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Original Research

Identification of Equine Herpesvirus 5 in Horses with Lymphoma

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ABSTRACT

Equine multinodular pulmonary fibrosis, equine herpesvirus 5 (EHV-5), and multicentric lymphoma were discovered in one patient. Review of gamma herpesvirus activity in humans revealed a propensity for lymphoproliferative disorders associated with infection. The objective was to determine the frequency of EHV-5 in lymphoma tissues and compare with the frequency found in the lymph nodes of clinically normal horses. Case control investigation of lymphoma-positive tissues and analysis via polymerase chain reaction (PCR) for EHV-5 was performed on 12 horses. Prospective collection and PCR analysis of lymph nodes (mesenteric or submandibular) for EHV-5 was performed on 21 control horses. Thirteen samples of lymphoma-positive tissues and fluid were submitted for PCR analysis for EHV-5. Of these, 67% was positive. In the control horse population, 14% was positive for EHV-5 ($P = .004$). Neoplastic samples positive for EHV-5 were classified as T-cell rich B-cell lymphoma (three), T-cell lymphoma (one), one was nondifferentiated, and two were not stained. Gamma herpesviruses in humans have been associated with lymphoproliferative diseases such as Kaposi sarcoma and Burkitt lymphoma. This study reveals an increased frequency of EHV-5 (gamma herpesvirus) in horses diagnosed with lymphoma compared with healthy control horses. Although the exact role this virus plays in the initiation or perpetuation of lymphoproliferative neoplasia is unknown, EHV-5 may be an etiologic agent associated with the development of some types of equine lymphoma.

1. Introduction

Equine lymphoma is a devastating, fatal disease that may affect horses of all ages. To date, there is no known infectious etiologic agent. A horse that presented to the Veterinary Health Center (VHC) at Kansas State University (KSU) was diagnosed with both multicentric lymphoma and equine multinodular pulmonary fibrosis (EMPF) and was positive on polymerase chain reaction (PCR) on pulmonary tissues and lymph node (horse 1, Table 1) for equine herpesvirus 5 (EHV-5), a gamma herpesvirus, which has been recently associated with EMPF [1]. Additionally, a horse with T-cell leukemia and EMPF, also positive for EHV-5 on PCR analysis of lung, lymph node, and bone marrow, was recently reported [2]. In humans, gamma herpesviruses, such as Epstein–Barr virus (EBV) and Kaposi sarcoma-associated herpes virus (KSHV), have been associated with malignant and nonmalignant lymphoproliferative conditions such as Burkitt lymphoma, multicentric Castleman disease, and Kaposi sarcoma [3–5]. Therefore, it

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Table 1
Twelve cases of lymphoma classified by type of lymphoma, IHC staining result, and result of PCR analysis for EHV-5

Horse	Type of lymphoma	IHC result	PCR result
1	Multicentric	T-cell rich, B-cell	Positive
2	Submandibular	T-cell rich, B-cell	Positive
3	Cutaneous	Not performed	Positive
4	Cutaneous	T-cell rich, B-cell	Positive
5	Mediastinal	Not performed	Negative
6	Multicentric	Not performed	Positive
7	Solitary tumor of extranodal site	T-cell rich, B-cell	Negative
8	Alimentary	T-cell	Negative
9	Solitary tumor of extranodal site	Nondifferentiated[a]	Negative
10	Multicentric	T-cell	Positive
11	Multicentric	T-cell rich, B-cell	Positive
12	Multicentric	Nondifferentiated[a]	Positive on pleural fluid, negative on lymph node

EHV, equine herpesvirus; IHC, immunohistochemistry; PCR, polymerase chain reaction.
[a] No stain uptake.

was the aim of our study to evaluate the frequency of EHV-5 in lymphoma cases compared with normal, healthy control cases.

