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Molecular screening of factor XI deficiency in Holstein cattle reared in Bulgaria

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ABSTRACT

Factor XI deficiency is a hereditary disease with an autosomal recessive mode of inheritance. The disorder is characterized by extended bleeding time and predisposition to various hemorrhagic conditions in affected animals. Despite the economic importance of the Holstein Friesian breed in dairy cattle breeding, the prevalence of the F11 gene mutation is still neglected by many farmers. The project aims to determine the frequency of the mutation in exon 12 of the F11 gene in 114 Holstein Friesian cows reared in private farms in Bulgaria. The PCR analyses confirmed that 104 animals were homozygous normal (91.23%), 9 were identified as heterozygous carriers (7.89%) and 1 was characterized as homozygous affected (0.88%). The calculated allele frequency of the mutant allele is 0.0482. Clinical data confirmed the association between homozygosity and reduced health status. The results obtained are consistent with reports from other countries and emphasize the need for genetic screening and selection control to limit the spread of the disease in dairy cattle.

Keywords: blood coagulation, Factor XI, Holstein-Friesian dairy cattle

INTRODUCTION

The plasma thromboplastin precursor (PTA), also known as Factor XI (FXI), is a plasma serine protease involved in the early stages of the blood coagulation pathway (Čítek et al. 2008). It is synthesized in the liver as a zymogen, circulates as a disulfide-linked homodimer, and is activated by proteolytic cleavage. The active form FXIa is involved in the conversion of factor IX to active factor IXa, promoting thrombin generation and fibrin clot formation (Windsor and Agerholm, 2009). Inherited FXI deficiency is a rare disease that has been described in humans, dogs, and cattle (Gentry 1984; Ghanem et al. 2005). Affected animals may exhibit symptoms such as

prolonged bleeding after injections, hematuria, bloody milk, mastitis, metritis, etc. (Liptrap et al. 1995).

The molecular basis of the deficiency in cattle was identified by Marron et al. (2004), who found that the cause is an insertion of a 76 bp polyadenylate fragment in exon 12 of the F11 gene. The mutation forms a premature stop codon and results in a truncated protein that lacks the serine protease domain encoded by exons 13–15. Another 15 bp insertion in exon 9, has also been described. The inserted fragment results in the replacement of the amino acid Phenylalanine with 6 amino acid residues in the fourth "apple" domain of the protein (Kunieda et al. 2005).

The mutation in exon 12 is inherited as an autosomal recessive trait (Mukhopadhyaya et al. 2006). The FXI activity in heterozygotes can be reduced, while animals that are homozygous for the recessive allele demonstrate complete absence of FXI activity (Avanus and Altinel. 2016). Therefore, identification of carriers is crucial for effective disease control (Mondal et al. 2016). The mutation in exon 9 follows an autosomal dominant manner of inheritance and so far, has been exclusively detected among the Japanese black cattle breed (Watanabe et al. 2006).

Diagnostic methods for FXI deficiency include biochemical tests such as activated partial thromboplastin time (APTT), which are reliable in identifying affected animals but are not sensitive in identifying heterozygous carriers due to overlap in reference values (Patel et al. 2007). PCR-based methods have been developed to accurately identify mutations in exons 9 and 12, and are considered the gold standard in breeding programs.

The present study aims to investigate the frequency of the mutation in exon 12, responsible for factor XI deficiency, in Holstein cattle bred in Bulgaria.

MATERIALS AND METHODS

Experimental animals and DNA extraction

The project genotyped a total of 114 Holstein Frisian cows, raised in three private farms located in different geographical regions of Bulgaria. All animals aged between 3 and 6 years, were randomly selected, with a preliminary pedigree check, aiming to exclude related individuals. Cattle with clinical manifestations such as mastitis or metritis were included in the study with priority. Blood samples were obtained from the tail vein (*vena coccygea*) and collected in vacuum tubes with EDTA. Genomic DNA was extracted from whole blood via commercial kit (QIAamp DNA Blood Kit, Qiagen), following manufacturer's protocol. Samples were stored at -20°C until analysis.

Molecular analyses

Genotyping of the mutation in exon 12 of the F11 gene was done using the method described by Marron et al. (2004), based on polymerase chain reaction (PCR) and subsequent differentiation of the amplicons by agarose gel electrophoresis. PCR primers had the following sequences: Forward: 5'-CCCACTGGCTAGGAATCGTT-3' and Reverse: 5'-CAAGGCAATGTCATATCCAC-3' (GenBank: M13142 and AA571040). The 25 µl reaction mix contained: 1U Taq polymerase (Invitrogen Platinum II Taq Hot-Start), 2.5 µl 10× PCR buffer, 1.5 mM $MgCl_2$, 50–100 ng DNA, 100 µM of each dNTP and 10 pmol of each primer. PCR was done in T100 PCR thermal cycler (Bio-Rad) under the following temperature profile: initial denaturation at 95°C for 5 min, followed by 38 cycles of 95°C for 1 min, 54°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. PCR products were detected via electrophoresis in a 2.5% agarose gel in TBE buffer and visualized by ethidium bromide. Allele determination was based on the length of the resulting fragments (Figure 1): a single 244 bp band in homozygous wild-type individuals (normal), a single 320 bp band in homozygous carriers of the mutation (Factor XI deficient), and presence of both fragments (244 and 320 bp) in heterozygous animals.

