Association of two newly recognized herpesviruses with interstitial pneumonia in donkeys (*Equus asinus*)

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Abstract.   Over a period of 6 years, antemortem and postmortem examinations were performed on a number of donkeys suffering from respiratory disease. For many cases, initial diagnostic efforts failed to identify an etiology consistent with the pathologic findings. However, retrospective examination of these cases using consensus primer polymerase chain reaction, designed to recognize herpesviruses from all 3 subfamilies of the Herpesviridae, amplified a fragment of the highly conserved herpesvirus DNA polymerase gene from a number of these animals. Two novel herpesviruses, herein designated asinine herpesvirus 4 (AHV4) and asinine herpesvirus 5 (AHV5), were consistently detected in lung tissue from donkeys in which the histopathology was characterized by interstitial pneumonia and marked syncytial cell formation but not in lung tissue from donkeys with evidence of bacterial or verminous pneumonia. Nucleotide sequence and phylogenetic analysis places these new viruses within the Gammaherpesvirinae subfamily and indicates that they are most closely related to the recently identified zebra herpesvirus and wildass herpesvirus as well as equine herpesviruses 2 and 5.

Respiratory pathogens are important causes of disease in equine species worldwide. Although multiple etiologies have been identified, viral pathogens are thought to play an important and often primary role in establishing equine respiratory disease. Potential viral etiologic agents implicated in respiratory disease of horses include equine herpesviruses 1 and 4, influenza A, and equine arteritis virus as well as equine adenovirus and equine rhinovirus.[7,29,30] Recently, members of the Paramyxoviridae family have been added to the list of potential equine respiratory pathogens.[25,32]

The Herpesviridae family of viruses contains a broad range of pathogens that have been grouped into the subfamilies Alpha-, Beta- and Gammaherpesvirinae based on both biologic and genomic characterization. Herpesviruses of all subfamilies can survive and persist in a population of animals due to the propensity of these pathogens to persist, often through latency, in individual hosts.[36] In latently infected hosts, virus may be periodically reactivated and shed. Latency is likely to be an important mechanism that has allowed herpesviruses to be perpetuated, even in small isolated host groups. A number of herpesviruses have been isolated or detected from a range of equine species, including donkeys. The most important of these pathogens are the alphaherpesviruses equine herpesviruses 1 and 4, which cause significant reproductive losses and respiratory disease worldwide.[24] Equine herpesviruses 2 and 5, members of the subfamily Gammaherpesvirinae,[41] are also widespread in the equine population.[24] However, the role of gammaherpesviruses as primary pathogens in horses is less clear.

Development of molecular diagnostic techniques has provided a new approach for the detection of a number of pathogens. For diseases caused by viral pathogens, molecular diagnostics are not dependent on the development of cell-culture systems for isolation and characterization of novel pathogens. Rather, molecular diagnostic assays can be designed based on regions of highly conserved DNA sequences that are present within a number of viral families. In this article, consensus primer polymerase chain reaction (PCR)[43] designed to recognize herpesviruses from all 3 subfamilies of the Herpesviridae was used to detect 2 novel herpesviruses in tissues from a number of donkeys suffering from interstitial pneumonia that was histologically characterized by marked syncytial cell formation. Phylogenetic analysis of these novel sequences places these newly recognized viruses within the Gammaherpesvirinae subfamily and indicates that they are most closely related to other gammaherpesviruses of equids.

Materials and methods

Cases. Between 1995 and 2000, 17 donkeys were submitted to the University of Missouri Veterinary Medical Teaching Hospital and/or the Veterinary Medical Diagnostic Laboratory for antemortem or necropsy examination from a total of 6 different premises in Missouri. All but 1 of the donkeys died or were euthanized. Moderate to severe respi-

Table 1. Oligonucleotide primers used for PCR amplification of the DNA polymerase gene fragment.