2. Materials and Methods

2.1. Study Design and Samples

A retrospective review of patient records of horses presenting to the University from 2005 to 2010 was performed to identify those with a histopathologic diagnosis of lymphoma. Additionally, all cases with a positive diagnosis of lymphoma presenting in 2010 and 2011 were prospectively selected, including one case from Kentucky and one case from Illinois. Diagnosis of lymphoma was made either ante- or postmortem with biopsy and/or histopathology of neoplastic lymph nodes or tissues. Information gathered from records included signalment, site of lymphoma, and immunophenotype of neoplastic cells, if performed.

Sample size was determined using the following assumptions: $\alpha = 0.05$, power $= 0.8$, and an expected difference in EHV-5 status between lymphoma and control animals of at least 40% (lymphoma >60% and control animals <20%). Twenty-one control horses, presented to the KSU VHC for euthanasia for reasons other than respiratory disease or lymphoma, were sequentially sampled and one lymphoid tissue (either submandibular or mesenteric) was submitted for PCR analysis after storage at −80°C. Gross postmortem examination was performed on control horses to ensure that overt respiratory or lymphoproliferative disease was not present. Lymphoid tissue was specifically isolated for EHV-5 PCR testing in control horses because of the propensity that this virus has for lymphocytes, which are localized to this tissue type.

2.2. DNA Extraction and PCR Assays

The methods used for DNA extraction were dependent on the sample available for testing. The neoplastic tissues from 12 horses were either formalin fixed or formalin fixed and paraffin embedded. To remove formalin, the formalin-fixed tissues not embedded in paraffin were soaked in phosphate buffered saline (PBS) solution at 4°C for 2 days with an exchange of PBS twice a day. The tissues were then minced with a razor blade, suspended in 3–4 mL of Bovarnick buffer, placed in a 15-mL conical centrifuge tube containing five–seven copper coated ball bearings (4.5 mm in diameter), and vortexed at full power for 1 minute. A 200-μL aliquot was removed and added to a 1.5-mL microcentrifuge tube containing 20 μL of proteinase K (Qiagen, Valencia, CA) and incubated at 55°C overnight. The remaining steps of DNA extraction were as recommended by the DNA extraction kit manufacturer (purification of DNA from animal tissues, DNeasy Tissue handbook, Qiagen, Valencia, CA). For formalin-fixed, paraffin-embedded tissues, the DNA was extracted as described previously [1].

Cells were pelleted from pleural fluid from one horse by centrifugation at 1700 rcf (relative centrifugal force) for 10 minutes. The pleural fluid was decanted and the cell pellet was suspended in 200 μL of PBS. The DNA was extracted from 30 μL of cell suspension using a rapid alkaline polyethylene glycol-based method that allowed direct PCR. For frozen tissues from the control horses, the DNA was extracted as recommended by the DNA extraction kit manufacturer (purification of DNA from animal tissues, DNeasy Tissue handbook; Qiagen, Valencia, CA).

The PCR assays used were gel based and specifically targeted the glycoprotein (g) H gene or the gB gene of EHV-5. The analytical sensitivity for both PCR assays was 10^{-1} cell culture infectious doses of EHV-5. The PCR primers used to detect the gH gene and the reaction conditions used were previously reported [1,6]. That PCR assay produced an amplicon of 344 base pairs and was used for detection of viral DNA in fresh tissues.

The PCR assay used to detect the gB gene of EHV-5 was used for fresh and formalin-fixed tissues and was designed from aligned sequences of the gB gene found in public data bases or derived in house. The nucleic acid sequence for the forward primer was TGATATGACGGCCAGATCACAC and CCAACCCCACACCATAGTCT for the reverse primer. Those PCR primers produce an amplicon that is 155 base pairs in length. The reaction conditions were one cycle at 94°C for 4 minutes; 10 cycles at 95°C for 30 seconds; 67°C for cycle 1 and then −1°C per cycle for 20 seconds; 72°C for 60 seconds; 40 cycles at 95°C for 30 seconds; 57°C for 20 seconds; 72°C for 60 seconds; and one cycle at 72°C for 7 minutes. The same commercially available reagent mixture was used for both PCR assays (Promega GoTaq Green; Promega, Madison, WI). Nucleic acid sequencing of amplicons from both PCR assays has been done, using diagnostic samples and experimentally derived samples, to verify detection of EHV-5.

