ABSTRACT

Background: Respiratory conditions are the leading cause of training disruption in racing horses. Molecular approaches to diagnose respiratory viruses have provided an opportunity for early and
subclinical pathogen detection, particularly in samples from the upper respiratory tract. Gammaherpesvius (EHV-2 and EHV-5) have variable presentations in horses. However, the infection can be asymptomatic and act as a co-factor for the development of other diseases. In this descriptive observational study, 10 healthy, young horses at regular training in Southern Brazil underwent clinical examination, videoendoscopy of the respiratory system, cytological evaluation of TA (tracheal aspirate) and BALF (bronchoalveolar lavage fluid), along with qPCR, in order to evaluate the presence of EHV-2 and EHV-5 in lower respiratory tract samples and compare with correspondent cytological and endoscopical findings.

Results: At least one abnormality per horse during endoscopy examination was observed, including, but not limited to, mucous secretion in the airways and pharyngeal lymphoid hyperplasia. The presence of EHV-2 and EHV-5 was detected by qPCR in three out of ten animals. One horse was positive for EHV-2 alone, one for EHV-5 alone, and one was positive for both viruses. No videoendoscopic finding correlated with each other neither predicts gammaherpesvirus status (positive or negative test). Additionally, there was no relationship between the percentage of cells in both TA and BALF and the probability to test positive for herpesvirus.

Conclusions: To the authors’ knowledge, this is the first molecular detection of EHV-2 and 5 in Brazilian Thoroughbred horses. These findings may provide new insights into the epidemiological situation of EHV-2 and 5 in Brazilian athletic young horses, evidencing the importance of the molecular investigation, early detection, and prevention of respiratory diseases.

Keywords: BALF, tracheal wash, endoscopy, gammaherpesvirus, PCR, equine.

BACKGROUND

Respiratory diseases are a well-known cause of poor athletic performance in horses. Different methods have been applied for early and precise diagnosis of respiratory diseases after recognition of poor performance, such as airway endoscopy, cytology, and lung function assessment (1). The ability to control or minimize the effects of some of these diseases depends on the diagnosis of possible etiological agents involved, especially viruses. Molecular biology techniques have been widely used to
detect active infections or to identify carriers of some important agents, such as equine influenza virus (EIV), equine rhinovirus, and equid herpesvirus (EHV) (2, 3).

Five out of nine identified species of equid herpesviruses infect the domestic horse. Two belong to the subfamily Gammaherpesvirinae (EHV-2 and EHV-5) and three to the subfamily Alphaherpesvirinae (EHV-1, EHV-3, and EHV-4). Except for EHV-3, which is a venereal pathogen, all herpesviruses cause upper or lower respiratory diseases in horses and are endemic worldwide (4-6).

The EHV-2 and EHV-5 have a variable presentation, from pharyngitis, lymphadenomegaly, fever, and anorexia to pneumonia or multinodular pulmonary fibrosis (EMPF). However, like other herpesviruses, the infection can lead to no clinical signs and latency, or act as a co-factor for the development of other diseases. The full pathogenic potential of gammaherpesviruses remains unclear (7-9).

In Brazil, the first equid herpesvirus isolation was described in 1966 (10). Cases and investigations on the current prevalence of gammaherpesvirus in Brazil are scarce, with one case report of EMPF (11) and one recent evaluation in two farms that detected EHV-2 and EHV-5 in samples from upper airway secretions of asymptomatic horses (12). Thus, this study aimed to investigate the presence of herpesviruses, especially EHV-2 and EHV-5, in samples from the lower respiratory tract (i.e., bronchoalveolar lavage fluid, BALF) and evaluate if the infection with these viruses cause alterations in the respiratory tract healthy athletic horses from Southern Brazil, assessed by videoendoscopy, complete blood count, and cytologic evaluation of BALF and tracheal aspirates (TA).