Assay and primer	Direction	Sequence (5′ to 3′)	Expected size of product
First round			
DFA	forward	GAYTTYGCNAGYYTNTAYCC	...
ILK	forward	TCCTGGACAAGCAGCARNYSGCNMTNAA	470 bp (ILK + KG1)
KG1	reverse	GTCTTGCTCACCAGNTCNACNCCYTT	725 bp (DFA + KG1)
Second round			
TGV	forward	TGTAACTCGGTGTAYGGNTTYACNGGNGT	...
IYG	reverse	CACAGAGTCCGTRTCNCCRTADAT	225 bp (TGV + IYG)

ratory disease was a prominent aspect of the clinical history for several animals, although some cases were submitted for diagnosis of unknown and/or unspecified disease or for diagnosis of a disease condition not related to the pulmonary system.

Virus isolation. Tissue homogenates of lung were prepared by mincing approximately 2–4 g tissue in 12–15 ml of Dulbecco's Modified Eagle Medium cell culture media containing 0.5 mg/ml gentamycin and 2.5 μg/ml amphotericin B. Homogenates were centrifuged at $6,000 \times g$ for 20 min, then filtered through a 0.45-μm filter. Filtered homogenates were adsorbed onto vero, rabbit kidney-13 (RK-13) or primary equine dermal cells for 60 min at 37 C, 5% CO_2 in a humidified chamber, after which the inoculum was removed and replaced with growth media. Cells were maintained under standard conditions for a minimum of 10 days and observed daily for the presence of viral-induced cytopathic effect.

Nucleic acid extraction and PCR amplification. Lung tissue collected at necropsy was homogenized in phosphate buffered saline (PBS) (pH 7.4; 2–4 g lung tissue per 5 ml). One milliliter of this homogenate was centrifuged at $12,000 \times g$ for 1 min, and the cell pellet was used for DNA extraction. Formalin-fixed, paraffin-embedded tissue sections of lung 2 sections per paraffin block, 20 μm thick for each section) were deparaffinized by 2 extractions with xylene, followed by centrifugation. Tissue pellets were then washed twice with 100% ethanol and dried for 10 min at room temperature. For DNA extraction from both fresh tissue and paraffin-embedded tissue sections, samples were digested with Proteinase K[a] in Buffer ATL[a] for a minimum of 12 hours at 55 C. The DNA extractions were performed using the QIAamp DNA mini kit[a] according to the manufacturer's instructions. For each of the DNA extraction steps, strict protocols were followed to avoid cross-contamination of samples. The DNA was stored at −80 C until used as the template for PCR amplification.

Amplification of a region of the herpesvirus DNA polymerase gene was performed with nested degenerate primers, shown in Table 1, targeted to highly conserved regions.[43] First-round amplification was performed using 2 upstream primers (DFA and ILK) and 1 downstream primer (KG1), each at a final concentration of 0.6 μM, in a 20-μl reaction with 0.5 units HotStarTaq[a] in the manufacturer's buffer containing 1.5 mM $MgCl_2$ and 0.2 mM (each) dNTPs. Thermocycling conditions for the first-round amplification were 95 C (12 min), followed by 10 cycles of denaturation (94

C, 30 sec), annealing (70 C, 30 sec), and extension (72 C, 90 sec), with the annealing temperature in these cycles reduced by 2 C each cycle. An additional 40 cycles of denaturation (94 C, 30 sec), annealing (50 C, 30 sec), and extension (72 C, 90 sec) were performed, followed by a final extension at 72 C for 7 min. Second-round amplification was performed using 2 μl of the first-round reaction with 1 upstream primer (TGV) and 1 downstream primer (IYG), each at a final concentration of 0.6 μM, in a 20-μl reaction with 0.5 units HotStarTaq in the manufacturer's buffer containing 1.5 mM $MgCl_2$ and 0.2 mM (each) dNTP. Thermocycling conditions for the second-round amplification were 95 C (12 min), followed by 10 cycles of denaturation (94 C, 30 sec), annealing (70 C, 30 sec), and extension (72 C, 60 sec), with the annealing temperature in these cycles reduced by 2 C each cycle. An additional 40 cycles of denaturation (94 C, 30 sec), annealing (50 C, 30 sec), and extension (72 C, 60 sec) were performed, followed by a final extension at 72 C for 7 min. For each step in DNA amplification, strict protocols were followed to prevent cross-contamination of samples. Second-round amplification products were visualized in a 2% agarose, $1 \times$ TAE gel by ethidium bromide staining and ultraviolet transillumination.[38]

