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Total hemosiderin score in the tracheal wash fluid when assessing exercise-induced pulmonary hemorrhage in thoroughbred racehorses

Escore total de hemossiderina no lavado traqueal para a avaliação da hemorragia pulmonar induzida pelo exercício em cavalos de corrida Puro-Sangue Inglês

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Abstract: Exercise-induced pulmonary hemorrhage (EIPH) is one of the main respiratory conditions that requires clinical follow-up in equine sports medicine. Post exertional airway endoscopy is the reference standard for diagnosing EIPH, however the severity score is based on the amount of blood that ascend the tracheobronchial tree, which might not accurately reflect lung injury. Although the total hemosiderin score (THS) in the bronchoalveolar lavage fluid (BALF) has been shown to have a good sensitivity to diagnose EIPH, racehorse owners and trainers are often reluctant to allow this procedure. The use of the tracheal wash fluid (TWF) to assess EIPH through THS has been demonstrated as an alternative diagnostic approach. The present study evaluated the sensitivity and specificity of THS when applied in TWF samples of Thoroughbred racehorses to establish diagnostic cut-off values. Horses were grouped according to their athletic development, EIPH endoscopic grade, and use of furosemide. Differential cell counts and mean THS by group and by endoscopic grade were compared by the Median Test. Sensitivity and specificity were assessed with a contingency table, and the receiver operating characteristic curve was used to define optimal cut-off values. The cut-off values 30, 138, and 246 were established to interpret THS results according to clinical history. A THS score of ≥246 can be interpreted as evidence of past bleeding and provides unequivocal confirmation of EIPH in healthy animals. This study demonstrates how defining appropriate cut-off values for THS in TWF samples can aid in the diagnosis and monitoring of EIPH in clinical practice.

Keywords: endoscopy; equine; hemosiderophages; pulmonary bleeding.

Resumo: A hemorragia pulmonar induzida pelo exercício (HPIE) é uma das principais condições respiratórias que requerem acompanhamento clínico na medicina esportiva equina. A traqueoscopia pós-esforço é o diagnóstico de referência, porém, o grau de severidade baseia-se na quantidade de sangue que ascende a árvore traqueobrônquica, podendo não retratar devidamente a injúria pulmonar. Apesar do escore total de hemossiderina (ETH) em lavado broncoalveolar (LBA) ter demonstrado boa sensibilidade para diagnosticar HPIE, proprietários e treinadores de cavalos de corrida costumam relutar em autorizar este procedimento. O uso do lavado traqueal (LT) para avaliar a HPIE pelo ETH foi demonstrado como método diagnóstico alternativo. O presente estudo avaliou a sensibilidade e especificidade do ETH no LT de cavalos Puro Sangue Inglês objetivando estabelecer valores de corte diagnósticos. Os cavalos foram agrupados de acordo com o desenvolvimento atlético, grau endoscópico de HPIE e uso de furosemida. A contagem diferencial de células e as médias de ETH por grupo e por grau endoscópico foram comparadas pelo Teste da Mediana. A sensibilidade e especificidade foram avaliadas por tabela de contingência, e a curva característica de operação do receptor definiu os valores de corte ótimos. Os valores 30, 138 e 246 foram estabelecidos para interpretar os resultados de ETH de acordo com o histórico clínico. ETHs ≥246 podem ser interpretados como evidência de hemorragia pretérita e confirma o diagnóstico de HPIE em animais saudáveis. Este estudo demonstra como a definição de valores de corte apropriados para ETH em LT podem auxiliar no diagnóstico e monitoramento clínico da HPIE.

Palavras-chave: endoscopia; equino; hemossiderófagos; sangramento pulmonar.

1. Introduction

The endoscopic assessment of the upper airways is a routine procedure in the racehorse industry. One of the main respiratory conditions requiring clinical follow-up is the Exercise-induced pulmonary hemorrhage (EIPH). The diagnosing EIPH is important because it not only has negative effects on the welfare of horses, but affects training schedules, requires pre-race medication (in countries where furosemide is permitted), or it can lead to penalization or disqualification from competition [1-5].