RESULTS

Laboratory data

Ten healthy Thoroughbred horses (2 to 3 years old) under regular training and housed in the Paraná State Jockey Club, Brazil, were evaluated. The CBC data is present in Table 1. The animal number 6 had mild evidence of an inflammatory process as determined by increased bands (0.28 x 10^9/L). There were two animals with a mild thrombocytosis (horses 5 and 8, 385 and 355 x 10^9/L, respectively) and two with marked thrombocytopenia (horses 3 and 6, 30.5 and 26.5 x 10^9/L, respectively). The remaining parameters were unremarkable. The comparison between positive and negative animals showed no statistical difference in any hematological variable among horses positive or negative for gammaherpesvirus.
Table 1. Hematologic parameters in ten young Thoroughbred horses under regular training in Southern Brazil.

| Parameters (unit)       | Mean ± standard deviation | Reference interval |
|-------------------------|---------------------------|--------------------|
| RBC (x 10¹²/L)          | 11.2 ± 0.6                | 6.8 – 12.9         |
| Hematocrit (L/L)        | 0.44 ± 0.02               | 0.32 – 0.53        |
| Hemoglobin (g/L)        | 153.2 ± 10.5              | 110 – 190          |
| Platelets (x 10⁹/L)     | 194.2 ± 122.9             | 100 – 350          |
| WBC (x 10⁹/L)           | 7.73 ± 1.03               | 5.5 – 14.3         |
| Neutrophils (x 10⁹/L)*  | 3.82 ± 0.89               | 2.26 – 8.58        |
| Bands (x 10⁹/L)*        | 0.06 ± 0.09               | 0 – 0.1            |
| Lymphocytes (x 10⁹/L)*  | 3.71 ± 0.78               | 1.5 – 7.7          |
| Monocytes (x 10⁹/L)*    | 0.04 ± 0.07               | 0 – 1              |
| Eosinophils (x 10⁹/L)*  | 0.08 ± 0.08               | 0 – 1              |
| Basophils (x 10⁹/L)*    | 0.01 ± 0.02               | 0 – 0.29           |
| Total plasma protein (g/L) | 66.2 ± 3.3                | 62.0 – 79.0        |
| Fibrinogen (g/L)        | 2.6 ± 1.3                 | 1.0 – 4.0          |

RBC, automated red blood cell count; WBC, automated white blood count; * based on manual differential cell counts.

Videoendoscopic examination

Table 2 contains compiled abnormalities observed in the videoendoscopic evaluation. Epistaxis, DDSP, and EE were not present in any horse, while PLH was present in all animals. Whenever secretion was observed in the nasal cavities, gullet pouches, pharynx, and trachea, it was mucoid and clear. All horses had mucous secretions in the trachea at different degrees, and one animal had a swollen carina. Nasal, pharyngeal, and gullet pouch secretions, and left RLN were observed in a few animals (Table 2). No videoendoscopic finding correlates with each other neither predicts gammaherpesvirus status (positive or negative test).
Table 2. Videoendoscopic findings and qPCR results for gammaherpesvirus from horses under regular training in Southern Brazil.

| Horse | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|-------|---|---|---|---|---|---|---|---|---|----|
| Nasal secretion | - | - | - | - | - | - | - | - | B |
| Guttural pouch secretion | - | B | R | B | L | - | - | - | - |
| Pharyngeal secretion | - | - | P | - | - | - | - | - | P |
| Pharyngeal lymphoid hyperplasia | P | P | P | P | P | P | P | P |
| Recurrent laryngeal neuropathy | - | - | - | - | - | L | - | - | - |
| Tracheal secretion score (0-4) | 1 | 3 | 2 | 1 | 2 | 1 | 2 | 1 | 3 |
| EHV-2 | - | - | - | P | - | P | - | - | - |
| EHV-5 | - | - | - | - | - | P | - | - | - |

:: absent/negative; P: present/positive; B: bilateral; R: right; L: left.

