animal health, herd management, the environment, and feeding. Consequently, heritability for reproductive functions in cows is usually extremely low (from 0.02 to 0.04), and only slightly higher in sire bulls (from 0.05 to 0.22) (BERRY et al., 2014). The genetic gain in Holstein cows of the American population (daughter pregnancy rate) had a negative digital value up to 2007 (from – 0.5 to 0), but since 2007 there has been a tendency of increasing genetic gain, and in 2015 this indicator was + 0.3 (GARCÍA-RUIZ et al., 2016). As a result of the high incidence of latent lethal mutations, dairy farms in the United States suffer great economic harm, with annual losses of $ 1,696,555 and $ 1,381,452 respectively, due to *HCD* and *HH3* fertility haplotypes in Holstein cows (COLE et al., 2016). Fourteen additional lethal haplotypes (*HH4-HH17*) were reported in HF, but in the meantime three have been resolved on the molecular level, *i.e. HH1 (APAF1), HH4 (GART)* and *HH5/HH6 (SLC35A3)* (FRITZ et al., 2013). Fertility haplotypes such as *HH0*, *HH1*, *HH2*, *HH3*, *HH4*, *HH5*, *HHC*, *HCD* are found in cattle of the American Holstein breed population, the prevalence of which is 2.76%, 1.92%, 1.66%, 2.95%, 0.37%, 2.22%, 1.37% and 2.50%, respectively (TAYLOR et al., 2018,). The ratio of carriers among bulls and cows of the Russian Holstein cattle population was, respectively, 2.89 and 4.13% for *FANCI*, 2.04 and 1.83% for *APAF1*, 1.14 and 2.98% for *SMC2*, 1.30 and 1.04% for *GART*, and 5.57 and 2.06% for *APOB* (ROMANENKOVA et al., 2017).

Genealogical analysis shows that the ancestor of the *HH1* genetic defect is a Pawnee Farm Arlinda Chief sire bull, who was born in 1962 in the United States, and has over 16,000 daughters. It was found that *APAF1* homozygous embryos die on the 16th day of pregnancy. This mutation has caused 525,000 spontaneous abortions worldwide over the past 35 years, with an economic loss of approximately US $ 420 million (ADAMS et al., 2016). The negative role of the mutant allele of the *APAF1* gene in the etiology of embryonic mortality was proven by studies (GHANEM et al., 2018), where 15 mummified Holstein fetuses were tested and 5 of them were homozygous carriers of the lethal *APAF1* mutation.

Very recently, a new effective method for detecting carriers of the HH1 haplotype was designed and proposed, using the following pair of primers: forward 5'-TTGGACGACAGCCATTTCCTA-3' reverse 5'-AATGCTTATTAAAAGTTCCAGCCC-3', which allows amplification of a 261 bp gene fragment (KAMIŃSKI, 2020; ADAMOV et al., 2020). The localization of a point mutation in the *SMC2* gene (*HH3* haplotype) was studied in detail by US scientists, who suggested that the cause of the *HH3* haplotype appearance in the Holstein breed is the identified point mutation in the F1135S position (HAYES et al., 2013). The variant strictly related to *HH3* is a non-synonymous SNP (T/C) within exon 24 of the Structural Maintenance of Chromosomes 2 (SMC2) on Chromosome 8 at position 95,410,507 (McCLURE et al., 2014).

The *HH4* fertility haplotype (Haplotype Holsteins 4, *GART* – glycinamide ribonucleotide formyltransferase) in the Holstein breed appeared as a result of a single nucleotide substitution at position (1277227) A → C, where this single nucleotide substitution led to the amino acid substitution Asn → Thr at position 290 and negatively affects the reproductive function, so pregnancy in cows is accompanied by early embryonic mortality (FRITZ et al., 2013). A method for detecting carriers of the *HH4* fertility haplotype in cattle involves the use of the *SspMI* restriction enzyme with a C/TAG recognition site to detect SNP polymorphism in the coding part of the *GART* gene, by introducing a single nucleotide substitution in the reverse primer (ROMANENKOVA et al., 2017).

