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Case report

Equine Multinodular Pulmonary Fibrosis Associated with Equine Herpesvirus 5 in a Horse in Brazil

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Abstract

This report describes the first diagnosed case of equine multinodular pulmonary fibrosis associated with equine herpesvirus 5 (EHV-5) in a horse in Brazil. A 7-year-old Thoroughbred mare from a stud farm in southern Brazil died after a chronic respiratory disease characterized by tachypnea, respiratory distress, and cough accompanied by weight loss, anorexia, and intermittent fever. Hematological findings included mild neutrophilia, lymphopenia, and hyperfibrinogenemia. At necropsy the cadaver was extremely emaciated and the lungs did not collapse when the rib cage was removed. Significant gross lesions were restricted to the lungs and consisted of numerous, firm, coalescing nodules widely distributed throughout the organ. The nodules were 1-5 cm in diameter, pale tan-white, and sharply demarcated from the scant normal lung parenchyma. Microscopically, the nodules consisted of marked expansion of the interstitium by well-organized, mature collagen and infiltration by lymphocytes, macrophages, neutrophils and occasional eosinophils. Cuboidal epithelial cells lined the alveoli in affected areas and their lumina were filled with a moderate to high number of neutrophils and foamy macrophages, which occasionally displayed oval amphophilic intranuclear inclusion bodies. Nucleic acid sequence analyses of amplicons from polymerase chain reaction assays targeting the viral gBand gHgenes showed closest homology with multiple corresponding sequences of the gB and gH genes of EHV 5 available in GenBank.
Key Words: EHV-5; equine multinodular pulmonary fibrosis; herpesviruses; horse; pneumonia; respiratory.

Case report

Equine multinodular pulmonary fibrosis (EMPF) is a chronic infectious disease characterized by interstitial fibrosis of the lung (1, 20, 22). It was originally reported in the United States (20) and has recently been documented in European countries (7, 13, 15, 17). The etiology of EMPF is associated with the infection by equine herpersvirus 5 (EHV-5) (20, 21). Affected horses tend to be middle aged or older, although a few cases involved young horses (1). The main clinical signs described in reports of the disease include lethargy, fever, weight loss, respiratory distress, and tachycardia (20, 21). Besides a few published anecdotal reports of individual horses recovering from EMPF, the disease usually has a fatal outcome (7). This study describes the first case of EMPF diagnosed in Brazil and, to the best of our knowledge, the first case of EMPF in South America.

The affected animal was a 7-year-old Thoroughbred mare from a stud farm located in southern Brazil (31° 19' 51" South 54° 06' 25" West). The mare died approximately one month after presenting weight loss, anorexia, intermittent fever (with peaks of temperature at dusk through the first hours of the evening), tachypnea, respiratory distress, and occasional episodes of

cough. On auscultation, the normal lung sounds were altered, indicating impaired inhalation. In the two weeks prior to death the mare lost approximately 100 kg. Hematological findings included mild neutrophilia (10,648 neutrophils/mm3; reference values: 2,260-8,580/mm3) with left shift (605 bands/mm3; reference values: 0-100/mm3), lymphopenia (726 lymphocytes/mm3; reference values: 1,500-7,700/mm3), and hyperfibrinogenemia (1,600 mg/dL; reference values: 100-400 mg/dL). Therapy with steroidal and non-steroidal inflammatory drugs did not improve the clinical disease.

At necropsy the cadaver was extremely emaciated and the lungs did not collapse when the rib cage was removed. The main gross lesions were restricted to the lungs and consisted of numerous, firm coalescing nodules widely distributed throughout the lung. The nodules ranged from 1 to 5 cm in diameter were pale tan/white, and sharply demarcated from the scant normal lung parenchyma (Figs. 1 and 2). On cut surface, the nodules were of uniform color, and slightly bulged from the surrounding lung tissue. Bronchial lymph nodes were enlarged.

