Characterization of a novel alphaherpesvirus associated with fatal infections of domestic rabbits

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A virus was found to be associated with a severe disease affecting rabbits on a farm near Anchorage, Alaska. Extracts from the skin of infected rabbits produced syncytia and cell lysis in cultured rabbit skin, rabbit kidney, and Vero cells. Examination of the infectious agent by electron microscopy revealed an icosahedral nucleocapsid surrounded by an envelope with a diameter of about 120 nm, suggesting that it was a herpesvirus. The viral genome was determined to be composed of double-stranded DNA of 120–130 kbp. PCR using degenerate primers to conserved herpesvirus genes was used to amplify sequences from purified viral DNA. Sequencing of these products allowed the design of specific primers so that complete sequence data for a number of genes could be determined. Analysis of these data indicated that the virus is most closely related to bovine herpesvirus 2. The next most closely related viruses are human herpesviruses 1 and 2, and a number of cercopithecine herpesviruses. Experimental exposure of domestic rabbits to the isolate resulted in severe clinical disease and necrosis in the spleen and lymph node. In addition, viral DNA was identified in a variety of tissues by PCR, consistent with a systemic infection. Taken together, these data suggest that this virus is highly pathogenic for domestic rabbits and belongs to the family Herpesviridae, subfamily Alphaherpesvirinae, genus Simplexvirus.

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Introduction

Three naturally occurring herpesviruses of rabbits and hares (leporids), called leporid herpesviruses 1, 2, and 3 (LHV-1, LHV-2, and LHV-3), have been identified (Roizman and Pellett, 2001). All three viruses are tentative members of the genus *Rhadinovirus* of the subfamily Gammapoxvirinae, whose members are often associated with lymphoproliferative diseases. The best characterized is LHV-3 (*Herpesvirus sylvilagus*), which is endemic in cottontail rabbits (*Sylvilagus floridanus*) and causes tumor-like lesions in lymph nodes, kidney, spleen, and liver (Hesselton et al., 1988; Hinze, 1971a,b; Spieker and Yuill, 1977; Swan et al., 1991). However, it does not cause disease in domestic rabbits (Cohrs and Rouhandeh, 1987; Dressler et al., 1988; Hesselton et al., 1988; Hinze, 1971a; Kramp et al., 1985). LHV-2 (*Herpesvirus cuniculi*) causes asymptomatic infections of domestic (New Zealand white) rabbits (*Oryctolagus cuniculus*) (Matsumaga and Yamazaki, 1976; Nii and Yasuda, 1975; Zygraich et al., 1972). LHV-1 is found in cottontail rabbits (Hesselton et al., 1988; Schmidt et al., 1992), and no disease has been reported to be associated with it in domestic rabbits. In addition, a herpesvirus infection, reported in wild eastern cottontail rabbits, was characterized by severe bronchiolitis and pneumonia and by the presence of intranuclear inclusion bodies in astrocytes and neurons in the brain (Schmidt et al., 1992). The identity of this virus was not determined.

Two outbreaks of an acute, fatal herpesvirus infection in domestic rabbits were reported in the early 1990s in Alberta and British Columbia, Canada (Onderka et al., 1992; Swan et al., 1991). These outbreaks resulted in high morbidity and mortality and were linked to infection by an uncharacterized herpesvirus. Lesions associated with the disease included hemorrhagic dermatitis on the face and back, interstitial pneumonia, and severe splenic necrosis. This fatal disease was reproduced in domestic rabbits by intramuscular injection of tissue culture fluid containing virus isolated from one of the outbreaks (Onderka et al., 1992; Swan et al., 1991).

In this report, we describe investigations of an uncharacterized herpesvirus associated with infections of domestic rabbits in Alaska (Jin et al., 2008). In July and August of 2006, mini Rex (a small rabbit breed used primarily as companion animals) and cross-bred New Zealand rabbits (raised primarily for meat), located on a farm about 70 km northeast of Anchorage near Wasilla, Alaska, developed a systemic illness that often began with conjunctivitis. This illness affected over half of the 55 rabbits on the premises, including adults and nursing young, and approximately 16 of the affected rabbits died...
or were euthanized due to severe illness within 7 days of the onset of clinical disease (Jin et al., 2008). The investigation that we describe in this report indicates that the Alaska outbreak was caused by a novel virus that we call leporid herpesvirus 4 (LHV-4). We recommend that this virus should be classified as a new member of the family Herpesviridae, subfamily Alphaherpesvirinae, genus Simplexvirus.