The reason for the fertility haplotype, and cholesterol deficiency (*HCD, APOB* gene) in the Holstein breed is insertion in the V exon part

of the 1299 bp *APOB* gene (MENZI et al., 2016; SCHÜTZ et al., 2016). Identification of all bulls was performed by means of the test described, by using 3 primers: forward primer starting from the wild sequence 5'-GGTGACCATCCTCTCTCTGC-3' and two primers discriminating the wild sequence from the mutant. The second wild reverse primer 5'-AGTGGAACCCAGCTCCATTA-3' ensured amplification of 249 bp, but the mutant forward primer 5'-CACCTTCCGCTATTCGAGAG-3' starting from the inserted LTR element, produced larger amplicon of 436 bp (KAMIŃSKI and RUŚĆ, 2016). A total of 138 Holstein bulls and 90 cows were tested for *HCD* using polymerase chain reaction amplification, and then seven bulls (5.07%) and one cow (1.11%) were identified as CD carriers (LI et al., 2019). It was experimentally proven that in cows as the heterozygous carriers of the *HCD* haplotype, the level of cholesterol and lipoprotein was lower than in healthy homozygous individuals (GROSS et al., 2019). According to the results of genetic testing of 1817 cows and 331 sire bulls of the Holstein breed, the occurrence of heterozygous carriers in cows was 8.09%, and 23.26% in bulls. Therefore, to reduce the prevalence of carriers of the *HCD* haplotype, Russian scientists recommend monitoring the breeding stock using PCR-RFLP analysis methods (POZOVNIKOVA et al., 2020). The aim of the study was to develop methods for detecting *HH1*, *HH3*, *HH*4, and *HCD* fertility haplotypes, and monitoring Holstein cows of foreign selection at the "Medeu Commerce" LLP breeding farm, for carriers of haplotypes.

Materials and methods

The experiments were carried out on 164 Holstein cows in the Canadian selection at the "Medeu Commerce" LLP breeding farm in the Karasay district of Almaty region in 2018-2020. The blood samples for research were taken from the jugular or tail vein into a vacuum tube with EDTA. DNA isolation was performed using the phenolic method. The polymerase chain reaction was carried out on an "Eppendorf" thermocycler, Germany. The PCR conditions for genotyping cows at the loci of the *HH1*, *HH3*, *HH4*, *HCD* haplotypes were performed using the first step - DNA denaturation at 94 °C for 5 minutes, the second step - denaturation at 94 °C for 45 sec, the primer annealing temperature at 60 °C for 45 sec, 56 °C for 30 sec, 59 °C for 30 sec, 60 °C for 30 sec, respectively for each haplotype, and elongation at 72 °C for 45 sec. The final elongation synthesis at 72 °C took 2-5 min, and the number of PCR cycles was 35. The composition of the PCR reaction mixture was: a total volume of 25 μL including, 1×PCR buffer, 4.0 μL 0.2 mM dNTP, 1.0 μL of each primer, 1.5 mM $MgCl_2$, 0.2μL of DNA *Taq-polymerase* with an activity of 10 units/μL and 2 μL of DNA template. The amplification results were checked in 3% agarose gel. The horizontal electrophoresis was performed at an electric field of 150 volts for 30 minutes. The *pUC19/MspI* DNA marker of fragment sizes was added to one of the wells of the gel, in the amount of 5 μL. The signal was photographed in the Infinity VX2 3026, WL/LC/26M X-Press gel documentation system (Vilber Lourmat, United States).