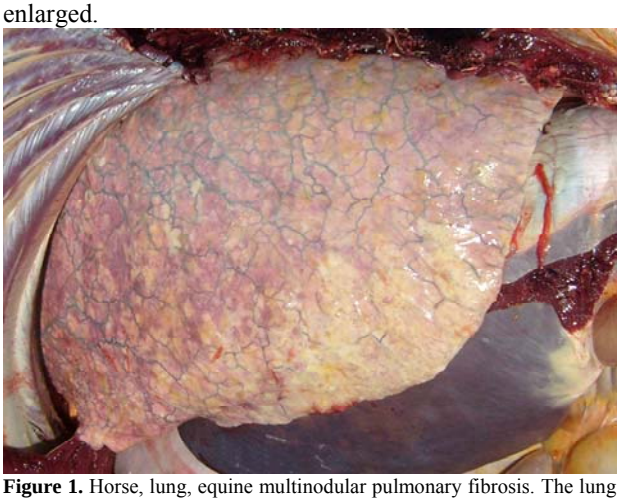

Figure 1. Horse, lung, equine multinodular pulmonary fibrosis. The lung is not collapsed and has an irregular pleural surface due to multifocal, pale tan/white nodules disseminated throughout the parenchyma.

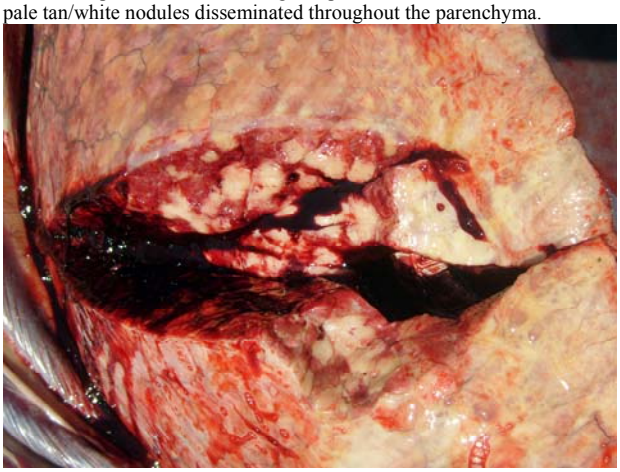

Figure 2. Horse, lung, equine multinodular pulmonary fibrosis. Cut section of lung that demonstrates the multifocal distribution and discreteness of nodules.

Histopathologically, the nodules were characterized by marked expansion of the interstitium by a large amount of well-organized mature collagen along with moderate infiltration of lymphocytes, macrophages, neutrophils and occasional eosinophils. The alveoli within nodular areas were lined by cuboidal epithelial cells and their lumina were filled with a moderate to high number of neutrophils and foamy macrophages. Occasionally, large intra-alveolar macrophages displayed oval and amphophilic intranuclear inclusion bodies (Fig. 3) that morphologically compatible with herpesviral inclusions.

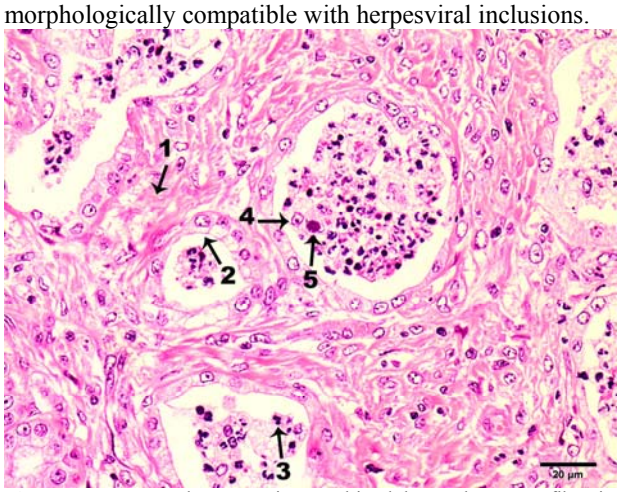

Figure 3. Horse, lung, equine multinodular pulmonary fibrosis. Histopathologically, the nodules consisted of marked interstitial expansion by collagen-rich connective tissue (1). Cuboidal epithelial cells line the alveoli (2), which have their lumina filled with a moderate to high number of neutrophils (3), and foamy macrophages (4). A large macrophage displays an oval amphophilic intranuclear inclusion body (5). Bar = 20 µm.

