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Short communication

PCR-RFLP method applied for identification of Warmblood Fragile Foal Syndrome carriers in Polish warmblood horses

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Abstract

The method for identifying the causative mutation for Warmblood Fragile Foal Syndrome (WFFS) involved PCR amplification of a 259-base pair fragment of the PLOD1 gene and its digestion with the restriction enzyme Aci I was developed, allowing for the clear detection of WFFS carriers. Eight WFFS carriers were detected among 308 warmblood horses kept in different farms across Poland, giving an overall frequency of 2.59%, which indicates a rather low frequency of the causative mutation for WFFS in Poland. Further research should be conducted on a larger number of horses, particularly those breeds in which WFFS carriers have already been identified or where there is a risk of carrier status based on the horse's pedigree.

Keywords: horse, genetic defect, fragile foal, carrier, PCR test

Introduction

Warmblood Fragile Foal Syndrome (WFFS) was studied as a subpart of the skin diseases described in humans as Ehlers-Danlos syndrome (EDS), linked with genetic defects affecting collagen or other extracellular matrix proteins (Lindgreen et al. 2020). According to Montboux et al. (2015), EDS-like diseases have been described in the cat, dog, rabbit, mink, cattle and sheep with variable heredity in the different species. WFFS is a monozygotic defect caused by a recessive lethal shift mutation (missense) in the lysine 2-oxoglutarate 1-oxoglutarate 5-dioxygenase (PLOD1, c.2032G>A) gene (Monthoux et al. 2015, Rowe et al. 2021). This nucleotide substitution triggers a swap of a single amino acid in the PLOD1 protein - at position 678, the glycine found in healthy individuals is replaced by arginine (p.Gly678Arg). A deficiency of PLOD1 enzyme results in a reduction in the number of bonds in the collagen molecule, differences in the arrangement of collagen

fibers weakening its structure and, as a result, a reduction in the strength of skin and tendons (Metzger et al. 2021, Ablondi et al. 2022). The predominant clinical sign in recessive homozygotes is death in the later stages of pregnancy or very poor condition of newborn foals requiring euthanasia (Aurich et al. 2019, Kehlbeck et al. 2024). Live-born specimens are characterized by excessively stretchy and thin skin that is easily torn and injured (Ablondi et al. 2022). The purpose of this study was to identify carriers of WFFS using a PCR-RFLP method and to assess its prevalence in Polish warmblood horses.

Materials and Methods

Hair follicles and blood samples taken from 308 warmblood horses were collected for routine veterinary control in the training centers, and according, to Polish Law, special permission was not required for this every day veterinary practice. Isolation of genomic DNA was carried out using a Sherlock AX universal kit (A&A Biotechnology) or a NucleoSpin Tissue Mini kit (Macherey-Nagel). Mutation c.2032G>A within the PLOD1 gene was identified using a Polymerase Chain Reaction (PCR) followed by digestion of a specific restriction enzyme. A pair of primers (forward: 5' GTGGCTCAGATGGGAGAATG 3' and reverse: 5' ATTAG GGATCGACGAAGGAGA 3') were designed using Primer3 software (Untergasser et al. 2012) and used to amplify a 259 bp fragment of the PLOD1 gene. The PCR thermal profile consisted of 35 cycles of 95°C for 25 s, 60°C for 25 s and 74°C for 30 s and a final extension at 74°C for 10 min. PCR was performed in a reaction mix containing 70-80 ng of genomic DNA, 0.4 µL (25 pM) of each primer, 2.0 µL PCR Buffer (10x, Biotools, B&M Labs), 1.2 µL dNTPs mix (2.0 mM each), 1 unit of Eurx Taq DNA Polymerase and deionized water added to reach a volume of 25 µL. Eight µL of specific PCR products were digested with 0.6 u of AciI restriction enzyme (recognition site: CCGC or GGCG depending on DNA strand reads) during 45 min incubation at 37 °C (Thermo Scientific).

Restriction fragments were electrophoresed in standard 2.5 % agarose gel stained with ethidium bromide (1 mg/ml). Amplicons obtained from DNA of WFFS GG and AG horses were cut out from the agarose gel, purified using a Gel-Out kit (A&A Biotechnology, Gdańsk, Poland) and sequenced using an Applied Biosystems sequencer (Genomed Ltd, Poland). The forward and reverse strands were analyzed using BioEdit v. 7.2.0 software.