PCR for infectious agents

No horse was positive for EAdV, EIV/H3N8, EHV-1, EHV-4, ERAV, ERBV, or Streptococcus spp. However, one horse was positive for EHV-2 (horse 4), one for EHV-5 (horse 10), and one for both EHV-2 and EHV-5 (horse 6). (Table 2)

TA and BALF cytology

Considering the BALF evaluation (Table 3), only one animal (horse 5) had a normal differential count (considering macrophages 60-80%, lymphocytes 20-35%, neutrophils <5%, mast cells <2%, and eosinophils <1% (13-15)). Nine horses had mildly to moderately increased neutrophils. Three horses that had mastocytosis also had increased percentages of neutrophils.

Considering the TA evaluation (Table 3), no animal had a normal differential count (considering macrophages 40-80%, neutrophils <20%, lymphocytes <10%, mast cells <1%, and eosinophils <1% (13, 14)), although three animals (horses 1, 2, and 5) had only mildly increased numbers of lymphocytes. Seven horses had more neutrophils in their TA than in their BALF. However, only two horses reached the cutoff for neutrophilic inflammation (>20%) (16); these two animals were positive for herpesvirus. One horse had a lymphocytic inflammation (lymphocyte was the predominant cell type).
There is no relationship between the percentage of cells in both TA and BALF and the probability to test positive for herpesvirus. However, the percentage of neutrophils in TA showed a statistical trend ($p = 0.0527$).

### Table 3. Differential cell counts of TA and BALF from horses under regular training in Southern Brazil.

| Horse | TA | BALF |
|-------|----|------|
|       | Mac | Lymph | Neut | Eos | MC | Mac | Lymph | Neut | Eos | MC |
| 1     | 69.6 | 24.0 | 6.4 | 0.0 | 0.0 | 65.6 | 13.6 | 13.4 | 3.7 | 3.7 |
| 2     | 68.9 | 15.8 | 14.7 | 0.3 | 0.3 | 36.9 | 20.6 | 38.2 | 1.7 | 2.7 |
| 3     | 57.8 | 24.8 | 15.4 | 0.9 | 1.2 | 71.8 | 21.5 | 6.0 | 0.3 | 0.5 |
| 4†    | 15.5 | 7.3 | 76.5 | 0.3 | 0.4 | 74.1 | 18.5 | 5.1 | 0.8 | 1.5 |
| 5     | 67.6 | 23.6 | 7.8 | 0.5 | 0.5 | 64.2 | 30.7 | 4.8 | 0.0 | 0.3 |
| 6‡‡   | 65.5 | 15.7 | 16.4 | 0.0 | 2.3 | 61.2 | 21.7 | 14.2 | 1.7 | 1.1 |
| 7     | 32.1 | 46.5 | 18.9 | 1.3 | 1.2 | 52.8 | 34.9 | 12.2 | 0.0 | 0.0 |
| 8     | 48.2 | 33.5 | 16.6 | 1.3 | 0.4 | 64.1 | 29.2 | 6.2 | 0.0 | 0.5 |
| 9     | 49.6 | 37.8 | 5.6 | 4.0 | 3.1 | 69.4 | 19.0 | 8.4 | 0.5 | 2.6 |
| 10‡   | 29.1 | 28.9 | 40.7 | 0.6 | 0.6 | 57.3 | 32.5 | 9.5 | 0.0 | 0.8 |
| Mean  | 50.41 | 25.79 | 21.90 | 0.91 | 0.99 | 61.75 | 24.22 | 11.80 | 0.85 | 1.37 |
| SD    | 19.15 | 11.52 | 21.61 | 1.18 | 0.99 | 10.83 | 7.06 | 9.90 | 1.19 | 1.24 |

TA: tracheal aspirate; BAL: bronchoalveolar lavage fluid; Mac: macrophages; Lymph: lymphocytes; Neut: neutrophils; Eos: eosinophils; MC: mast cells; †: EHV-2 positive; ‡: EHV-5 positive; †‡: positive for both EHV-2 and EHV-5.