DNA was extracted from 3 sections (5 µm thick) of paraffin embedded lung using a commercial DNA extraction kit[a] with minor modifications of the kit instructions. The sections of lung were placed in a 1.5 ml sterile microcentrifuge tube, then 180 µl of ATL buffer and 20 µl of proteinase K (both reagents supplied in the kit) were added to the tube. The tube was incubated at 56°C for 3 to 20 hours until the paraffin melted and the embedded tissue was completely lysed. The tissue lysate was aspirated from under the melted paraffin and the lysate was transferred into a sterile microcentrifuge tube. Then 200 µl of AL buffer (supplied in the kit) was added to the lysate, the contents of the tube were mixed using a vortex for 15 sec; and 200 µl of 100% ethanol was added to the tube. The remainder of the extraction procedure followed the kit manufacturer's instructions until the final DNA elution step, which was done using 50 µl of AE buffer (supplied in the kit).

Two PCR assays were performed, which amplified either a 344 bp segment of the glycoprotein H (gH) gene or a 155 bp segment of the glycoprotein B (gB) gene from EHV 5. The PCR primers for the gH gene were forward 5'-TAACCTCCGCGACACGTTTTCA-3' and reverse 5'-TAGACATCACCGCAGAAACCACAA-3',

and the PCR primers for the gB gene were forward 5'-TGATATGACGGCCAGATCACAC-3' and reverse 5'-CCAACCCCACACCATAGTCT -3'. The reaction mixture consisted of 3 µl DNA that had been extracted from paraffin embedded tissue, 12.5 µl of a commercial PCR mastermix reagent[b], 8.9 µl molecular biology grade water, and 0.3 µl each (25 pmole per µl) of forward and reverse PCR primers. The reaction conditions were 1 cycle of 94° C for 4 min; 5 cycles of touch down at 95° C for 30 sec, 65° C (-1 °C per cycle) for 30 sec, 72° C for 30 sec; 35 cycles of 95° C for 30 sec, 60° C for 30 sec, 72° C for 30 sec; and 1 cycle of 72° C for 5 min. The PCR amplicons were visualized in ethidium bromide-stained 1.5% agarose gels after electrophoresis in sodium borate buffer (5mM disodium borate decahydrate, pH adjusted to 8.5 with boric acid) (3). PCR products of the appropriate size were excised from the gel, purified using a commercial gel extraction kit[c], and eluted in 30 µl of molecular biology grade water. The eluted DNA was sent to the Research Technology Support Facility, Michigan State University where it was sequenced in each direction using the aforementioned PCR primers. The sequences were edited with a sequence editing software[d], trimmed to remove primer sites, and analyzed with BLAST (Basic Local Alignment Search Tool), available through the National Center for Biotechnology Information.

Nucleic acid sequence analyses showed the amplification product from the gB gene was most closely related (96% to 100% similarity) to multiple sequences available for EHV 5 in GenBank. The nucleic acid sequence from the gB gene of the EHV 5 analyzed in the current study was identical to the North America isolate SJ of EHV-5 (KC715731). The nucleic acid sequence from the gH gene was most closely related at 99%, 81% and 78% similarity to nucleic acid sequences available in GenBank for EHV 5 submissions DQ504440, GQ325594, and GQ325595; respectively. Additionally, the nucleic acid sequence from the gH gene of the EHV 5 analyzed in the current study was identical to the sequences of three North American EHV 5 analyzed at the Diagnostic Center for Population and Animal Health, Michigan State University (unpublished data). The nucleic acid sequence from the gH and gB genes only showed less than 76% similarity to corresponding sequences of Equine herpesvirus 2, another gammaherpesvirus commonly found in equine.