Amplification products of the appropriate size (approximately 225 bp) were excised from the gel and purified with the Qiaex II gel extraction kit.[a] Purified products were either sequenced directly using the herpesvirus IYG primer or cloned using the pCR-Blunt TOPO II system.[b] When amplified fragments were sequenced directly, a minimum of 3 sequencing reactions was performed for each product. When amplified fragments were cloned, a minimum of 3 clones was sequenced using both the universal forward and reverse sequencing primers. Nucleotide sequencing was performed by the DNA Core facility, University of Missouri–Columbia. Nucleotide sequence analysis was performed with GeneTool, version 1.0 C, and BLAST.[2] The herpesviral DNA polymerase sequences obtained in this study have been deposited in the National Center for Biotechnology Information database and have been assigned GenBank accession numbers AY054990 (AHV4-1), AY054991 (AHV4-2), AY054992 (AHV4-3), AY054993 (AHV5-1), AY054994 (AHV5-2), AY054995 (AHV5-3), and AY054996 (AHV5a). For the animals from which no herpesvirus PCR product was detected, DNA was extracted and amplified as described above from a total of 3 independent samples of tissue.

Alignments and phylogenetic analysis. DNA and deduced amino acid sequences of amplified herpesvirus PCR prod-
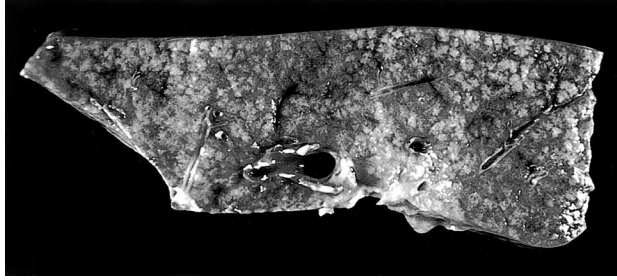
Figure 1. Section of lung from a donkey with interstitial pneumonia. The cut surface of the lung contains numerous military pale foci. The entire lung was affected.

ucts, excluding the primer sequences, were aligned with CLUSTAL W (1.74) software. Sequences for comparison were obtained from GenBank and corresponded to amino acid positions 691–745 of the DNA polymerase protein (or nucleotides 37,158–37,323 of the DNA polymerase protein gene) from equine herpesvirus 2 (GenBank accession no. NC_001650). Phylogenetic analyses of DNA and amino acid alignments were performed using maximum parsimony methods (DNAPARS or PROTPARS) and distance matrix methods (DNADIST or PROTDIST followed by NEIGHBOR) within the PHYLIP software package.[16] Data sets were subjected to bootstrap analysis,[15] based on 100 resamplings of the original data set, using the SEQBOOT program to produce a majority-rule consensus tree. Completed tree files were visualized using TreeView 1.5.[27]

Results

Gross lesions and histopathology. Gross necropsy examination of the pulmonary system for cases that were subsequently PCR positive for herpesvirus typically revealed lungs that failed to collapse and contained patchy areas of grey, tan, or reddish discoloration predominantly located in anterior ventral lobes. Gross necropsy examination in one of these animals revealed diffusely edematous lungs that failed to collapse and were diffusely mottled pink to light tan. The
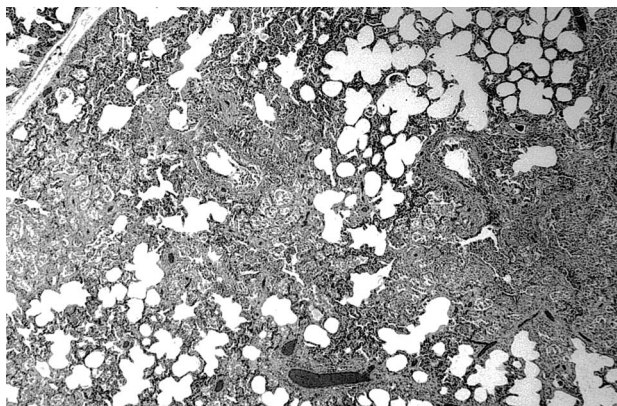

Figure 3. Bronchiolar epithelium is denuded and the lumen contains sloughed epithelium, macrophages, and syncytial cells. There is mild to moderate fibrosis of the adjacent interstitium. HE.

cut surface of this lung contained numerous, multifocal to coalescing, 1–3-mm in-diameter tan nodules scattered throughout the parenchyma (Fig. 1). Greater than 90% of the lung was affected in this case.