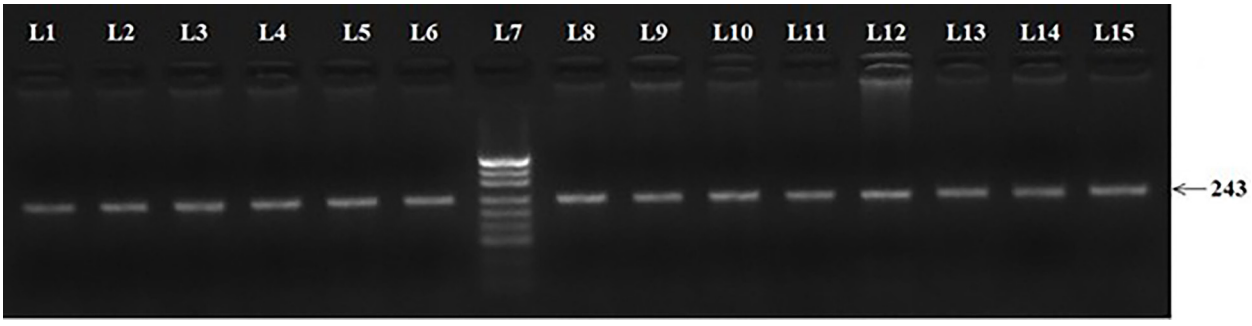

Fig. 1. Electropherogram of the amplified PCR product of the *APAF1/HH1* gene, 3% agarose, size 243 bp, Lines 1-6, 8-15 PCR product, Line 7 - *pUC19/MspI* DNA marker

Investigation of APAF1/HH1, GART/HH4 markers using the PCR-RFLP method. The PCR-RFLP genotypic profile of *APAF1/HH1* gene was performed using the *BstC8I* restriction enzyme. As a result of restriction enzyme (RE) incubation of the PCR products (243 bp) (Fig. 1), the following RE fragments of 176 bp, 12 bp, and 55 bp were obtained, of which the 176 bp and 55 bp fragments were well visualized on the electropherogram (Fig. 2).

Using the Primer 3,0 computer program, the primer sequences of *GART/HH4* haplotype were designed as: forward F 5'-TTTAATGAAGGTGTCCTCTATGC-3'andreverse R 5'- TTTCAAGGCTGAAAAATCCTAAG-3', to obtain an amplified PCR product of the *GART/HH4* gene with a size of 155 bp (Fig. 3).

The PCR-RFLP genotypic profile of the *APAF1/HH1* gene and *GART/HH4* gene was carried out

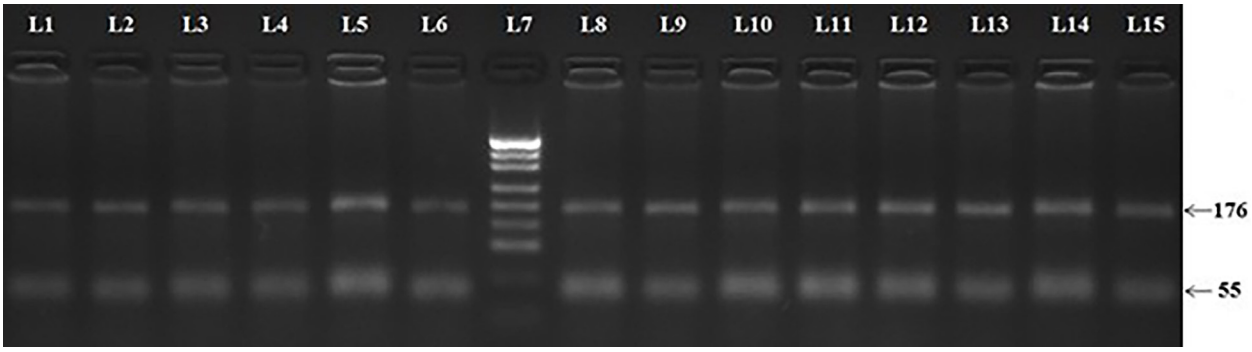

Fig. 2. Electropherogram of the amplified PCR product of the *APAF1/HH1* gene after incubation with restriction enzyme *BstC8I endonuclease*, in 3% agarose gel. Lines 1-6, 8-15 were DNA samples from healthy homozygous animals, showing 176 bp and 55 bp. Line 7 - *pUC19/MspI* DNA marker.