The diagnosis of EMPF in this case was based on clinical signs, characteristic gross and histopathological findings, and the detection by PCR of nucleic acid sequence from the gBand gHgenes of the EHV 5 (12, 13, 15, 17, 20, 22). Although there is no clear demonstrable predilection of EMPF for either sex or breed, Thoroughbreds seem to be at a higher risk (15, 20, 22). The hyperfibrinogenemia, neutrophilia, and lymphopenia seen in this study have also been described as clinical pathologic findings by other authors (8, 12, 22). The relative low number of cases of EMPF precludes an association of these laboratory findings with an ante mortem diagnosis of the disease (12). The direct role of viral infection in the development of these hematological abnormalities is unclear. Studies using human mesenchymal stem cells demonstrate that cytomegalovirus directly causes an immune-mediated destruction of mature cells in the peripheral circulation or hematopoietic precursors in the bone marrow and leading to cytopenia (14).

Two patterns of gross lesions are described in the lung of horses with EMPF (20): numerous coalescing pale tan-white 1-5 cm in diameter nodules of fibrosis, and a less common presentation of multiple discrete larger nodules (up to 10 cm in diameter) separated by grossly unaffected lung. Our case could be included in the former. Usually, macroscopic lesions are confined to the lungs, but bronchial, (13, 17, 20) and occasionally retropharyngeal lymph nodes, may be enlarged (17).

The involvement of EHV-5 with the lung lesion in this case was confirmed by PCR technique. The use of PCR for detection of viral DNA consists of one of the most useful diagnostic methods for EMPF. The use of this technique in either fresh or paraffin embedded lung tissues yielded herpesviral DNA polymerase gene in approximately 80% of affected horses and in only 8% of the control horses (13, 15, 17, 20, 22).

EMPF has been experimentally induced by inoculation of EHV-5 in the lungs of healthy horses and the fibrosis apparently occurs while the virus is latent within the lung (21).

The mechanism of herpesvirus-induced fibrosis is unknown, however, a murine gamma-herpesvirus has been shown to induce fibrosis in mice in the presence a co-factor (bleomycin) (10). In humans with idiopathic pulmonary fibrosis, a T helper 2 (Th2)-like immune response is implicated in the pathogenesis that leads to fibrosis (18). Activated Th cells can produce many different cytokines and the modulatory properties of herpesviruses might induce a cytokine response that drives the lung towards development of chronic pulmonary lung fibrosis (2, 11). Changes in the immune response profile of horses with EMPF have not been described yet. The infrequent occurrence of EMPF or any other form of clinically important pulmonary fibrosis in horses suggests that specific and rare conditions are involved in the induction of significant fibrosis in this species (7).

There are similarities between the infection of Gammaherpesvirinae in other species and EHV-5 in horses. Gammaherpesviruses are associated with both pulmonary fibrosis and neoplasia. A relationship between Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus is suspected in humans (9). EHV-5 has been identified by PCR in six out of seven horses diagnosed with lymphoma; one of the lymphoma bearing horses also developed EMPF. In the lymphoid tissue of 20 control cases, EHV-5 could only be identified in 15% of cases (16).

Pulmonary interstitial fibrosis is an uncommon disease in horses and has often been described as being idiopathic or caused by pathogens other than EHV-5 (4, 5, 6, 19). There is no available data of EHV-5 infection in Brazil, but it is possible that similar lesions in the lung in the past could have gone misdiagnosed due to unawareness of EMPF and the unavailability of current molecular diagnostic methods.

This is the first report of EMPF in Brazil. As EMPF is being increasingly recognized in other countries, additional cases are likely to be reported from other regions of Brazil in the future. EMPF should be included in the differential diagnosis list of cases of horses with anorexia accompanied by respiratory distress, persistent or recurrent pyrexia, hyperfibrinogenemia, neutrophilia, and lymphopenia.

Source and manufactures

a. DNeasy® Blood & Tissue Kit (QiagenInc, Valencia, CA);
b. GoTaq® Green Master Mix (Promega, Madison, WI);
c. QIAquick Gel Extraction Kit (Qiagen, Valencia, CA);
d. Sequencher software (Gene Codes Corp, Ann Arbor, MI);

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