Histologic findings in cases that were subsequently PCR positive for herpesvirus ranged from mild, lymphohistiocytic, and suppurative bronchiolitis with peribronchiolar interstitial inflammatory cell infiltrates to lungs that contained marked, diffuse accumulations of histiocytes and neutrophils with areas of interstitial fibrosis and necrotizing bronchiolitis (Figs. 2, 3). Bronchioles often contained abundant mucus mixed with neutrophils and cell debris. All herpesviral PCR-positive cases had syncytial cells that contained up to 20 visible nuclei (Fig. 4). In most severely affected cases, alveolar spaces were obliterated by accumulations of histiocytes, neutrophils, edema, and fibrin. Hypertrophic alveolar epithelium lined affected alveoli. Viral inclusions were not observed.

Control cases (6 animals) were from additional donkeys, often originating from the same premises, with

Figure 2. Photomicrograph of lung from Fig. 1. There are multifocal to coalescing accumulations of mononuclear inflammatory cells that obliterate and fill alveolar spaces. HE.
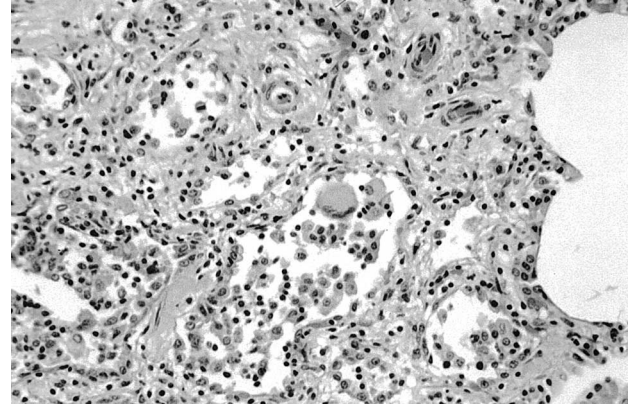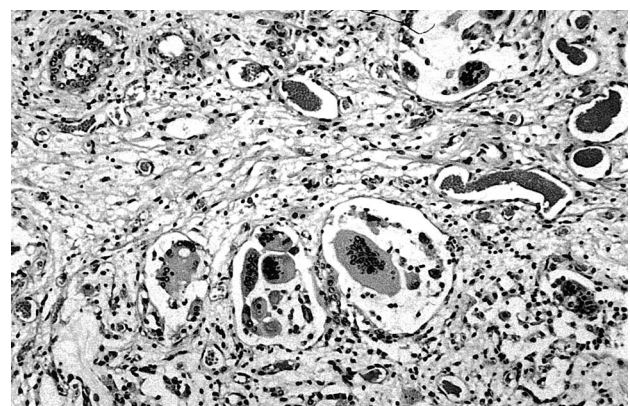

Figure 4. Areas of marked interstitial fibrosis that contained numerous syncytial cells were typical of the lesions seen in affected donkeys. HE.

respiratory disease that was histologically consistent with either a bacterial or verminous pneumonia. Cases that were interpreted as principally bacterial pneumonia contained marked, diffuse accumulations of neutrophils, often associated with areas of coagulative necrosis. Cases of verminous pneumonia were characterized by the presence of nematode larvae in alveolar spaces and peribronchilar accumulations of lymphocytes and eosinophils. Herpesviral genomic DNA was not detected in lung samples from any donkeys with either bacterial or verminous pneumonia.

Virus isolation and bacteriology. Virus isolation was attempted on lung homogenates from 7 animals (5 of which were subsequently herpesvirus PCR positive and 2 of which were PCR negative) using both cultured mammalian cells and embryonated chicken eggs. No cytopathic agents were detected for any of the samples tested. Additionally, each lung homogenate prepared for virus isolation was tested for influenza A antigen using a commercially available ELISA[d] and found to be negative. Bacterial isolation was attempted on lung homogenates from 4 animals (all of which were subsequently herpesvirus PCR positive). No bacteria were recovered from 1 animal, *Streptococcus equi* subsp. *zooepidemicus* was recovered from 1 animal, both *Streptococcus equi* subspecies *zooepidemicus* and *Staphylococcus aureus* were recovered from 1 animal, and both *Staphylococcus aureus* and *Mycoplasma* spp. were recovered from 1 animal.