Furosemide, a potent loop diuretic, is commonly used to manage EIPH in racehorses as it acts reducing pulmonary capillary intraluminal pressures, which is considered the underlying cause for hemorrhagic events [6]. Despite the apparent benefits of furosemide, the exact mechanism by which the drug mitigates EIPH is unresolved, and its possible side effects on the athletic performance of the animals that receive it are still debatable [7,8].

EIPH is currently almost exclusively diagnosed through a tracheobronchoscopy, performed from 30 up to 120 min after racing or strenuous exercise and bleeding severity is assessed and graded from 0 to 4 [1,9]. Despite its practicality and low invasiveness, airway endoscopy alone does not highlight any bleeding that does not ascend the tracheobronchial tree. This issue has been addressed by many authors who have shown that some horses considered as EIPH-negative at the endoscopic examination had a considerable number of hemosiderophages in their airway cytology [10-13].

Consensus on the diagnostic criteria when evaluating hemosiderophages has not yet been reached, however Doucet and Viel [10] adapted the total hemosiderin score (THS) [16] to the bronchoalveolar lavage fluid (BALF) of Standardbred horses, demonstrating its sensitivity to

detect EIPH. Despite its good sensitivity [10], racehorse owners and trainers are often reluctant to allow bronchoalveolar lavage (BAL) procedures. To date, the diagnostic accuracy of THS has not yet been demonstrated in tracheal wash fluid (TWF) samples. The aim of the present study was to evaluate the sensitivity and specificity of THS when applied to TWF samples of Thoroughbred racehorses and determine the optimal diagnostic cut-off values for screening EIPH.

2. Material and methods

2.1 Ethics

This research was approved by the Ethics Committee for the Use of Animals (Comissão de Ética no Uso de Animais - CEUA) of the Universidade Federal Fluminense**,** Rio de Janeiro, Brazil (#7207290519, 13 June 2019, and #3213230821, 9 December 2021).

2.2 Study population

In 2019 and 2022 a randomized clinical trial with an initial population of 71 Thoroughbred horses, untrained or in competition, aged from 2 to 5 years, were randomly selected. At the time of collection, the animals were stalled in the Brazilian Jockey Club of Rio de Janeiro, where they received food and water supplies at similar volumes, were regularly dewormed, and periodically vaccinated against influenza, rhinopneumonitis, Eastern and Western encephalomyelitis, rabies, and tetanus.

The exclusion criteria were: history of respiratory illnesses (e.g. pneumonia) other than EIPH within three weeks prior to the collection date; abnormalities upon the clinical examination (i.e. dehydration, lameness, mucopurulent nasal discharge, epistaxis, positive doping test, increased cardiorespiratory recovery time, and other injuries); laboratory abnormalities; recent use of any drug treatment other than furosemide; and horses that were uncooperative during endoscopy and tracheal wash (TW) procedure despite the use of a nose twitch.

Nine horses were excluded during the trial: 2 fillies and one colt with clinical signs of respiratory infection, 3 horses with hyperfibrinogemia, one non-compliant mare, and 2 other horses that yielded unsuitable TWF samples. The 62 final selected animals were classified by groups (0, 1, 2 and 3) according to their athletic development (untrained or in competition), EIPH endoscopic grade [9], and as users or non-users of pre-race medication (0.5 mg/kg IV of furosemide 4 h before race time).

2.3 Chronology and grouping

Group 0 (n = 15) included two-year-old yearlings that were still being broken in, while other groups were formed according to the EIPH clinical history of the animals that had already started their racing careers or were on debut on the selection day. The endoscopic

assessment and TWF collection from Group 0 were performed in the morning after their return from the daily breaking-in routine, while the remaining ones were evaluated from 30 to 60 min after competing in an official race performed on turf or sand oval tracks at the Jockey Club Brasileiro (JCB), in Rio de Janeiro city, Brazil (Fig. 1).