Allele frequency calculations

The allele frequency of F11 gene in the analyzed population was calculated according to the Hardy-Weinberg principle:

$$p = \frac{2(\mathrm{AA}) + (\mathrm{Aa})}{2N}; \quad q = 1 - p$$

where "p" is the frequency of the normal allele, "q" is the frequency of the recessive (mutant) allele, N is the total number of tested animals, "AA" is the number of animals that are homozygous for the normal allele and "Aa" is the number of animals that are heterozygous carriers of the mutant allele for Factor XI deficiency.

RESULTS AND DISCUSSION

Results

The molecular analysis revealed the presence of the mutant allele of the F11 gene, associated with coagulation factor XI deficiency. A single animal (0.88%) was detected to be homozygous for the recessive mutant allele (ff), nine (7.89%) were heterozygous carriers (Ff), and the remaining 104 (91.23%) were homozygous for the normal (wild type) dominant allele (FF).

Based on this data, the calculated frequency of the normal allele (F) is approximately 0.9518 [(2 × number of FF + number of Ff) / (2 × total number of animals) = (2×104 + 9) / (2×114) = 217 / 228 ≈ 0.9518)], and of the mutant

allele (f) – 0.0482 [(2 × number of ff + number of Ff) / (2 × total number of animals) = (2×1 + 9) / (2×114) = 11 / 228 ≈ 0.0482]. The expected genotype frequencies according to the Hardy–Weinberg law (p2 +2pq + q2)=1 are respectively: 90.6% for FF [$(0.9518)^2$ ≈ 0.906], 9.18% for Ff [2 × 0.9518 × 0.0482 ≈ 0.0918] and 0.23% for ff [$(0.0482)^2$ ≈ 0.0023]. When multiplied by the total number of animals (N=114), these proportions correspond to approximately 103.4, 10.5 and 0.26 animals for each genotype.

To statistically assess if the observed genotype frequencies (104 FF, 9 Ff, 1 ff) deviate from the expected values, a chi-square (χ^2) test was executed (Table 1). The χ^2 was calculated as $\Sigma\ (O-E)^2/E$, where O represents the observed animal count and E is the expected count. This results into the following χ^2 values: FF: $(104 - 103.4)^2 / 103.4 = (0.6)^2 / 103.4 \approx 0.0035$; Ff: $(9 - 10.5)^2 / 10.5 = (-1.5)^2 / 10.5 \approx 0.2143$ and ff: $(1 - 0.26)^2 / 0.26 = (0.74)^2 / 0.26 \approx 2.105$. The total $\chi^2 = 2.32$. With one degree of freedom (df = number of genotypes − number of alleles = 3 − 2 = 1), the critical χ^2 value at the 0.05 significance level is 3.84. Since the calculated χ^2 (2.32) is below this threshold, the differences between observed and expected distributions were considered as not statistically significant. These results confirm that the population studied is in Hardy–Weinberg equilibrium with respect to the F11 locus. The insignificant deviation in the frequency of heterozygous individuals could be a result of the limited sample size or to selection practices in farms.

Table 1. Observed and expected genotype distributions for the F11 gene mutation in Holstein cattle reared in Bulgaria, and results of the chi-square (χ^2) test for Hardy–Weinberg equilibrium.

Genotype	Observed (O)	Expected (E)	$(O-E)^2/E$
FF	104	103.4	0.0035
Ff	9	10.5	0.2143
ff	1	0.26	2.105
Total χ^2			**2.32**

df = 1; χ^2 critical (0.05) = 3.84

The animal with genotype ff is three years old and, according to the farmer, has repeatedly developed clinical mastitis and periodically secretes bloody milk. These symptoms are typical of factor XI deficiency and are well described by other authors. Due to its young age, it is not possible to make a definitive assessment whether reproductive disorders, such as repeated miscarriages or reduced fertility, will also occur.

The calf born from this cow was also subjected to PCR analysis and was found to be a heterozygous carrier (Ff). In addition, 8 more cows in the sample showed heterozygous carrier status. Two of them originated from the same farm and six from the other farms. This data confirms that although in very low frequency the mutant allele is present in all tested farms. According to the

farmers, the productivity and health status of heterozygous carriers do not differ from homozygous dominant animals, which is consistent with the well-known principle of "hidden carrier". Hence heterozygous individuals do not show clinical symptoms of the disease.