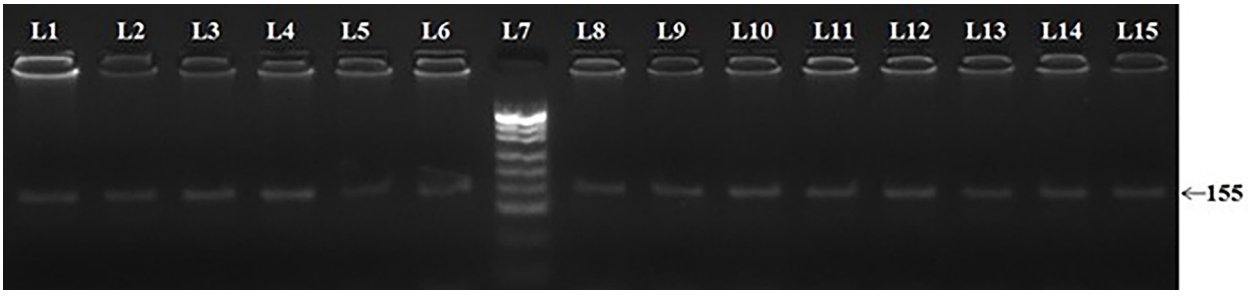

Fig. 3. Electropherogram of the amplified PCR product of the *GART/HH4* gene, 3% agarose, Lines 1,2,3,4,5,6,8,9,10,11,12,13,14,15 amplified PCR product size of 155 bp, Line 7- *pUC19/MspI* DNA marker

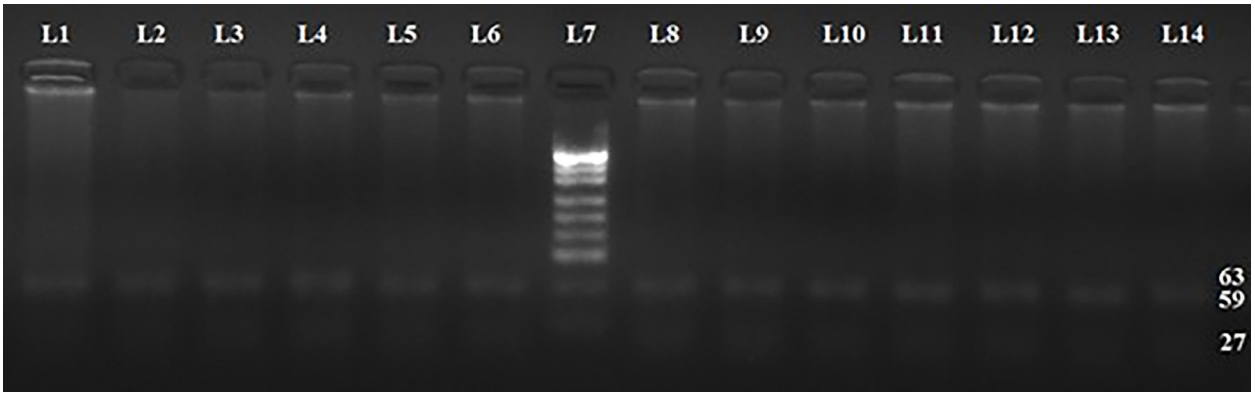

Fig. 4. Electropherogram of amplified PCR product of the *GART*/*HH4* gene after restriction with *Tru9I* endonuclease, in 3% agarose, showing fragments of 63 bp, 59 bp and 27 bp, Lines 1-6, 8-15 homozygous individuals, Line 7- *pUC19/MspI* DNA marker

using the *Tru9I* / *MseI* restriction enzymes with the T/TAA recognition site. After incubation of the amplified PCR product (155 bp) with this restriction enzyme, the following RE fragments of 63 bp, 59 bp, 27 bp and 6 bp, were obtained. Depending on the genotype of the animals; fragments of 63 bp, 59 bp and 27 bp are informative (Fig. 4).

