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Validation of high-resolution melting analysis as a diagnostic tool for endothelin receptor B mutation in American Paint horses and allele frequency estimation

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ABSTRACT

Overo lethal white foal syndrome (OLWFS) is a genetic disorder caused by a dinucleotide mutation in the endothelin receptor type B (*EDNRB*) gene leading to the death of affected foals shortly after birth. The use of rapid and reliable genetic testing is imperative for the early diagnosis of the mutation avoiding, therefore, either additional suffering or the production of affected animals. In the present study, we developed and validated a high-resolution melting (HRM) genotyping assay to detect the OLWFS causative mutation, and we also determined the frequency of heterozygotes among American Paint horses in Brazil. The HRM genotyping assay resulted in a high sensitivity, specificity, and positive and negative predictive values. The overall estimated frequency of heterozygotes was 21.6%; however, this frequency increased to 89.5% when considering only overo horses. The HRM assay optimized here was a reliable and suitable method for the detection of the dinucleotide mutation observed in the *EDNRB* gene resulting in a fast, accurate, and precise diagnostic tool. The causative gene mutation of OLWFS is present in heterozygosity in the American Paint Horse population in Brazil and is highly frequent among overo horses.

Among the many coat color patterns in the American Paint Horse (APH) breed, the overo and tobiano are two generally accepted types of white spotting patterns. Horses with the overo pattern usually exhibit a white-spotting pattern that does not cross the back, with extensive white spots on the head and some dark color on feet and legs, whereas the white-spotting pattern in tobiano horses crosses the back and covers the feet, varying portions of the legs, and a small portion of the head [1]. Mutations associated with white-spotted patterns in horses have been identified in *EDNRB, KIT, MITF, PAX3* and *TRPM1* genes [2–10]. The frame overo spotting pattern occurs in heterozygous horses due to a missense mutation in the first exon of the endothelin receptor type B (*EDNRB*) gene [5–7,11]. Pleiotropic effects such as a white or nearly complete white coat and ileocolonic aganglionosis have been reported as the result of the homozygous mutation [5–7,12]. The condition known as overo lethal white foal syndrome (OLWFS) is a genetic disorder inherited in an incomplete dominant, homozygous lethal fashion. Affected foals exhibit severe colic episodes due to functional intestinal obstruction shortly after birth and invariably die from condition [13,14]. Therefore, the rapid diagnosis is imperative to avoid any additional suffering of the animals. More importantly, foreknowledge of the progenitors' genotypes and mating selection are more rational approaches to prevent the production of affected animals. In any case, genetic testing is a useful and auxiliary tool for the early diagnosis of the disease and guidance in the mating selection.

Previous studies used allele-specific PCR approaches either to determine the causative DNA mutation of OLWFS [5–7,15] or to estimate the frequency of heterozygotes [16,17]. Although genotyping PCR-based methods are simple and reliable [18], the technical challenges to obtain optimal PCR primers may cause low PCR efficiency [19]. Also, the post-PCR handling to resolve the genotypes increases the time to

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obtain the result and may provide a source of post-PCR contamination. High resolution melting (HRM) of DNA for genotyping purposes relies on the analysis of the melting temperature to differentiate among genotypes; therefore, a robust and specific PCR technique is critical for the analysis [20]. The HRM analysis also is a closed-tube method, which eliminates processing between amplification and analysis and prevents the risk of cross-contamination [20]. In addition, HRM assay is a simple, rapid, and low-cost analytical technology providing reliable, accurate, and precise genotyping results [20–23].

The objective of the present study was two-fold, first to develop and optimize an HRM genotyping assay to detect the dinucleotide mutation in the *EDNRB* gene; and, secondly, to determine the frequency of heterozygotes for the causative mutation among American Paint horses in Brazil.

This study was approved by the São Paulo State University Institutional Animal Care and Use Committee (protocol n.30/2010). To estimate the frequency of heterozygotes among APHs in Brazil, the sample size was calculated using an open source software (OpenEpi version 3.03, available in www.OpenEpi.com) considering the Brazilian population size of 17,174 APHs [24], anticipated frequency of heterozygotes of 50%, absolute precision of 10%, 95% confidence interval, and random sampling. Based on this calculation a minimum of 96 horses was necessary to estimate the frequency of heterozygotes in this population. Blood was collected from 99 horses, and they were classified phenotypically regarding their coat color pattern as previously described [16]. Thirteen extra blood samples that had been collected throughout the years were used as controls (i.e., three samples from affected foals [homozygous dominant], five samples from frame overo horses [obligate heterozygotes], and five samples from solid-colored horses [homozygous recessive]) for the optimization of the real-time PCR and HRM assays. Genomic DNA was isolated using a commercially available kit (Illustra Blood GenomicPrep Mini Spin Kit, GE Healthcare Life Sciences, PA, USA). The relative purity and quality of the isolated DNA were determined by spectrometry (Nanodrop™, 2000 Spectrophotometer, Thermo Scientific™, MA, USA). All purified DNA samples were immediately stored at −80 °C until processing.