All horses in this study had their blood collected into vacuum tubes (Vacutainer, Becton Dickinson) containing 7.2 mg ethylenediaminetetraacetic acid (EDTA) for complete blood count, protein, and fibrinogen measurements. This collection was performed on the same day as the tracheal washings.

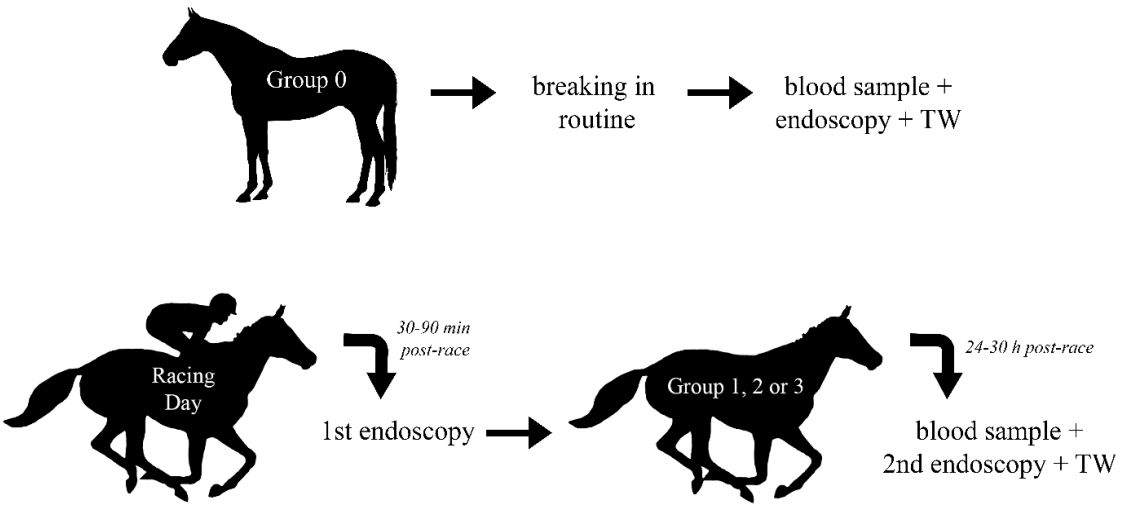

Figure 1. Chronological flowchart of the experiment

In all animals, the presence of blood [9] and mucus [17] was checked in the pharynx, larynx, trachea, and main bronchi, as well as signs of airway inflammation (i.e., airway hyperresponsiveness, hyperemia, mucosal and carina oedema). The Endoscopic examination was carried out by two examiners consecutively, on the same day, using a fiberscope (CF-10L, Olympus Optical do Brasil) with 168 cm working length, 13.3 mm outer diameter, and 3.2 mm working channel. No iatrogenic hemorrhage occurred during the endoscopy.

According to the post-race examinations, grouping was organized as follows: Group 1 (*n* = 15) comprised animals with no history of EIPH and that had grade 0 at endoscopy; Group 2 (*n* = 15) had positive endoscopic grades for EIPH and were not receiving furosemide; Group 3 (*n* = 17) were horses with a history of EIPH in recent races and were being treated with furosemide as established by the Brazilian racing regulations [3].

After the racing day selection, trainers were asked to maintain horses at rest, only giving them food and water. TW was performed 24-30 h post-race, which is the minimal time interval for hemosiderophages to appear in the airway fluid after a pulmonary bleeding [18]. This time interval was also necessary to obtain the systemic clearance of furosemide [19] in animals from Group 3. TWF collection followed the method described by Whitwell and Greet [20]. With the endoscope introduced in the horse's nostril, 20 mL of 0.9% sterile saline solution was instilled

into the distal trachea, and immediately aspirated using a delivery catheter (EDC190, Mila Supplies) through the working channel. Samples were considered adequate if any turbidity was classified as at least score 2, i.e., a clear, or slightly opaque fluid with moderate suspended particles/mucus flecks [21].