Investigation of SMC2/HH3, and HCD markers using our own design PCR markers: The genotypic profile of the *SMC2/HH3* gene was performed using the following own design primers sequences, notably: F5'- TTAGTGGCTCTGTCATTAATCCTG -3' and R5'-ATACTGACCATTACTAAAGAATAG-3' (outer primers sequences), as well as the inner primer sequences: F5'-TGGACATATGCTACGTACTCATTCC-3' and R5'-TTGGTTCTTACCTGAGAATGTGTGA-3'. The genotypic profile of homozygous healthy animals were identified by the presence of 219 bp and 155 bp fragments, while for the heterozygous individuals they were identified by the presence of 219 bp, 155 bp. and 112 bp fragments (Fig. 5).

The genotypic profile of the *HCD/APOB* gene was performed using our own-design forward primers for wild-type alleles F-5'-GGTGACCATCCTCTCTCTGC-3' and reverse common primers R-5'-AGTGGAACCCAGCTCCATTA-3', to amplify 249 bp of PCR product. To identify heterozygous carriers, we used forward primers for the mutant allele type F-5'-CACCTTCCGCTATTCGAGAG-3' and reverse common primers R-5'-AGTGGAACCCAGCTCCATTA-3' to amplify 436 bp of the PCR product. On the electrophoretogram (Fig. 6), the presence of the 249 bp fragment represented homozygous healthy animals, and the presence of 436 bp and 249 bp fragments represented heterozygous carriers.

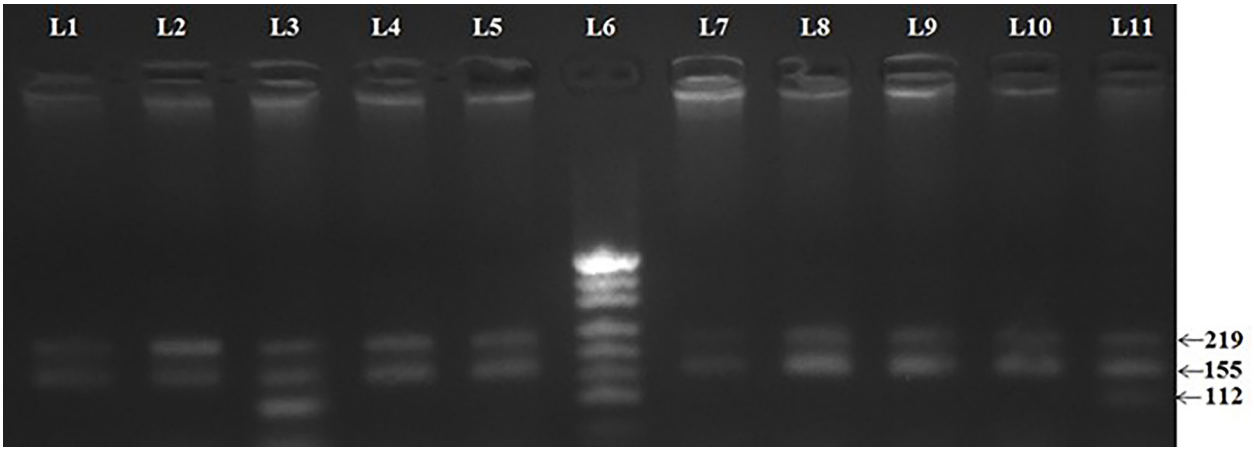

Fig. 5. Electropherogram of the amplified PCR product of the *SMC2/HH3* gene, 3% agarose, Lines 1, 2, 4, 5, 7, 8, 9, 10 for homozygous healthy animals, Lines 3, 11 for heterozygous carriers, Line 6 - *pUC19/MspI* DNA marker.