The genotypes of the control samples were confirmed before the optimization of the real-time PCR and HRM assays. Briefly, PCR was performed in duplicate to amplify a 175 base pair (bp) amplicon of the equine *EDNRB* gene (Gene ID: 100033875) containing the dinucleotide mutation. The PCR reaction contained 10 μL of a commercial master mix (GoTaq® Green Master Mix, Promega, WI, USA), 0.3 μM of each forward (5′-GAACCATCGAGATCAAGGAGAC-3′) and reverse (5′-TGCA GCAGAGTCTCCCAGAGC-3′) primers [5], 2 μL of template DNA, and nuclease-free water (q.s.p 20 μL). The amplification was performed with an initial denaturation step at 95 °C for 2 min. The samples were then subjected to 35 cycles of the following conditions: 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 7 min. The amplified PCR products were purified and sequenced for genotype confirmation using PCR primers and Sanger sequencing.

Four primer sets were designed (Primer Express® Software 3.0, Applied Biosystems®, CA, USA) to amplify fragments smaller than 150 bp of the equine *EDNRB* gene (Gene ID: 100033875) containing the dinucleotide polymorphism (Table 1) [5]. Assay development and optimization were carried out using the control samples and HPLC-purified primers under several concentrations (0.1 μM–0.5 μM). The adequate primer set amplified a specific fragment with no primer dimer generation and standard deviation (SD) of the raw Ct values ≤ 0.25 in the real-time PCR allowing accurate genotype call by the HRM software. The selected primer set amplified an 86 bp amplicon (Table 1). The DNA concentration of all unknown and control samples was normalized to 20 ng/μL. All reactions were carried out in triplicate in a total of 20 μL each, containing 0.2 μM of each forward and reverse primer, 2 μL of template DNA, 10 μL of master mix (MeltDoctor™ HRM Master Mix, Applied Biosystems®, CA, USA) and nuclease-free water q.s.p. In addition, a no template control and at least one control sample of each genotype were included in triplicate on each plate. PCR conditions were: initial denaturation at 95 °C for 10 min and 40 cycles at 95 °C for 15 s and 60 °C for 60 s, followed by a high resolution melting curve from 65 °C to 95 °C with 0.1 °C increase at each step. The amplification of specific alleles was confirmed by melting curve analysis.

High resolution melting analysis was performed with an HRM software (HRM Software 2.0.1, Applied Biosystems®, CA, USA). Active melt region was set between 0% fluorescence intensity at post-melting region and 100% fluorescence intensity at the pre-melting region. The pre-melting and post-melting regions were set between 75.25 °C and 75.75 °C and 82.0 °C and 82.5 °C, respectively. The HRM software algorithm used those parameters to place all unknown samples into one of the three possible DNA variants. All samples were also sequenced to confirm the HRM assay results using the conventional PCR protocol described above. Validation of the HRM technique was carried out by analysis of the sensitivity, specificity, repeatability, and reproducibility as previously described [21]. Sensitivity and specificity were calculated by comparing the HRM against sequencing results. Repeatability was analyzed by placing ten replicates of each control genotype within the same run. Reproducibility was analyzed as described for repeatability on five different days.

All primer sets designed for real-time PCR-HRM assay amplified specific fragments with no primer dimer generation and allowed for accurate genotype call in the HRM analysis. Although all primer sets amplified products with SD of Ct values ≤ 0.5, only one of them achieved the cutoff value established.

Validation of the HRM-based genotyping assay and estimation of the frequency of heterozygotes were performed in 97 out of 99 sampled horses. Those two excluded samples, which were obtained from homozygous recessive horses (confirmed by sequencing), were not submitted to HRM analysis because they obtained higher SD values than the minimum established in our laboratory for real-time PCR-HRM assay. Other real-time PCR approaches, such as gene expression assays or probe-based genotyping assays, are usually less restrictive regarding the SD of technical replicates accepting values up to 0.5 or even higher in some cases. This criterion may be more rigorous than usually practiced for HRM-genotyping assays, but it ensures that HRM analysis is always performed in high-quality real-time PCR data.

Despite the good integrity and apparent purity of the excluded samples (data not shown), the real-time PCR assay used in the present study for HRM analysis resulted in an analytical failure rate of 2% (2/99). Previous studies have demonstrated analytical failure rate of the real-time PCR-HRM analysis between 1.6% and 5.4% [21,25]. In one of

Table 1
Primer sets designed for the development and optimization of the real-time PCR-HRM assay.

Primer set	Forward (5' – 3') sequence	Reverse (5' – 3') sequence	Product size (bp)
1[a]	CAGTAGTGTCCTGCCTAGTGTTCGT	CCGCATGCACTTGTTCTTGT	86
2	CAGTAGTGTCCTGCCTAGTGTTCGT	CAGGCTGGCGATCAAGATATTAG	116
3	GAAAGAACCATCGAGATCAAGGA	CCGCATGCACTTGTTCTTGT	129
4	CCCGTGCGAAAGAACCAT	CGTTCCGCATGCACTTGTT	140

[a] Selected primer set utilized in the genotyping assay.