TWFs were conditioned into 50 mL sterile polypropylene centrifuge tubes, which were then immediately taken to be centrifuged at 1.200x *g* for 10 min. The supernatant was then discarded, and the remaining cell pellet was used to make linear smears. The smear slides were air-dried and fixed in absolute methanol for 5 min. For each horse, at least one slide was stained with the Diff-Quick rapid panoptic stain (Laborclin) for differential cell count. This cytological analysis was performed by counting 300 cells under a microscope (DM750, Leica Microsystems) with a 100x oil immersion objective. Neutrophilic inflammation was considered as present when the percentage of neutrophils was ≥30% [22], which was also used as an exclusion criterion for animal enrolment in the study. Other slides were submitted to the Perl's Prussian blue (PPB) staining protocol for counting and scoring of macrophages according to cytoplasm stain intensity (from 0 to 4) [16]. A total of 100 macrophages were assessed for each horse, and THS was calculated based on Doucet and Viel [10].

2.4 Statistical methods

The Kolmogorov-Smirnov test was performed to verify the normality of the endoscopic grades, THS values and cytological variables (mucus, cell degeneration, erythrocytes, hemosiderin, and percentages of macrophages, neutrophils, lymphocytes, eosinophils, and epithelial cells). THS was the variable that had a normal distribution ($P > 0.05$) to the groups 0, 2, and 3 and was thus assessed by ANOVA and *t*-test. Group 1 had a non-normal distribution (P = 0.013) and was then compared with the other groups by the Mann-Whitney test. The other variables had a non-normal distribution and were assessed using the Median test.

Differential cell counts between groups were compared by the Median test. Mean THS values by group and endoscopic grade were also compared by the Median test. Through categorical analysis of endoscopy results test performance criteria were established with a contingency table [23] to assess the sensitivity and specificity of THS in the TWF samples. The receiver operating characteristic (ROC) curve was used to define optimal cut-off values for THS in TWF samples. These analyses were carried out using IBM SPSS Modeler v. 15.0, and P-values ≤0.05 were considered as significant.

3. Results

The final study population comprised 62 Thoroughbred horses, 36 males (58%) and 26 females (42%), aged from 2 to 5 years, with different owners and trainers. The endoscopic examinations had 100% consensus among the examiners. EIPH endoscopic grades were no higher than 3 in all groups. The result for differential cell counts showed no statistically significant differences ($P < 0.05$) between groups (Table 1). However, eight horses showed

evidence of neutrophilic inflammation, with only one animal (G1-11 in Supplementary Table S1) having grade 1 mucus in both endoscopic examinations [14].

Table 1. Descriptive statistics (median, maximum, and minimum) values for differential cell counts (%) in tracheal wash samples according to the observational groups.

	Macrophages	Neutrophils	Lymphocytes	Eosinophils
Group 0	88.0[a] (21.0-97.0)	8.0[b] (2.0-32.0)	2.0[c] (0.0-7.0)	0.0[d] (0.0-2.0)
Group 1	82.3[a] (35.7-94.0)	11.0[b] (3.0-58.0)	5.0[c] (0.0-13.0)	0.0[d] (0.0-10.0)
Group 2	80.3[a] (23.0-95.0)	12.3[b] (3.0-76.0)	2.7[c] (0.0-9.0)	0.0[d] (0.0-1.0)
Group 3	83.7[a] (20.0-97.0)	14.3[b] (2.0-74.0)	3.0[c] (0.0-8.0)	0.0[d] (0.0-1.3)

*Group 0: two-year-old healthy yearlings that were still in the breaking in process; Group 1: horses with no EIPH history and grade 0 at the endoscopic examination; Group 2: horses with positive endoscopic grades for EIPH and not receiving furosemide; Group 3: horses with a recent EIPH history receiving furosemide. Median Test, **P**>0.05.*

Irrespective of the group, hemosiderophages were observed in all samples. Mean THS values by endoscopic bleeding grade (Table 2) and by group (Table 3) showed that Group 0 was significantly different ($P \leq 0.05$) from the other groups.

