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Evaluation of variants in the *ENTPD1* and *ENTPD2* genes in athletic horses with exercise-induced pulmonary haemorrhage

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Abstract

Background Exercise-induced pulmonary haemorrhage (EIPH) in athletic horses is characterized by the presence of blood from the lungs in the tracheobronchial tree after intense exercise. Despite the high prevalence of EIPH in horses, the primary aetiology remains unknown. Variants in the genes encoding CD39 and CD39L1 (*ENTPD1* and *ENTPD2*, respectively) were previously reported as potential genetic causes involved in EIPH pathogenesis. However, the role of these variants in haemostatic functions is unknown.

Results To investigate the association between EIPH and missense variants in the *ENTPD1* (rs1152296272, rs68621348, and rs68621347) and *ENTPD2* genes (rs782872967), 76 Thoroughbred horses diagnosed with EIPH and 56 without clinical signs of EIPH (control group) by trachea-bronchial endoscopy were genotyped. The rs1152296272 and rs68621347 variants were linked, which explained why the same results were found in all horses. Approximately 96% and 95% of the EIPH and control horses, respectively, carried at least one nonreference allele for these variants. In contrast, 100% of the control horses and 96% of the EIPH horses were homozygous for the reference allele for the rs68621348 variant. In the EIPH group, 1.5% of the horses were homozygotes and 24% were heterozygous for the nonreference allele of the rs782872967 variant. In the control group, the nonreference allele of this variant was observed only in heterozygotes (16%). There were no significant differences between groups for any of the variants.

Conclusions The variants previously described in the genes encoding the CD39 and CD39L1 enzymes were highly present in the studied population. However, no association was found between the occurrence of EIPH and the presence of these variants in Thoroughbred horses in this study.

Keywords Athletic horse, Epistaxis, Genetic disease, Haemorrhage, Haemostasis

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Background

Exercise-induced pulmonary haemorrhage (EIPH) is the presence of blood in the tracheobronchial tree and/or red blood cells in the bronchoalveolar lavage fluid after intense exercise and has affected athletic horses for centuries [1]. Studies suggest several hypotheses for EIPH aetiology, such as increased pressure in the pulmonary capillaries, hypoxia, exercise-induced blood hyperviscosity, continuous mechanical trauma, and genetics [2, 3]. EIPH is frequently described in racehorses, especially Thoroughbreds [4], and raises concerns worldwide due to the financial implications resulting from decreased performance, the suspension of affected animals, the loss of training days and medication costs, which can represent significant economic losses for the horse industry [5].

Despite advances in recognizing the conditions predisposing horses to this disease, its progression and impacts on performance, the definitive aetiology of EIPH remains unknown [4]. Theories about the possible causes of EIPH often diverge and lead to important discussions in the literature. It has been suggested that there exists high variability in the intensity of bleeding among horses; moreover, not all horses present EIPH under the same conditions of stress, pressure and speed, as some bleed with submaximal effort [6]. In addition, studies have shown that the physiological responses to exercise on a treadmill and experimental conditions cannot replicate the responses that occur in field studies [7].

Variants in the genes encoding CD39 and CD39L1 (*ENTPD1* and *ENTPD2*, respectively) have been previously reported as potential genetic causes involved in EIPH pathogenesis [8]. These enzymes, known as ENTPDase-1 and ENTPDase-2, respectively, are ectonucleotidases that hydrolyse nucleoside diphosphates and triphosphates and play a role in nucleotide metabolism related to haemostasis, such as nucleoside diphosphates (ADP) [9]. There was no difference between haemostatic variables related to coagulation and fibrinolysis in horses with and without EIPH. However, platelet responsiveness to ADP is decreased in horses with EIPH compared to that in healthy horses [2, 10, 11].

Boudreaux et al. [8] evaluated the genes encoding the ectonucleotidases CD39 and CD39L1 in horses with and without a history of pulmonary haemorrhage and suggested that pathogenic variants in these genes may play at least a partial role in the predisposition of some horses to EIPH. Therefore, the aim of this study was to evaluate the missense variants in *ENTPD1* the (rs1152296272, rs68621348, and rs68621347) and *ENTPD2* (rs782872967) genes in high-performance equine athletes with EIPH diagnosed by trachea-bronchial endoscopy.

Results

Regarding variants of the *ENTPD1* gene, in the control group, 66% (37/56) were homozygous for nonreference alleles of the rs1152296272 and rs68621347 variants, 29% (16/56) were heterozygous, and 5% (3/56) were homozygous for the reference alleles. In contrast, 57% (43/76) of horses in the EIPH group were homozygous for the nonreference alleles, 40% (31/76) were heterozygous, and 4% (3/76) were homozygous for the reference alleles. There was no significant difference between the control and EIPH groups when comparing the homozygous genotype for the nonreference alleles ($p=0.3211$) and the heterozygous genotypes ($p=0.1866$). All horses in the control group (56/56) were homozygous for the reference allele of the rs68621348 variant. Similarly, 96% (74/76) of EIPH horses were homozygous for the reference allele, while only 1% (1/76) were homozygous and 3% (2/76) were heterozygous for the nonreference alleles ($p=0.7806$).