PCR amplification and sequence analysis. Retrospective examination of previous cases was performed with DNA either held at −80 C or extracted from formalin-fixed, paraffin-embedded tissue blocks, some of which had been stored at room temperature for up to 6 years. Amplification products were detected following consensus primer PCR for herpesvirus in samples from 11 of the 17 donkeys from which lung tissue was tested. All animals that were herpesvirus PCR positive had evidence of interstitial pneumonia with syncytial cell formation as a prominent component of the lung histopathology. Herpesvirus genomic DNA was not detected in lung samples from the control group, which had evidence of either a verminous or bacterial pneumonia.

Nucleotide sequencing of the herpesvirus PCR products identified putative genomic DNA of 2 novel but closely related herpesviruses, herein designated asinine herpesvirus 4 (AHV4) and asinine herpesvirus 5 (AHV5). The AHV4 was detected in lung samples from 5 donkeys. Three single-base substitutions were found within the sequences identified as AHV4, and the nucleotide sequence from these samples have been designated AHV4-1, AHV4-2, and AHV4-3 (Fig. 5A). AHV4-1 had a thymidine substituted for adenine at position 34, and AHV4-2 had 2 adenine residues substituted for cytosine and guanine at positions 13 and 16, respectively. However, each of the substitutions in AHV4-1 and AHV4-2 were translationally silent, and thus a single deduced amino acid sequence is shown for AHV4 in Fig. 5B. The AHV5 was detected in lung samples from 6 donkeys. Three single-base substitutions were found within the sequences identified as AHV5, and the nucleotide sequence from these samples have been designated AHV5-1, AHV5-2, AHV5-3, and AHV5a (Fig. 5A). The AHV5-2 had a thymidine substituted for cytosine at position 130, and AHV5-3 had adenine substituted for guanine at position 67. These 2 substitutions were translationally silent. The AHV5a had a thymidine substituted for cytosine at position 117, resulting in a semiconserved amino acid change (Fig. 5B). Compared with each other, AHV4 and AHV5 (and AHV5a) were 84% identical at the amino acid level and 88% identical at the nucleotide level (Tables 2, 3), with the most important difference being a 3-nucleotide insertion in AHV5 and AHV5a (positions 127–129) relative to AHV4, resulting in a single amino acid addition.

Pairwise alignment of both the nucleotide and deduced amino acid sequences to other herpesvirus sequences indicated that AHV4 and AHV5 were most closely related to zebra herpesvirus (ZHV) and wildass herpesvirus (WAHV), respectively[12] (Fig. 5A, 5B; Tables 2, 3). Two amino acid substitutions were present in AHV4 compared with ZHV; however, both of the substitutions were conserved changes. The most significant difference between AHV5 and AHV5a compared with WAHV was a 3-base insertion found in WAHV (bases 132–134), which resulted in a single amino acid addition. Pairwise alignments also demonstrated a reasonably high level of similarity to EHV5 and a slightly lower degree of similarity to EHV2, 2 widely recognized gammaherpesviruses of equids (Fig. 5A, 5B; Tables 2, 3).

Phylogenetic trees were constructed based on amino acid sequence alignments of known, representative alpha-, beta-, and gammaherpesvirus DNA polymerase proteins. The trees produced using both parsimony and distance methods were very similar. The known herpesvirus DNA polymerase sequences clustered into the predicted herpesvirus subfamilies (Fig. 6), as has been demonstrated by others using similar phylogenetic methodologies.[13,14,23,37,43] Inclusion of AHV4-, AHV5-, and AHV5a- deduced amino acid sequences in this phylogenetic analysis demonstrated that these viruses are members of the Gammaherpesvirinae subfamily and that AHV4, AHV5, and AHV5a are distinct from, but most closely related to, other gammaherpesviruses of equids.