Results and Discussion

An example of genotyping for PLOD1 missense mutation A>G is shown in Fig 1. Within the entire amplicon (259 bp), in the case of guanine in the polymorphic site (G allele), the Aci I enzyme was able to recognize this site and another two non-polymorphic sites and cut the 259 bp amplicon into four smaller fragments: 121, 107, 21 and 10 bp. In the case of adenine (A allele), the polymorphic site was not recognized, giving three restriction fragments (228, 21 and 10 bp). When the individual was heterozygous (AG), five fragments occurred: 228, 121, 107, 21 and 10 bp. In other words, the Aci I enzyme always cut the amplicon twice (in a non-polymorphic site) when the animal was affected (AA genotype) or three times when the animal was non-affected (GG genotype). The amplicon from the WFFS carrier (AG genotype) was cut twice by Aci I (from one parent) and three times (from another parent), showing a total of five restriction fragments. Among the analyzed horses, eight WFFS carriers (2.59%) were identified. The reproducibility of WFFS genotyping by our PCR-RFLP method was 94%. Inefficient PCR amplification was the main reason to repeat genotyping, especially when genomic DNA was isolated from hair. Optimizing DNA content in the PCR mix increased the success rate of the method to 100%. Compared to sequencing, real-time PCR (Flanagan et al. 2021) and other PCR-RFLP techniques (Ayad et al. 2022), the current method is equally reliable, fast and cheap. It has an advantage over a similar method described by Ayad et al. (2022) because the three restriction sites for the Aci I enzyme introduce internal control of the digestion process (proof that the restriction enzyme is active); it is also fast since the total genotyping time takes only 6-7 hours, and it is inexpensive since the reagents used are low in price and easily available. Another advantage of the PCR-RFLP method is that genotyping can be set for one or many individuals without significant additional cost/per sample. These features make the method described available to an average diagnostic laboratory with a PCR thermocycler either for screening a population of horses or a single horse which is suspected to be a potential WFFS carrier. The results obtained in the current study confirm the occurrence of WFFS carriers in warmblood horse farms in Poland. The obtained frequency of alleles and number of carries in the current study are lower than the data cited for warmblood horses – 9.5-15% (Wobbe et al. 2022) and higher than data for closely connected thoroughbred horses (Bellone et al. 2020, Grillos et al. 2021). In the work of Reiter et al. (2020), several breeds kept in Poland were analyzed. In that study, the highest frequency of carriers was found in the Silesian breed

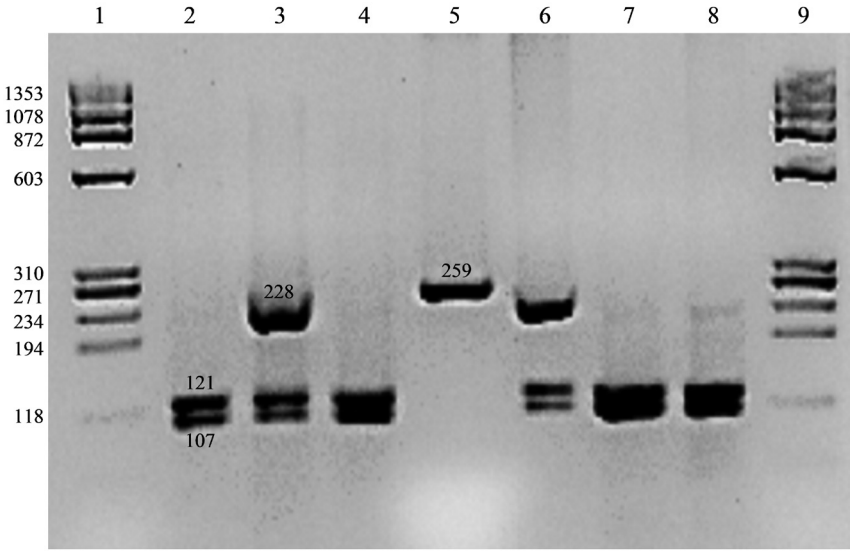

Fig. 1. Example of electrophoresis of 259 bp amplicons of PLOD1 gene containing a polymorphic site A>G causing Warmblood Fragile Foal Syndrome (WFFS) detected by the use of the Aci I restriction enzyme.
Lines 2, 4, 7 and 8 – horses WFFS free - with GG genotype (121 and 107 bp); lines 3 and 6 – WFFS carriers - with genotype AG (228, 121 and 107 bp); line 5 – amplicon 259 uncut by restriction enzyme Aci I; Lines 1 and 9 – DNA size marker PhiX174/Hae III. Fragments 21 and 10 bp diffused from the gel and are invisible.