Regarding the rs782872967 variant in the *ENTPD2* gene, the control group comprised 45 horses, and the EIPH group comprised 68 horses. In the control group, 84% (38/45) of the genotypes were homozygous for the reference allele, 16% (7/45) were heterozygous, and none were homozygous for non-reference allele. In the EIPH group, 75% (51/68) were homozygous for the reference allele, 2% (1/68) were homozygous for the nonreference allele, and 24% (16/58) were heterozygous. The p value was 0.4566 for the reference allele genotype and 0.2064 for the heterozygous genotype, with no significant difference between groups. The results are summarized in Table 1.

Table 1 Comparison of genotype frequencies (in percentages) between the control and EIPH groups

Variants	Genotype	Control (%)	EIPH (%)	*P* value
rs1152296272 and rs68621347	Non-reference + He*	95.0	96.0	0.9193
	Reference	5.0	4.0	0.6221
rs68621348	Non-reference + He	--	4.0	--
	Reference	100.0	96.0	0.7806
rs782872967	Non-reference + He	16.0	25.0	0.1401
	Reference	84.0	75.0	0.4566

*Heterozygous

Discussion

Studies have shown that the physiological responses to exercise on a treadmill and experimental conditions cannot replicate the responses in the field [7]. The current study presents favourable points that include the use of high-performance athletic horses, considered a risk group for EIPH, evaluated under the real conditions of official races and, consequently, under the maximum level of effort requirements. In addition, endoscopy exams and genetic tests were performed.

Conducting field studies provides the opportunity to evaluate real stress conditions among healthy athletic animals with high-performance capacities. However, due to the high zootechnical value of horses, using more sensitive techniques that can be performed in vivo, such as bronchoalveolar lavage, is a challenge. Because these techniques are more invasive, they demand substantial collaboration between trainers, owners, jockeys and the scientific community; thus, it is not feasible for the individuals involved to make their animals available for analysis [10, 11]. For this reason, trachea-bronchial endoscopy was used in this study, which is a widely used technique for diagnosing EIPH [12–14]. The genotype frequencies of the rs1152296272 and rs68621347 variants were equal across all horses, regardless of genotype. This finding reinforces the possible existence of a link between these two variants, and was consistent with a previous study [8]. The results revealed a high prevalence of nonreference alleles for the rs1152296272 and rs68621347 variants, where 96% of horses in the EIPH group carried at least one nonreference allele. However, 95% of the horses in the control group analysed in the present study also carried at least one nonreference allele for these same variants, while the result found in the control group in the previous study was 53%. A possible explanation for this finding is the use of different diagnostic methods for EIPH; Boudreaux et al. [8] used histopathological samples, while the present study used trachea-bronchial endoscopy. In the present study, we opted to use endoscopy because, although widely utilized, this method provides a macroscopic view of the bleeding, which could potentially lead to the inclusion of animals with only a mild degree of bleeding at the histopathological level in the control group.

In the present study, none of the animals in the control group and 4% of horses in the EIPH group carried nonreference allele for the rs68621348 variant. On the other hand, the horse population assessed in a previous study presented a prevalence of at least one mutated allele in 4.5% of horses with other types of bleeding and 38% of EIPH horses [8]. Thus, the authors of the study cited above suggested that there may be a negative selection for this alteration. Furthermore, the rs782872967 variant in the *ENTPD2* gene was less frequent in the control and the EIPH groups. These data corroborate the findings of a previous study [8] in which a high prevalence of reference genotypes was observed in affected (62%) and healthy (71%) animals; the occurrence of negative selection for this polymorphism was also suggested by these authors.

Notably, the genetic base of the Thoroughbred horse is narrow, with 78% of the alleles in the current population deriving from 30 sires, of which 27 are males, with a single stallion representing 95% of sire lines and 10 mares representing 72% of dam lines [15].

The breeds in which the four variants studied by Boudreaux et al. [8] were found at a higher frequency were Thoroughbreds and Quarter Horses, which are genetically related. In contrast, despite the small number of evaluated animals of other breeds, rs1152296272 and rs68621347 nonreference variants were not found in Rocky Mountain or Belgian horses; the rs68621348 nonreference variant was not present in Oldenburg, Paint, Saddlebred, Tennessee Walking Horse, Warmblood or Belgian horses, and the rs782872967 nonreference variant was not found in Oldenburg, Paint, Rocky Mountain, Saddlebred, Standardbred or Tennessee Walking Horse breeds [8]. Hence, the prevalence of these nonreference variants might be affected by breed-related factors, particularly in breeds closely related genetically to Thoroughbreds. This fact could explain the high rate of bleeding in Thoroughbred horses and the high prevalence of nonreference variants found in this study. However, it was not possible to affirm this hypothesis since we only assessed Thoroughbreds, the breed most affected by EIPH.