A)

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                   10        20        30        40        50        60        70        80        90       100
                    *         *         *         *         *         *         *         *         *         *
EHV5       GGCCTCGGGCATCCTGCCGTGCCTAAAGATAGCAGAGACGGTCACCTTTCAGGGGAGGAGGATGCTAGAGAGGTCCAAGCGCTACATAGAGGCCGTGACC
AHV4-1*    ...................C.....C......T........G...........C...........G.............A.G...........G......
AHV4-2     ...........A..A..C.....C.................G...........C...........G.............A.G...........G......
AHV4-3†    ...................C.....C.................G...........C...........G.............A.G...........G......
ZHV        ...................C.....C.............C..G.....C.....C...........G......................G......
WAHV       ...................C.....C........C.....C..G...........C...........G......................G......
AHV5-1‡    ...................C.....C........C...C..G..C...........G......................G......
AHV5-2     ...................C.....C........C...C..G..C...........G......................G......
AHV5-3     ...................C.....C........C...C..G..C...........G......................G......
AHV5a      ...................C.....C........C...C..G..C...........G......................G......
EHV2       ...................C..T..C.......C...............C.....C...C.C.....G....AC..................GG......

                  110       120       130       140       150       160
                    *         *         *         *         *         *
EHV5       CCCGAGGGGTTGGCTGCCATCTTGCA---GAGGCCCGTGGCGGCCTGCGACCCCGAGGCCAGCTTTAAGGTC 169
AHV4-1     ..G......C....C.............---C......---CC.T........C...G..C.............. 166
AHV4-2     ..G......C....C.............---C......---CC.T........C...G..C.............. 166
AHV4-3     ..G......C....C.............---C......---CC.T........C...G..C.............. 166
ZHV        .........C.C..G......C...G---C.......---CC.T........CT..................... 166
WAHV       .........C.C..G......C..GGGGGCC...GGCCC.T................................. 172
AHV5-1     .........C.C..G......C..GGGGGCC...---CC.T................................. 169
AHV5-2     .........C.C..G......C..GGGGGTC...---CC.T................................. 169
AHV5-3     .........C.C..G......C..GGGGGCC...---CC.T................................. 169
AHV5a      .........C.C..G.T....C..GGGGGCC...---CC.T................................. 169
EHV2       .........C....A.A...A...GG---CA...---G..T..AG....C......T.....T......... 166
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B)

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                   10        20        30        40        50
                    *         *         *         *         *
EHV5       ASGILPCLKIAETVTFQGRRMLERSKRYIEAVTPEGLAAILQR-PVAACDPEASFKV 56
AHV4§      ..........................................H.-...-.A.D..... 55
ZHV        ..........................................R.-...-.A....... 55
WAHV       ......................................GGRRPV.......... 57
AHV5‖      ..........................................GGR-PV......... 56
AHV5a      .......................................V..GGR-PV......... 56
EHV2       ......................N......G.......D..G-R-RVE.A.D..... 55
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Figure 5. A, Alignment of the nucleotide sequences from the herpesvirus DNA polymerase gene. Only nucleotides that differ from the sequence of EHV5 are shown. Identical bases are shown by a "." and gaps are shown by a "-". Sequences for EHV5, ZHV, WAHV, and EHV2 were obtained from GenBank. * † sequence amplified from 2 donkeys; ‡ sequence amplified from 3 donkeys. **B**, Alignment of the deduced amino acid sequences of the same region of the herpesvirus DNA polymerase gene shown above. § deduced amino acid sequence from amplification products of 5 donkeys and corresponding to the nucleotide sequences from AHV4-1, -2, and -3. ‖ deduced amino acid sequence from amplification products of 5 donkeys and corresponding to the nucleotide sequences from AHV5-1, -2, and -3.

Discussion

Over a period of 6 years (1995–2000), 17 donkeys originating from 6 different premises within the state of Missouri were presented to the University of Missouri's Veterinary Medical Diagnostic Laboratory or Veterinary Medical Teaching Hospital for diagnosis of disease. A morphological diagnosis of interstitial pneumonia with marked syncytial cell formation was observed in the lungs of 11 of the donkeys. At the time of presentation or necropsy examination, a viral etiology was suspected for these animals, but diagnostic testing failed to identify any etiologic agent consistent with the clinical signs and/or observed gross and histologic lesions of interstitial pneumonia. How-

Table 2. Percent nucleotide identity of the DNA polymerase gene fragment between gammaherpesviruses of equids.