2.3. Immunohistochemistry

Immunohistochemical staining was performed on lymphoma-positive tissues using anti-CD3 (T cell), -CD79α (B cell), and/or -CD20 (B cell) antibodies. Because this was a retrospective clinical study, only samples submitted for histology in lymphoma cases were analyzed. Except for horse 12, this resulted in one sample per horse.

2.4. Statistical Analysis

A one-sided Fisher exact test was used to determine if there was an association between EHV-5 PCR status and lymphoma status. An odds ratio was also performed to determine the strength of an association if it was found to be significant. A $P < .05$ was used to determine statistical significance. MedCalc 12.2.1 (MedCalc Software, Mariakerke, Belgium) was used to analyze the data.

3. Results

Twelve cases of lymphoma were identified through review of patient records and prospective recruitment. Mean age was 11.25 ± 6.88 years. There were five mares, five geldings, and two stallions. Breeds identified included Quarter Horse (four), Paint (two), Thoroughbred (two), Warmblood, Arabian, Clydesdale, and Morgan and were representative of the hospital population. The paraffin-embedded (four) and formalin-fixed (eight) histopathology samples that were submitted for PCR analysis included submandibular and thoracic lymph nodes, fibrotic lung tissue, a subcutaneous mass, a thoracic mass, and a skin biopsy. Additionally, a sample of pleural fluid obtained antemortem from horse 12 was submitted for PCR analysis. Type of lymphoma, immunohistochemical staining results, and results of the PCR analysis are presented in Table 1. Eight lymphoma-positive tissues were positive for EHV-5 (67%).

The mean age of the control horses was 13.6 ± 7.67 years and this was not significantly different from case cohorts ($P = .4$). There were 13 mares and eight geldings. Breeds included in the control population included Quarter Horse (13), Thoroughbred (four), Arabian, Paint, Morgan, and Percheron. Reasons for euthanasia were identified as colic (seven), donation because of chronic illness, neurologic condition, or lameness (nine), catastrophic musculoskeletal injury (three), old age (one), and neurologic disease (one). Three control horses were positive for EHV-5 (14%). There was a significant difference between the number of positive EHV-5 samples in the lymphoma group compared with the number of positive samples in the control group ($P = .004$). The odds of a horse with lymphoma testing positive for EHV-5 was 12 times greater than a healthy horse without lymphoma (95% confidence interval: 2.16–66.56).

4. Discussion

Of the lymphoproliferative disorders encountered in horses, lymphoma is most common, with an overall incidence of approximately 1.3%–2.8% of all equine tumors and a prevalence of 0.002%–0.5% in the equine population [7,8]. There is no gender, breed, or age predilection, and etiologies have only been speculated but not conclusively determined [9–11]. Lymphoma is classified into multicentric, alimentary, mediastinal, cutaneous, and solitary tumors of extranodal sites. Immunohistochemistry is a sensitive and versatile method widely used to investigate the antigenic determinants on cells that can lead to more reliable interpretation of neoplastic versus reactive proliferations of lymphocytes and nonlymphoid disorders. Three of the samples included in this study were classified as T-cell rich large B-cell origin, where the B cells are considered malignant and the T cells are in high concentration but are benign in character. T-cell lymphoma is overall a more common finding in equine patients with lymphoma [12]; however, this was not reflected in this small subset of cases. Gamma herpesviruses tend to infect and proliferate in either B cells, such as with EBV [13–15], or T cells, such as with herpesvirus saimiri 2 [16]. Because of the small number of cases that had typing performed and the variety of lymphoma types present in the study, it cannot be determined which lymphoid cells the equine herpesviruses tend to infiltrate. Although most gamma herpesviruses are not tumorigenic, some are associated with lymphoproliferative diseases that result in cancerous growths in either their natural host or in a phylogenetically related host. The virus genome replicates in lymphoblastoid cells, but infection is frequently not productive. Two samples remain undifferentiated. This could be because of CD79a and CD20 stain uptake variability in the equine lymphocyte.