Table 2. Descriptive statistics of mean total hemosiderin score (THS) according to the endoscopic bleeding grades on the first endoscopic examination.

Endoscopic bleeding grade	N	Group	Mean THS ± SD
Grade 0	37	G0, G1, G3	96.7[a] ± 86.3
Grade 1	9	G2, G3	156.2[b] ± 86.3
Grade 2	8	G2, G3	134.3[a, b] ± 65.3
Grade 3	8	G2, G3	215.3[b] ± 78.7
Total	62		125.5 ± 87.7

*SD, Standard Deviation. [a, b] Different letters mean statistical significance (**P** ≤0.05) by ANOVA and **t**-test for independent samples.*

Table 3. Mean total hemosiderin score (THS) according to group.

Groups	N	Mean ± SD
Group 0	15	17.9[a] ±7.6
Group 1	15	13.8[b] ± 73.8
Group 2	15	15.9[b] ± 64.7
Group 3	17	181.0[b] ±75.2
Total	62	125.5 ± 87.7

*Group 0: two-year-old healthy yearlings that were still in the breaking in process; Group 1: horses with no EIPH history and grade 0 at the endoscopic examination; Group 2: horses with positive endoscopic grades for EIPH and not receiving furosemide; Group 3: horses with a recent EIPH history receiving furosemide.SD, Standard Deviation.[a, b] Different letters indicate statistically significant difference by ANOVA and **t**-test between groups 0, 2, and 3 (**P**<0.05). [a, b] Different letters indicate statistically significant difference by Mann-Whitney between groups 1 and 0, 1 and 2, and 1 and 3 (**P**<0.05).*

Two ROC curves (Fig. 2) derived from the overlapping of THS and endoscopy results considered the following: 1) Group 0 as true EIPH-negative horses as they had never been challenged with strenuous exercise and had no visible bleeding at endoscopy; 2) groups 0 and 1 as EIPH-negative animals according to endoscopy. Both inferences showed a high global performance (*P*<0.001) when considering different cut-off values. Group 0 alone had an area under the curve (AUC) of 0.99, while the combination of groups 0 and 1 presented an AUC of 0.82 (Fig. 2). Despite this, both inferences were statistically different (P ≤0.05; Table 4).

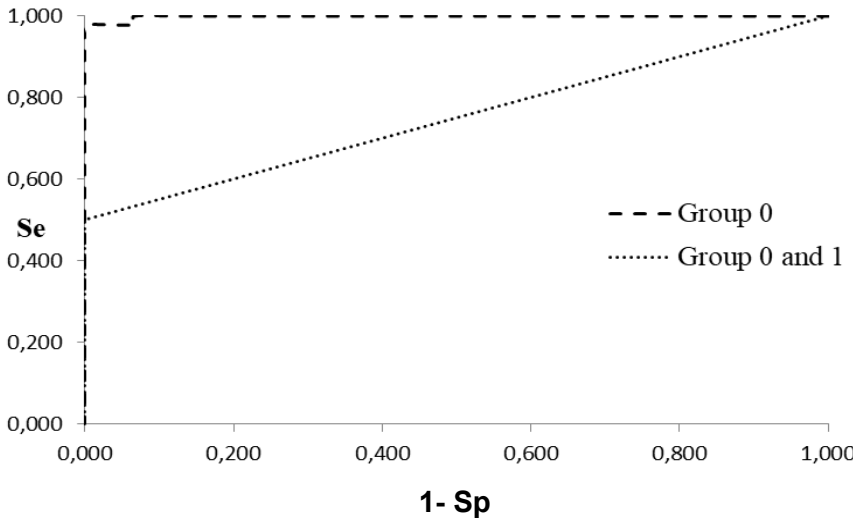

Figure 2. Receiver operating characteristic (ROC) curves derived from the overlapping of total hemosiderin score (THS) and endoscopy results (sensitivity versus 1-specificity).