### DISCUSSION

We described the presence of clinically healthy, asymptomatic, athletic Thoroughbred horses positive for EHV-2 and EHV-5, detected by qPCR in samples from the lower respiratory tract (i.e., BALF), housed in the Paraná State Jockey Club, in Southern Brazil. A recent study detected 14 positives out of 18 sampled Appaloosa horses for gammaherpesvirus. Nine animals were positive for EHV-5, four for EHV-2, and one was double-positive. Although no details about the age of the animals, their athletic condition, and the percentage of animals sampled from the whole farm, the animals were...
said to be asymptomatic adults. Given this report showing that the virus is indeed circulating in the
country, and more specifically in the state, we found it to be essential to evaluate the possible presence
in the Jockey Club. Animals in the Jockey club live at a high density and, occasionally, travel interstate,
which could increase the risk of contamination and spreading the disease. A recent study analyzed the
same infectious agents evaluated herein in animals imported into the United States. In 167 animals
assessed using PCR from nasal swabs during 20 months, 48 were positive for EHV-2, 68 for EHV-5,
and in 27 were double-positive for gammaherpesvirus, in which 72 were asymptomatic and 44 had
various symptoms (e.g., fever, tachypnea, cough, lymphadenopathy, and nasal discharge) (17). It
emphasizes the frequent and silent presence of gammaherpesvirus in equine populations.

In our study, the animal positive for EHV-2 only (horse 4) had an unremarkable CBC and BALF,
but the most severe neutrophilic inflammation in the TA among all animals. However, he only had a
small amount of tracheal secretion and bilateral mucus in both gullet pouches, indicating an
unremarkable inflammatory reaction in the respiratory tract. In contrast, the animal positive for EHV-5
only (horse 10) had a mild and moderate increased number of neutrophils in BALF and TA, respectively,
but marked presence of tracheal mucus, in addition to nasal and pharyngeal secretion. The single horse
double-positive (horse 6), had neutrophilic inflammation in its BALF, but unremarkable TA, and only a
small amount of tracheal secretion. Interestingly, this was the animal with severe thrombocytopenia and
a mild inflammatory leukogram. These inconsistent videoendoscopic and cytologic findings highlight
the lack of correlation and predictive value of these evaluations to help to identify infections by
gammaherpesvirus, emphasizing the importance of molecular diagnostics.

The most consistent videoendoscopic finding in all animals was PLH, which is significantly
higher in younger, asymptomatic racehorses when compared to older ones. Despite its high prevalence
in Thoroughbreds (63%), PLH does not appear to affect performance (18-20). A second constant finding
was tracheal secretion at various degrees in all horses. Although it may be a sign of inflammatory airway
disease (IAD), it is not sufficient for the diagnosis of this condition, since none of the horses had any
history of chronic cough or poor performance. Additionally, considering the higher cutoffs for BALF
differential counts currently proposed for IAD diagnosis (>10% neutrophils, >5% eosinophils, and >5%
mast cells) (1), this disease was not present in our study population.
The presence of neutrophils in TA, although not significantly correlated with infection by gammaherpesvirus, appeared to be the closest indicator of a possible presence of the virus in the respiratory tract. However, athletic horses can have transient increases in cells as an adaptation to intensive training, which has been reported to alter the innate immune response in the lung and the systemic circulation (21). The overall poor correlation between TA and BALF in our study has been previously described, indicating the distinct response of tracheal and bronchioalveolar epithelium to stimuli. Additionally, the trachea is the natural exit from inflammatory debris from the lower bronchial tree (20, 22). Therefore, an inflammatory process may be solved in the lower respiratory tree and be currently “passing through” the trachea. Besides, both tissues may be concurrently affected, or infections can progress from upper to lower tract.