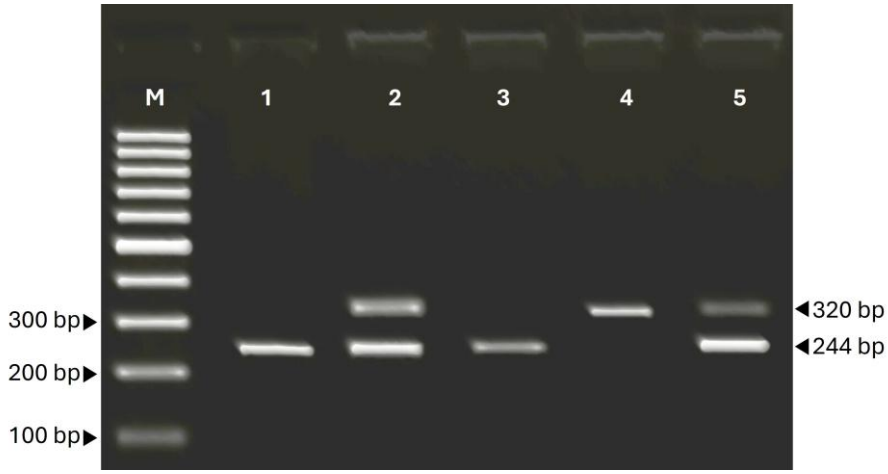

Figure 1. Gel electrophoresis of PCR genotyping of F11 gene in Holstein cows reared in Bulgaria. Lane M - DNA Ladder (Thermo Scientific™ GeneRuler™ 100 bp); Lanes 1 and 3 – homozygous dominant (FF - normal); Lanes 2 and 5 – heterozygous (Ff - carrier); Lane 4 – homozygous recessive (ff - deficient).

Discussion

This is the first study in Bulgaria focused on the prevalence of the mutation in exon 12 of the F11 gene, leading to coagulation factor XI deficiency in Holstein Friesian cattle. The identification of 9 heterozygous carriers (7.89%) and 1 homozygous affected animal (0.88%) out of 114 cows indicates an alarming presence of the mutant allele in the population. The calculated allele frequency (0.0482) is close to the results reported in Turkey – 0.054 (Meydan et al. 2009), Poland – 0.043 (Gurgul et al. 2009), and India – 0.033 (Kanishk et al. 2025) among the Holstein populations, which confirms the global prevalence of the mutation in this breed. Possible reasons for the variations between countries include local breeding practices, use of limited numbers of bulls, and lack of systematic genetic control of the spread of the mutation (Rahim et al. 2019).

Clinical manifestations in the homozygous animal, including recurrent mastitis and bloody milk discharge, are consistent with the symptoms described in literature. In Turkey, Hacıhasanoğlu-Çakaloz et al. (2016) reported bleeding after routine manipulations and difficulty in hemostasis in affected cows. Similarly, Akyüz et al. (2012) reported serious reproductive problems, including reduced fertility and repeated miscarriages. In Bulgaria,

this animal is still young (3 years), which limits the available data of possible reproductive disorders, but farm data already indicates reduced productivity and health status.

Heterozygous carriers (Ff), on the other hand, did not show any clinical signs and did not differ significantly in productivity and health status from the homozygous dominant animals (genotype FF). This explains why carriers are usually used for reproduction which allows them to transmit the mutation to the next generation (Meydan et al. 2009; Kumar et al. 2011). A similar case was described by Gurgul et al. (2009), where three generations (mother, daughter and granddaughter) were identified as carriers of the mutation. This family cluster confirms that the mutation can be maintained in the population through heterozygous transmission, without obvious clinical manifestation. The presence of heterozygous carriers in different farms in our study highlights the need to control the spread of the mutant allele through targeted genetic selection.

The insignificant differences between observed and expected Hardy–Weinberg frequencies suggests that there is no strong selection pressure at the locus in question. In support of this assumption, the performed chi-square (χ^2) test (Table 1) did not reveal statistically significant differences between observed and expected frequencies for all genotypes ($\chi^2 = 2.32$, df = 1, $p > 0.05$). This result confirms that the studied population is in Hardy–Weinberg equilibrium with respect to the F11 locus. This indicates that the observed distribution is consistent with random mating and is not influenced by strong selection pressure. However, intensive artificial insemination practices, widely used in dairy cattle breeding, pose a risk of increasing homozygosity and the prevalence of recessive diseases (Kaya et al. 2021). Therefore, the introduction of marker assisted selection targeting the FXI deficiency in routine breeding programs is a key element in the strategy for limiting this genetic disease in cattle (Gurgul et al. 2009; Marron et al. 2004). An additional advantage of applying PCR-based methods is their high sensitivity and specificity in distinguishing between normal (FF), heterozygous (Ff) and homozygous for the mutation individuals (ff). This is especially important since biochemical tests such as APTT have limited ability to identify carriers (Zhang et al. 2012). From an epidemiological point of view, the results confirm the importance of genetic screening in dairy cattle breeding, particularly considering the fact that heterozygous carriers do not show clinical symptoms (Korkmaz Agaoğlu et al. 2015).

CONCLUSION

The present study, as the first of its kind in Bulgaria, provides valuable information on the frequency of the mutation in the F11 gene and emphasizes

the need for dedicated breeding programs, aiming to eradicate this genetic disease from the population.

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