Table 4. Results of the area under the curve, standard error, significance, and confidence interval of receiver operating characteristic (ROC) curves.

EIPH Negative	AUC	SE	*P*	CI 95% ROC curve	
				Lower boundary	Upper boundary
Group 0	0.999	0.002	<0.001	0.994	1.000
Group 0 + Group 1	0.825	0.056	<0.001	0.716	0.934

Group 0: two-year-old healthy yearlings that were still in the breaking-in process; Group 1: horses with no EIPH history and grade 0 at the endoscopic examination. AUC, area under curve; CI, confidence interval; EIPH, Exercise-Induced Pulmonary Hemorrhage; SE, standard error.

It was possible to establish three cut-off values: 30, 138, and 246 (Table 5; Supplementary Table S2). The cut-off value 30 corresponds to the highest specificity when the sensitivity was 1.0; the sensitivity and specificity presented the highest values at the cut-off value of 138; and the cut-off value 246 described the highest sensitivity when the specificity was 1.0.

Table 5. Variation of diagnostic parameters according to groups and cut-off values for exercise-induced pulmonary hemorrhage (EIPH) diagnosis in Thoroughbred racehorses.

Cut-off value	Parameter	Group 0			Group 0 and 1		
		Value	LB	UB	Value	LB	UB
138	SEN	0,553[a]	0,450	0,656	0,656[a]	0,558	0,754
	SPC	1,000[a]	1,000	1,000	0,833[b]	0,756	0,910
	PPV	1,000[a]	1,000	1,000	0,808[b]	0,726	0,889
	NPV	0,417[a]	0,315	0,519	0,694[b]	0,599	0,790
	KAP	0,661[a]	0,564	0,759	0,742[a]	0,652	0,832

Group 0: two-year-old healthy yearlings that were still in the breaking-in process; Group 1: horses with no EIPH history and grade 0 at the endoscopic examination. KAP, kappa index; LB, lower boundary; NPV, negative predictive value; PPV, positive predictive value; SEN, sensitivity; SPC, specificity; UB, upper boundary. [a,b] Different letters between columns indicate statistical difference with a confidence interval of 95%.

4. Discussion

Since Doucet and Viel [10] first proposed using THS as a complementary diagnostic parameter for EIPH, few studies have explored its use in clinical practice [12,24,25] or in TWF samples [11, 14, 15]. Given that THS is a non-specific diagnostic parameter with low specificity in BALF samples [10], we thus focused on group homogeneity to achieve our study goal. Using a single breed, an age range that fits the period of athletic development and career performance, and by selecting only healthy horses (Table 1), this in situ study achieved a high-test accuracy.

Our results showed that among competing animals with EIPH grade 0 at endoscopy (Group 1), some had THS values associated with recurrent EIPH episodes (Table 2, Supplementary Table S1). As hemosiderophages can persist in the lungs for up to three months [26], THS should be treated as a follow-up diagnostic parameter similarly to endoscopy. However, unlike endoscopy, establishing a specific collection time for airway fluid samples for THS evaluation might not be appropriate in all circumstances. Yet, the short diagnostic window of endoscopy presents as a limitation for identifying EIPH. The evidence of past bleeding in Group 1 could be interpreted as evidence that at least some of these horses had EIPH within the past few months. Variables inherent in the individual training stage and schedule as well as the competition calendar should be considered when using both diagnostic parameters.