Moreover, although the low reactivity to ADP shown by horses affected by EIPH has been reported for at least 39 years [2, 16, 17], one of the main challenges in this study was the limitation of data available in the literature about the role of nucleotides in the haemostatic changes that occur in this disease. Therefore, the emphasis of this study was on the possible association between phenotype and genotype for variants in the *ENTPD1* and *ENTPD2* genes since these genes encode important enzymes in haemostasis regulation in the context of ADP metabolism.

Conclusion

In conclusion, phenotypes are determined by complex interactions between genes and the environment [18]; thus, although investigating many of the factors linked to the pathogenesis that sustain the occurrence of EIPH in horses has improved the clarity of the literature, the possible molecular mechanisms involved remain incompletely understood. The mutations previously described in the genes encoding CD39 and CD39L1 enzymes are highly present in the studied population. However, no association was found between the occurrence of EIPH

Table 2 PCR and sequencing primer sets used in this study

Primer sets	Primer sequences	Product	Melting	Location
JPOF_CD39F	CTGAGACCAGTTAGGAAGGTTATG	522 bp[1]	62 ºC	chr1:33477089 to 33,477,112
JPOF_CD39R	TAGGCAAGTGTGGCTTTCTC			chr1:33476610 to 33,476,591
JPOF_CD39L1F	CCACATGCCCTCCACAG	463 bp	62 ºC	chr25:39220500 to 39,220,484
JPOF_CD39L1R	CTGGCTGTCTCAAAGGTGAT			chr25:39220057 to 39,220,038

[1]Base pairs

and the presence of these variants in the *ENTPD1* and *ENTPD2* genes in Thoroughbred horses assessed in this study. However, if there are genetic determinants associated with EIPH, they may exist elsewhere in the genome or there may be other regulatory elements that were not evaluated in the present study.

Materials and methods

A total of 122 high-performance athletic Thoroughbred horses, both male and female, that were active in their respective sports activities were grouped according to the endoscopic diagnosis of EIPH into the EIPH ($n=76$) and control ($n=56$) groups. Endoscopy examination and bleeding grading were performed by experienced veterinarians 40 min post-race. After endoscopic evaluation, blood samples were collected from the jugular vein of the horses using vacuum tubes with EDTA, followed by DNA purification. The horses were genotyped for four previously described missenses variants [8]: rs1152296272; rs68621348; rs68621347; and rs782872967. The variant nomenclatures were updated from the original report to align with the current reference and nomenclature according to the EquCab3.0 genome [19]. Polymerase chain reactions (PCR) analyses were performed using DNA purified from the collected blood samples with specific primers designed from the transcript sequences for *ENTPD1* (XM_001500628.6) and *ENTPD2* (XM_023629424.1) using an online tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Table 2).

The PCR (25 µL) contained 2.5 µL of template DNA, 0.3 µM each of the forwards and reverse primers, 12.5 µL of PCR Master Mix (Promega, CA, USA), and 8.5 µL of nuclease-free water. In addition, a no-template control reaction was performed to check for contamination in the PCR preparations. The amplification conditions were as follows: initial denaturation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 30 s, 64 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 5 min. The PCR products were purified and subjected to Sanger sequencing. The obtained sequences and electropherograms were analysed using Geneious© software (Biomatters©, Auckland-New Zealand) and compared with four investigated variants (rs1152296272; rs68621348; rs68621347; and rs782872967) using the EquCab3.0 genome sequences [19].

For the statistical analysis, the genotypes frequencies for each variant were compared across different variants within the groups using the chi-square test. Statistical significance was set at $p<0.05$.

Author contributions

Conceptualization: R.L. and J.P.O-F.; Methodology: R.O.L., L.G.A., R.C., F.C. and J.P.O-F.; Formal Analysis: R.L., J.P.O-F. and A.S.B.; Investigation: R.O.L., L.G.A., L.M.S.S., R.C., F.C. and J.P.O-F.; Writing: R.O.L., and J.P.O-F.; Review and Editing: R.O.L., L.M.S.S., L.G.A., J.P.O-F and A.S.B.; Supervision: J.P.O-F.; Funding Acquisition: A.S.B. and J.P.O-F.

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Data availability

Data used in this study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All the samples were collected with permission of the owners. All methods were performed in accordance with the relevant guidelines and approval by the Animal Care and Use Committee from the School of Veterinary Medicine and Animal Science of the São Paulo State University, on 19 May 2021 (063/2021-CEUA-UNESP).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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