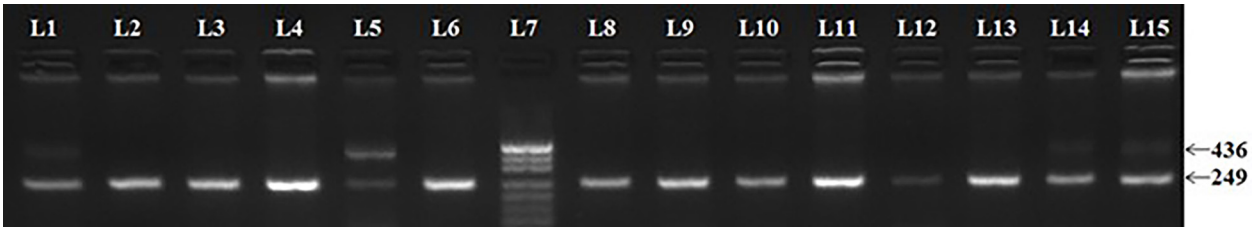

Fig. 6. Electropherogram of the amplified PCR product of the *HCD* gene, in 3% agarose, Lines 2, 3, 4, 6, 8, 9,10, 11, 12,13 for homozygous healthy animals, Lines 1, 5, 14, 15 for heterozygous carriers, Line 7 - *pUC19/MspI* DNA marker.

Results and discussion

Identification of monomorphic fertility haplotype markers. The PCR-RFLP genotypic profile of the *APAF1/HH1* gene in all the investigated (n = 164) Canadian Holstein cows revealed the presence of 176 bp and 55 bp fragments (normal homozygous genotype), therefore they were identified as the monomorphic marker for the recessive fertility defects in Canadian Holstein cows (Fig. 2). Similarly, the PCR-RFLP genotypic profile of the *GART/HH4* gene in all the investigated (n = 164) Canadian Holstein cows revealed the presence of 63 bp, 59 bp, and 27 bp fragments (normal homozygous genotype), therefore they were identified as the monomorphic marker for recessive fertility defects in the Canadian Holstein cows (Fig. 4).

Identification of polymorphic fertility haplotype markers. The genotypic profile of the *SMC2/HH3* gene in all the investigated (n = 164) Canadian Holstein cows revealed the presence of 219 bp and 155 bp fragments (normal homozygous genotype), and 219 bp, 155 bp and 112 bp fragments (normal heterozygous genotype), respectively. Detection of mutant and wild-type alleles was performed using inner and outer primers. For the wild type of allele, the amplification was performed with forward outer and reverse inner primers, to obtain the 155 bp fragment, and for the mutant allele type, amplification was performed with reverse outer and inner forward primers, to obtain a 112 bp fragment. In the investigated cow population, we identified five Holstein cows heterozygous for the *SMC2/HH3* gene (Table 1). Therefore, we considered *SMC2/HH3* gene as a polymorphic marker for recessive fertility defects in Canadian Holstein cows (Fig. 5). Similarly, the genotypic profile of the *HCD* gene in all the investigated (n = 164) Canadian Holstein cows revealed the presence of a 249 bp fragment (normal homozygous genotype), and 436 bp and 249 bp fragments (normal heterozygous genotype), respectively. In the investigated cow population, we identified nineteen Holstein cows heterozygous for the *HCD* gene (Table 1). Therefore, we considered the *HCD* gene as a polymorphic marker for the recessive fertility defects in Canadian Holstein cows (Fig. 6).

Analysis of Table 1 showed that there were no heterozygous carriers of the *APAF1/HH1,* and *GART/HH4* haplotypes in the studied population of imported Canadian Holstein cows, although according to the literature, the prevalence of the *APAF1/HH1* haplotypes carriers is 2.1% (ADAMS et al., 2016), and for *GART/HH4* 3.6% (FRITZ et al., 2013). Moreover, according to the results of other studies, the frequency of the mutant type of the alleles of the *APAF1/HH1* and *GART/HH4* genes in Holstein cattle was 1.92% and 0.37%, respectively (COLE et al., 2016).