In our previous study [14], although unmedicated horses with grade 0 in the post-race endoscopy had a much lower mean THS value than other groups (bleeders and furosemide users), no statistical difference was found between groups. Here, hemosiderophages were also present in the TWF samples of all animals, but there was a clear statistical difference between the mean THS values (Table 3) of horses that had not yet been challenged with strenuous exercise (Group 0) and those that already started their athletic careers (Groups 1, 2, and 3). This was corroborated by the AUC of 0.99 (P<0.05; Fig. 2, Table 4) when Group 0 was plotted in the ROC curve as the only true EIPH-negative group (i.e., unchallenged by strenuous exercise). Our results are in line with Almeida et al. [27], who demonstrated how

the initial months of race training influence the airway health of Thoroughbred horses, with hemosiderophages appearing in the TWF since the very beginning of training, which suggests that some degree of pulmonary hemorrhage occurs even with sub-maximal exercise and reinforces how racehorses are susceptible to EIPH [1,28,29].

The second curve, where groups 0 and 1 are considered as EIPH-negative, yielded an AUC of 0.82 (P<0.05; Fig. 2, Table 4). From both ROC curves, three THS cut-off values – 30, 138 and 246 – were established for evaluating TWF samples of healthy animals (Table 5; Supplementary Table S2). The cut-off value of 30 should indicate that EIPH is starting to emerge or that furosemide is being effective (see animal G3-6 in Supplementary Table S1). Conversely, animals already diagnosed with EIPH will likely present a cut-off value of ≥138. This value provided the best compromise between false-positive and false-negative rates and may indicate that recurrent EIPH episodes are occurring despite the presence of visible blood in the trachea on post-exercise endoscopy. Finally, the cut-off value of 246 was the only value that differentiated between the two diagnostic tools (Table 5; Figure 2) and provides unequivocal evidence of EIPH despite the endoscopic diagnosis. In our study, most animals that were within the 246-cut-off value were in groups 2 and 3 (Supplementary Table S1).

Although endoscopy is a valuable and low-cost diagnostic tool in equine practice, as demonstrated here and in previous EIPH studies [10,13,25], this method has a high rate of false-negative results. When comparing the mean THS values by endoscopic grade (Table 2) and by group (Table 3), animals assigned to grade 0 at endoscopy had a higher mean THS value than those from Group 0, which reinforces how endoscopy alone cannot detect the early onset of EIPH. Also, Group 1 comprised horses with no clinical history of EIPH, however their THS values were significantly higher than those of Group 0 and closer to those of groups 2 and 3 (Table 3; Supplementary Table S1).

While endoscopy is necessary to assess a horse's immediate response to exercise, THS provides an additional complementary diagnostic parameter for EIPH as it provides a more in-depth examination. It is worth to note that assessing the horse's overall health status is of key importance to provide an accurate interpretation of THS and to better plan the most suitable cardiopulmonary conditioning. Finally, animals that present or are recovering from any disease with an increased risk of pulmonary bleeding or that have recently undergone surgery should be assessed carefully and differential cell counts should be included.

5. Conclusion

The present study demonstrates the importance of defining appropriate cut-off values when using the THS method to evaluate TWF samples. The cut-off values indicated here should be regarded as ancillary parameters to better diagnose EIPH based on previous clinical and endoscopic examinations. THS provides an additional perspective to the diagnosis and monitoring of EIPH in clinical practice.

Supplementary material

Table S1. Individual variables organized by endoscopic group.

Table S2. Sensitivity and specificity variation according to the cut-off value for total hemosiderin score (THS)when considering groups 0 and 1.

Conflict of interest statement

The authors declare no conflict of interest.

Data availability statement

The data will be provided upon request.

Author contributions

Conceptualization: D. A. B. Lessa and N. X. de Alencar. Formal analysis: G. N. de Souza. Funding acquisition: D. A. B. Lessa and N. X. de Alencar. Investigation: B. Cascardo, C. Bernardes and N. R. Pires. Methodology: D. A. B. Lessa and N. X. de Alencar. Project administration: D. A. B. Lessa and N. X. de Alencar. Supervision: D. A. B. Lessa. Visualization: D. A. B. Lessa and B. Cascardo. Writing (original draft): B. Cascardo and C. Bernardes. Writing (proofreading and editing): D. A. B. Lessa, N. X. de Alencar and C. Bernardes.

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