	EHV5	AHV4*	ZHV	WAHV	AHV5†	AHV5a	EHV2
EHV5	100	86.9	86.9	85.4	85.6	84.8	82.2
AHV4	...	100	92.1	87.2	88.7	88.1	85.5
ZHV	...	...	100	91.8	94.6	94.0	86.7
WAHV	...	...	...	100	96.7	96.5	82.5
AHV5	...	...	...	...	100	99.0	85.2
AHV5a	...	...	...	...	...	100	85.2
EHV2	...	...	...	...	...	...	100

* Represents the mean of identity values from AHV4-1, -2, -3.
† Represents the mean of identity values from AVH5-1, -2, -3.

Table 3. Percent amino acid identity of the DNA polymerase gene fragment between gammaherpesviruses of equids.

	EHV5	AHV4*	ZHV	WAHV	AHV5†	AHV5a	EHV2
EHV5	100	92.8	94.6	89.4	89.4	87.7	78.9
AHV4	...	100	96.3	84.2	84.2	82.4	82.4
ZHV	...	...	100	85.9	85.9	84.2	80.7
WAHV	...	...	...	100	98.2	96.4	84.2
AHV5	...	...	...	...	100	98.2	85.7
AHV5a	...	...	...	...	...	100	85.7
EHV2	...	...	...	...	...	...	100

* Represents the mean of identity values from AHV4-1, -2, -3.
† Represents the mean of identity values from AVH5-1, -2, -3.

ever, retrospective use of a PCR assay, designed to amplify a fragment of the highly conserved herpesviral DNA polymerase gene,[43] followed by nucleotide sequencing of the PCR products detected herpesviral genomic DNA in all cases with interstitial pneumonia and syncytial cell formation. Herpesviral genomic DNA was not detected in a similarly aged group of control donkeys that had evidence of either verminous or bacterial pneumonia. Recently, others have used PCR amplification of the highly conserved DNA polymerase gene as a means of detecting novel members of the Herpesviridae family in diseased tissue.[8,12–14,21,22,31,34,37] The ability to perform PCR amplification of pathogens from formalin-fixed, paraffin-embedded tissues[10,17,18,26,42] has provided the opportunity to retrospectively examine samples from cases with similar lesions. In the present study, DNA was extracted and herpesvirus DNA was successfully amplified from formalin-fixed, paraffin-embedded tissue blocks that had been held under ambient conditions for up to 6 years.

Nucleotide sequence and phylogenetic analyses of herpesviral PCR products from these cases demonstrated that 2 novel but closely related herpesviruses had been identified. The 2 putative novel pathogens, designated asinine herpesvirus 4 (AHV4) and asinine herpesvirus 5 (AHV5), were found to be related to other gammaherpesviruses of equids, including equine herpesviruses 2 and 5.[41] Based on both nucleotide and deduced amino acid sequence alignments, the herpesviruses most closely related to AHV4 and AHV5 (and AHV5a) were those detected in peripheral blood mononuclear cells from free-ranging mountain zebras in Namibia and a captive Somali wildass,[12] respectively. The donkeys from which AHV4, AHV5, and AHV5a were detected originated from 5 different premises, all within the state of Missouri. Although previous contact between these individual animals or herdmates cannot be ruled out, the presence of AHV4 and AHV5 in several herds suggests that these viruses may be fairly widespread within donkeys and potentially among other equids. The finding that the 2 most closely related herpesviruses, ZHV and WAHV, were detected from equids located in Africa and Europe, respectively, suggests a worldwide distribution for these related viruses, similar to that described for EHV2 and EHV5.

