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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.