The role gammaherpesviruses play in the development of respiratory disease remains poorly understood. However, molecular diagnostic techniques such as qPCR have been pivotal tools for early and fast detection of infectious diseases (23). Nonetheless, several epidemiologic investigations on the prevalence of herpesvirus may be affected by the type of sample utilized. For instance, whole blood, peripheral blood mononuclear cells, conjunctival swab, nasal swabs, and lower respiratory secretions (i.e., TA and BALF, as in our study) have been described, which can potentially affect the sensitivity of the test (2, 7, 24, 25). To our knowledge, the sensitivity for each sample type has not been determined to date. Moreover, the higher gammaherpesvirus incidence in comparison with alphaherpesvirus found by us and others (17, 23, 25) is likely due to the unavailability of vaccines for EHV-2 and -5, although experimental immunization of foals against EHV-2 has been reported (26).

**CONCLUSIONS**

Regardless of the small sample size of our study, we demonstrated a 30% prevalence of gammaherpesvirus in horses that did not present any pathognomonic clinical sign, relevant hematologic, videoendoscopic, or cytologic finding, neither the parameters evaluated would reliably predict the viral infection. The detection of these viruses in places with high transit of animals, such as the Paraná State Jockey Club in our case, reinforces the value of molecular techniques to identify potential reservoirs and disseminators. Investigations of asymptomatic or subclinical conditions in racehorses can potentially minimize considerable costs associated with poor performance.
METHODS

Horse population

Ten Thoroughbred horses with ages ranging from 2 to 3 years old (mean 2.7 ± 0.5), eight males and two females, housed in the Paraná State Jockey Club, Brazil, determined to be healthy by standard physical examination were included in this study. Horses were housed in individual stables with a sawdust bed, fed with approximately 6 kg whole oat groats, and 2 kg commercial concentrate (Supra Alisul Foods, São Paulo, Brazil), in addition to hay and water ad libitum.

Blood sampling

Approximately 10 mL of whole blood was collected from the jugular vein of each horse and immediately transferred to EDTA (ethylenediaminetetraacetic acid) tubes for complete blood counts (CBC). After sampling, horses were transferred back to their individual stables. Blood samples were stored on ice packs, shipped to the clinical pathology laboratory, and analyzed within 24 hours after collection. The CBC was performed in an automated cell counter (BC-2800Vet, Mindray Bio-Medical Electronics Co., Shenzhen Shi, China) followed by a manual differential count. Total plasma protein and fibrinogen, using the heat-precipitation method, were determined by refractometry.

Videoendoscopic examination

The animals underwent a one-hour training approximately 24 hours before blood collection, videoendoscopy, and related procedures. After intravenous sedation (1 mg/kg xylazine, Rompun, Bayer Animal Health, Leverkusen, Germany), a flexible videoendoscope (Karl Storz, Endoscopy-America, Inc, El Segundo, CA, USA) was passed through the right nostril and advanced along the ventral meatus, caudally to the nasopharynx. Structural and functional aspects of the nasal cavity, nasopharynx, guttural pouches, and larynx were examined, including the presence or absence of secretion, pharyngeal lymphoid hyperplasia (PLH), dorsal displacement of the soft palate (DDSP), epiglottic entrapment (EE), and recurrent laryngeal neuropathy (RLN). The endoscope was advanced caudally into the trachea to the level of the carina. At that time, the presence and amount of mucus and blood in the trachea were assessed. An adapted grading score to assess the quantity and quality of secretion present in the trachea from 0 to 4 was used, where 0 is the absence of secretion and 4 is abundant secretion (27-29).

Tracheal aspirate technique
By the end of the examination described above, a single lumen polypropylene catheter was
advanced through the endoscope biopsy channel until the catheter tip was positioned immediately
proximal to the tracheal bifurcation. The tracheal fluid was aspirated and processed in the laboratory
within 30 minutes of collection. Two evaluators counted at least 400 cells in cytocentrifuge (CT14
model, Teklab, Piracicaba, SP, Brazil) preparations (1488 x g for 10 minutes) stained with May-
Grünwald Giemsa (MGG).