Human gamma herpesviruses, such as human herpesvirus (HHV) 4 (HHV-4 [EBV]) and 8 (HHV-8 [KSHV]), have been positively associated with lymphoproliferative disorders such as Burkitt lymphoma, Kaposi sarcoma, and primary effusion lymphoma. The horse is the natural host of five recognized herpes viruses: EHV-1 (alpha herpesvirus), EHV-2 (gamma herpesvirus), EHV-3 (alpha herpesvirus), EHV-4 (alpha herpesvirus), and EHV-5 (gamma herpesvirus). Equine herpesvirus 5 was first isolated in Australia in 1987 [17], and its prevalence in the equine population is variable depending on specific geographical region tested, ranging from 3% in respiratory liquids (bronchoalveolar lavage or transtracheal wash) of horses in France [18] to 64% in nasal secretions in horses in California [19]. In addition, lower prevalence rates have been found in older versus younger horses [20]. There are no data on the prevalence of EHV-5 in other tissues in horses. Although EHV-5 has recently been found to be associated with EMPF [1] and there have been an increasing number of reported cases of EHV-5-associated fibrotic lung diseases, there has only been one report of lymphoproliferative disease associated with EMPF [2]. It is unknown why some horses develop EMPF, some develop lymphoma, and some have no evidence of disease at all but still harbor the latent virus. The same is true for humans who are infected with latent herpesviruses and may or may not develop lymphoproliferative disorders.

Limitations of this study are reflected in its partially retrospective nature. The availability of samples to be evaluated by PCR analysis was limited by the number of samples initially submitted for histopathology when the case presented or was submitted for postmortem examination. Because this was usually limited to one sample, the control and prospective samples were collected and analyzed in the same manner. We were only able to recruit 12 lymphoma-positive animals; however, the sample size was sufficient to detect a difference, thus ruling out the risk of a type II error, and we were able to conclude, posthoc, that our power was sufficient. It is notable that among the lymphoma-positive cases, there were 12 samples where DNA was extracted from formalin fixed or formalin-fixed

and paraffin-embedded samples. Among these samples, there were seven that were positive for EHV-5. As a result of DNA extraction methodology, test sensitivity was reduced; therefore, it would be appropriate to consider that the actual number of positive cases may have actually exceeded the reported 64%.

Investigation into the association of EHV-5 and equine lymphoma may lead to further treatment options, including long-term antiviral therapy. Two recent case reports [21,22] of human patients with primary effusion lymphoma (associated with HHV-8) that were unresponsive to traditional intensive chemotherapy were finally moved to remission with antiviral therapy. With the discovery of a positive association between EHV-5 and equine lymphoma, the response to potential antiviral therapies can be investigated. This theory led to the attempted treatment in one horse (horse 7), first by complete removal of the lymphomatous lymph node chain (submandibular lymph nodes) and chemotherapy for one month. This resulted in a change in cell type from T-cell rich, B-cell to T-cell lymphoma. Acyclovir was then administered for 4 months [23]. Cytology and histopathology revealed complete remission of the lymphoma [24].

In conclusion, EHV-5 has been found more frequently in lymphoma tissues as compared with control lymph nodes in this population. Additional study is required to determine if this frequency is influenced by region. Quantitative PCR may also allow detection of the level of target DNA in the tissues. Further investigation is also required to determine the in vitro sensitivity of EHV-5 to antiviral agents.

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