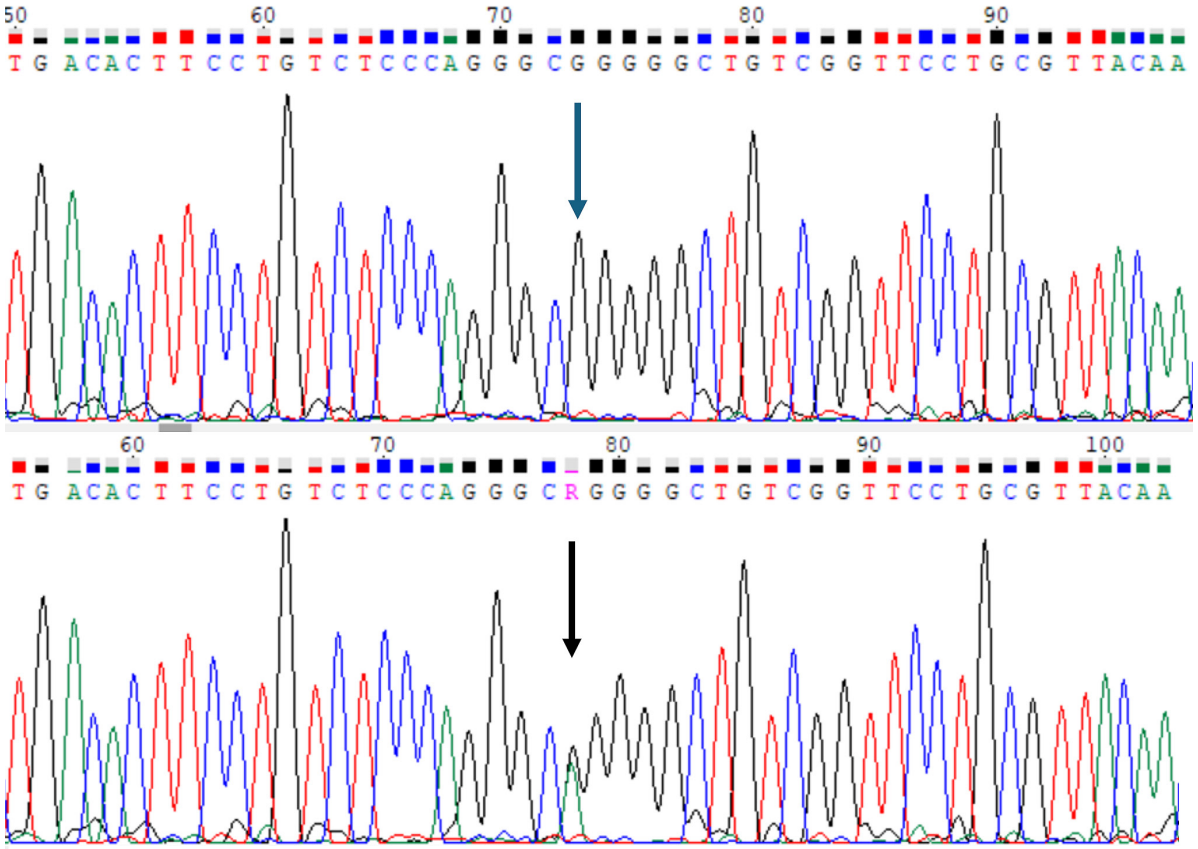

Fig. 2. Fragment of a 259 bp amplicon sequence of the PLOD1 gene obtained from horses genotyped by the PCR-RFLP method. The polymorphic site is indicated by an arrow. Horse free of WFFS has GG genotype (upper) and horse being WFFS carrier (down) has AG genotype. Only forward strands are shown. R indicates heterozygote (G or A).

(12 carriers out of 96 horses examined), as well as in the warmblood horses (3 carriers out of 157 horses examined). Our test can be used by veterinarians to verify their diagnosis of WFFS cases and consequently can be very helpful in breeding strategies, avoiding mating between WFFS carriers.

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