**Bronchoalveolar lavage fluid collection technique**

Briefly, a bronchoalveolar lavage tube (11 mm x 245 cm, VBAL30, Bivona, Smiths Medical,
Dublin, OH, USA) was passed blindly into the distal airway. After the infusion of 20 mL of 2%
lidocaine (Bravet, Laboratory Bravet, Rio de Janeiro, Brazil) to desensitize the airways, the tube was
gently advanced into the bronchial tree. Two-50 mL aliquots of sterile 0.9% saline were infused into
the alveolar space and gently aspirated. The average percentage of recovered fluid was 36.5% of the
original volume infused. All BALF were kept on ice and processed within 12 hours of collection.
Cytocentrifuge preparations were prepared and evaluated as the TA samples. Approximately 1 mL of
BALF was frozen at -20 ºC at the time of sampling and sent to the IDEXX Laboratories (Sacramento,
CA, USA) on ice packs within 24-48 h from the collection.

**Polymerase chain reaction (PCR)**

Quantitative PCR (qPCR, Comprehensive Equine Respiratory RealPCR Panel, IDEXX
Laboratories, Inc., Westbrook, ME, USA) was used for the detection of EHV-2 and EHV-5.
Additionally, the test provides detection of Equine Adenovirus (EAdV), Equine Influenza Virus
(EIV/H3N8), EHV-1, EHV-4, Equine Rhinovirus type A (ERAV) and type B (ERBV), in addition to the
bacteria *Streptococcus equi* subsp. *equi*, *S. dysgalactiae* subsp. *equisimilis*, and *S. equi* subsp.
zooepidemicus. All assays were designed and validated according to industry standards, and details
are of proprietary rights.

**Statistical analysis**

All data were tested for normal distribution with D'Agostino & Pearson omnibus test. Animals
positive and negative for gammaherpesvirus were compared using Mann-Whitney test or unpaired t-
test with Welch's correction; the null hypothesis was that the distribution or means of both groups
were identical, respectively. Linear logistic regression was run to calculate the probability of predicting
positivity for gammaherpesvirus based on the percentage of cells present in both BALF and TA. The correlation among videoendoscopic findings and positivity for gammaherpesvirus using Spearman’s test was also performed. Alpha was set in 0.05 (GraphPad Prism 8.0.2, GraphPad, San Diego, CA, USA).

**Abbreviations**

BALF: bronchoalveolar lavage fluid; CBC: complete blood counts; DDSP: dorsal displacement of the soft palate; EAdV: equine adenovirus; EDTA: ethylenediaminetetraacetic acid; EE: epiglottic entrapment; EHV: equid herpesvirus; EIV/H3N8: equine influenza virus strain H3N8; EMPF: equine multinodular pulmonary fibrosis; ERAV: equine rhinovirus type A; ERBV: equine rhinovirus type B IAD: inflammatory airway disease; MGG: May-Grünwald Giemsa; PLH: pharyngeal lymphoid hyperplasia; qPCR: quantitative polymerase chain reaction; RBC: red blood cells; RLN: recurrent laryngeal neuropathy; TA: tracheal aspirate; WBC: white blood cells.

**DECLARATIONS**

**Ethics approval and consent to participate**

The study was approved by the Regional Animal Ethics Committee (042/2012). Owners volunteer signed the consent for their animals’ inclusion in the study.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.
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Authors’ contributions

MAF, JSB, PTD, JHP, AWB, and IRBF contributed to study design, sample collection. MAF, JSB, LSU, PBSS, APS, AWB, CML, and IRBF contributed to data analysis and interpretation, manuscript preparation, and manuscript revision. All authors approved the final version of the manuscript.

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