Isolation of 3 herpesviruses from donkeys has been previously reported.[5] These viruses have been designated asinine herpesviruses 1, 2, and 3. Asinine herpesvirus 1 (AHV1) was isolated from a donkey with vesicular and erosive lesions on the muzzle of a foal and the external genitalia and udder of the dam. While these lesions are similar to those caused by the alphaherpesvirus EHV3, restriction endonuclease and Southern blot hybridization analysis demonstrated that, although AHV 1 was related to EHV3, it was genetically distinct. Asinine herpesvirus 2 (AHV2) was isolated from the blood of an apparently healthy donkey, and similar analysis found that it was most closely related to the gammaherpesviruses EHV2 and EHV5, although the overall levels of similarity were estimated by Southern blot hybridization to be relatively low. Asinine herpesvirus 3 (AHV3) was isolated from healthy donkeys only after treatment with steroids, suggesting that the donkeys had been latently rather than actively infected. The AHV3 demonstrated considerable sequence similarity to the alphaherpesviruses EHV1 and, to a lesser extent, EHV4. Experimental inoculation of AHV3 into juvenile donkeys that were seronegative for EHV1 and EHV4 resulted in a transient afebrile rhinitis. Comparison of the restriction endonuclease digestion patterns of AHV1, AHV2, and AHV3 viral genomic DNA has demonstrated that each of these asinine herpesviruses is distinct from each other as well as from other herpesviruses. Based on the similarities found in Southern blot hybridizations, it is likely that AHV1 and AHV3 are members of the Alphaherpesvirinae subfamily while AHV2 is a member of the Gammaherpesvirinae subfamily. Unfortunately, however, nucleotide sequence data have not been reported for AHV1, AHV2, or AHV3, and thus their phylogenetic relationship to other herpesviruses, as well as to those detected in the present study, cannot be assessed at this time.

The role of gammaherpesviruses in equine disease has not been well established. Equine herpesvirus 2 (EHV2) has been linked to respiratory disease, conjunctivitis, and general malaise in horses.[4,9,19,20,28,39–41] However, infection with EHV2 appears to be widespread, and several studies have also found EHV2 in the respiratory tract and peripheral blood leukocytes of horses without clinical disease, with isolation and/or PCR detection rates ranging from 31 to 89%.[4,19,33,35,39] Following experimental inoculation of EHV2 into both juvenile and adult horses, chronic pharyngitis was observed in the young horses while the adult horses remained clinically normal.[3] As with other gammaherpesviruses, latency in lymphocytes has been demonstrated for EHV2.[11] A second gammaherpesvirus of horses, EHV5, has been isolated but is less well characterized and has not yet been associated with disease.[1,6,41] Like EHV2, EHV5 also appears to be widespread, although somewhat less prevalent.[33] For the 2 recently identified gammaherpesviruses of equids ZHV and WAHV, no disease symptoms were reported in the animals from which these viruses were detected.[12] For animals from which ZHV was identified, blood from a total of 20 free-ranging zebras from Namibia was initially tested and found positive for anti-

bodies to EHV2. Additional testing using PCR and nucleotide sequencing found that 6 of the 20 zebra showed evidence of infection with ZHV. Wildass herpesvirus was detected from a clinically normal Somali wildass housed in a zoo, presumably in Europe. In contrast with the animals from which ZHV and WAHV were detected, the donkeys examined in this report had acute and typically fatal respiratory disease as a prominent feature of the clinical history. Furthermore, at least 2 of the herds from which animals were examined (and were subsequently identified as positive for AHV4 or AHV5) had a history of recent epizootics of fatal respiratory disease among other donkeys in the herd. Unfortunately, additional animals were not consistently submitted for necropsy examination in these epizootics.

In summary, 2 novel putative herpesviral pathogens have been detected in the lungs of donkeys. These viruses are most closely related to other gammaherpesviruses of equids. A correlation was made between the presence of viral genomic DNA and interstitial pneumonia with marked syncytial cell formation. Although the possibility that these animals were latently infected cannot be ruled out, the severity of lesions observed suggests a severe, active clinical viral infection. This article offers evidence for an important role of these gammaherpesviruses in equine respiratory disease. Future efforts will focus on directly linking the presence of these viruses with the lesions observed, through techniques such as in situ hybridization or PCR, and on the isolation of AHV4 and/or AHV5 in cell culture. Successful isolation of these putative pathogens will allow experimental inoculation of naive animals to directly assess the pathogenesis of these viruses in equids.

Sources and manufacturers

a. Qiagen, Inc., Valencia, CA.
b. Invitrogen, Inc., Carlsbad, CA.
c. BioTools, Inc., Oakland, CA.
d. Directigen Flu A, Becton Dickinson Microbiology Systems, Sparks, MD.

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