In our study, we discovered that among the 164 animals tested, five Holstein cows turned out to be heterozygous carriers of the *SMC2/HH3* fertility haplotype, which accounted for 3.0% of the studied population. Heterozygous carriers of cholesterol deficiency were found in high frequency among cows on the "Medeu Commerce" LLP breeding farm. A total of 19 out of 164 cows were heterozygous carriers of *HCD*, which was 11% of the studied population. According to the results of genetic monitoring of Holstein cattle worldwide, the prevalence of heterozygous carriers of the *SMC2/HH3,* haplotype is 2.95% (COLE et al., 2016). Similar results were obtained in Holstein

Table 1. Prevalence of carriers of the *APAF1/HH1, SMC2/HH3, GART/HH4* and *HCD* fertility haplotypes in Canadian Holstein cows reared on the "Medeu Commerce" LLP farm.

Group of animals	Fertility haplotype name			
	APAF1/HH1	*GART/HH4*	*SMC2/HH3*	*HCD*
Homozygous healthy, cows (%)	164	164	159 (97%)	145 (89%)
Heterozygous carriers, cows (%)	0	0	5 (3%)	19 (11%)
Total cows	164	164	164	164

cows, where the occurrence of heterozygous carriers of the *SMC2/HH3* haplotype was 2.9% (DAETWYLER et al., 2014; McCLURE et al., 2014). However, information on the prevalence of the *HCD* haplotype was contradictory; for example, the prevalence of heterozygous carriers in Russian Holstein cattle ranged from 8.09% to 23.26% in cows and sire bulls, respectively (POZOVNIKOVA et al., 2020). The prevalence of cholesterol deficiency in the Chinese Holstein breed was 5.07% in sire bulls and 1.11% in cows (LI et al., 2019).

Conclusions

This population study revealed that the PCR-RFLP test of *APAF1/HH1 and GART/HH4* candidate genes for recessive fertility defects showed them to be monomorphic, therefore they should not be considered as suitable diagnostic markers for the investigated Canadian Holstein cows. However, polymorphic *SMC2/HH3* and *HCD* candidate genes for recessive fertility defects can be considered suitable diagnostic markers in the investigated Canadian Holstein cows. Finally, the study concluded that the large breeding population of Canadian Holstein cows in Kazakhstan should be investigated for genetic monitoring of recessive fertility defects using the *SMC2/HH3* and *HCD* diagnostic markers. This may make the timely identification of carriers of harmful lethal mutations possible, and control the fertility haplotype elimination process.

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SAŽETAK

U krava holštajnske pasmine, uvezenih iz Kanade i uzgojenih na poljoprivrednom gospodarstvu *Medeu Commerce* LLP u Kazahstanu, istraživani su haplotipovi slijedećih kandidatnih gena za koje se smatra da su povezani s poremećajima (gubitkom) plodnosti: faktor koji aktivira apoptotsku proteazu 1 (APAF1 p.Q579X ili HH1), glicinamid-ribonukleotidna formiltransferaza (GART ili HH4), strukturno održavanje kromosoma 2 (SMC2 ili HH3) i haplotip za manjak kolesterola (HCD). Genotipsko profiliranje nositelja haplotipova *APAF1/HH1, GART/HH4* provedeno je metodom PCR-RFLP uz upotrebu *BstC8I* i *Tru9I* te *MseI,* dok je genotipsko profiliranje nositelja haplotipova *SMC2/HH3,* i *HCD* provedeno vlastitim primerima, oblikovanim internim metodama. Rezultati su pokazali da su metodom PCR-RFLP dijagnostički markeri *APAF1/HH1* i *GART/HH4* za recesivne poremećaje plodnosti u istraživanih krava bili monomorfni. Istovremeno, dijagnostički markeri nositelja haplotipova *SMC2/HH3* i *HCD* (oblikovani prema vlastitim metodama) bili su polimorfni, s učestalošću od 3 % i 11 %. Zaključeno je da genetski nadzor recesivnih poremećaja plodnosti omogućuje pravodobno identificiranje nositelja štetnih letalnih mutacija i kontrolu eliminacije haplotipova koji štetno utječu na plodnost.

Ključne riječi: gubitak plodnosti; mutacije; SNP; genetski poremećaji; haplotipovi; PCR-RFLP; *APAF1*; *HH1*; *SMC2*; *HH3*; *GART*; *HH4*; *HCD*; *APOB*; krave holštajnske pasmine