Results

Virus isolation and cytopathic effects

LHV-4 was obtained from infected rabbit skin tissue and plaque purified (Jin et al., 2008) and used to infect rabbit skin (RS) and Vero (monkey kidney) cells. By 24 h post-infection [hpi], cytopathic effects

(CPE) such as syncytium formation, cell enlargement, and cell lysis was observed in both cell lines (Fig. 1) and became more extensive as the infection progressed. The CPE was similar to that of herpesviruses such as human herpesvirus type 1 (HHV-1). However, syncytium formation in RS cells (Fig. 1B) was more extensive than that in Vero cells (Fig. 1D). In addition, the plaque size in RS cells was 2 to 3 times larger than that of HHV-1 (Fig. 2).

Growth curve analysis

In order to analyze further the infectivity of LHV-4 in vitro, growth curve analyses were performed in rabbit kidney (RK), RS, and Vero cells and were compared to concurrent growth curves of HHV-1 (Fig. 3). The highest titers of LHV-4 \(1 \times 10^7\) plaque-forming units (PFU/ml) were produced in Vero cells; although, the infection was delayed compared to HHV-1. In contrast, the yield of LHV-4 was 2–3 logs lower than that of HHV-1 in RK and RS cells, with the titer particularly reduced in RK cells. Differing kinetics of replication were also evident. The LHV-4 titer peaked at around 2 days post-infection (dpi) in RS and RK cells, but at 3 dpi in Vero cells.

Electron microscopy

To compare the morphology of LHV-4 with that of HHV-1, purified virions were subjected to negative staining and examined by electron microscopy (Fig. 4). LHV-4 core particles were similar in size and structure to those of HHV-1. In addition, the viral capsid contained a relatively small, asymmetrical, electron-dense region, which probably
represents the condensed DNA core. Many of the capsids observed were surrounded by a tightly fitting envelope (solid arrow, Fig. 4D), and some were surrounded by a looser structure, which may represent a ruptured envelope (Fig. 4B–C, open arrow). To confirm the presence of an envelope structure, virions isolated from cell culture media were subjected to treatment with a detergent (0.1% Triton X-100), which removes the envelope of herpesviruses and causes a reduction in titer (Davison, 1992; Hersberger et al., 2004; Prince et al., 2000). This treatment reduced the titer of LHV-4 from 1×10⁵ PFU/ml to undetectable levels (Fig. 5), a result that is consistent with the presence of an envelope in the virion. However, this treatment did not affect infectivity of the non-enveloped virus, Bovine Adenovirus 3 (BAdV-3) (Fig. 5), which is consistent with a non-enveloped virus.

Virus genome characterization and phylogeny

Initial characterization of nucleic acid extracted from purified LHV-4 virions by restriction enzyme digestion suggested the presence of a relatively large, double-stranded DNA genome not closely related to that of HHV-1 (Jin et al., 2008). To further examine the size of the genome, field inversion gel electrophoresis (FIGE) was employed. A genome size of 120–130 kbp was observed (data not shown).

To examine the sequence of LHV-4, degenerate PCR primers were selected based on one of the most highly conserved genes in the alphaherpesviruses that encodes the large subunit of ribonucleotide reductase (RR1). Using degenerate oligomers designed from domains in the RR1 amino acid sequence that are conserved among alphaherpesviruses, a PCR product of the size predicted was amplified. Sequence analysis of this product revealed the presence of the targeted region of the RR1 gene. Primers specific to the LHV-4 genome were then designed from this sequence, and additional sequence information was acquired, including the complete RR1 gene, the complete gene encoding the small subunit of ribonucleotide reductase (RR2), and the flanking sequences of about 1300 bp upstream and 600 bp downstream of this gene pair. A diagram of the organization of this region compared to that of HHV-1 and bovine herpesvirus 2 (BoHV-2) is shown in Fig. 6A. As in HHV-1 and BoHV-2, the LHV-4 RR1 and RR2 genes are adjacent and oriented in the same direction, and are located between the genes (for which partial sequences were obtained) encoding orthologs of the virion host shutoff factor (VHS) downstream and the UL38 capsid protein upstream (Fig. 6A). Although the RR2 subunits are similar in size at about 300 amino acid residues, the RR1 subunits differ significantly in size. The HHV-1 RR1 subunit is substantially longer, at over 1100 residues, than the homologs from LHV-4 and BoHV-2. This difference is located predominantly in the N-terminal region, where HHV-1 contains an additional 318 residues. The size variation of the LHV-4 and BoHV-2 RR1 subunits is also concentrated in the N-terminal region, where the LHV-4 protein has an additional 28 residues.

To investigate phylogenetic relatedness, the amino acid sequences of LHV-4 RR1 and RR2 were combined and compared to the combined orthologous sequences from representative members of the four alphaherpesvirus genera, *Iltovirus*, *Simplexvirus*, *Varicellovirus* and *Mardivirus* (Fig. 6B). This analysis revealed that the LHV-4 RR1 and

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**Fig. 4.** Electron micrographs of LHV-4 and HHV-1 virions harvested from infected RK cells and negatively stained. (A–E) LHV-4; (F) HHV-1. The arrow indicates the envelope surrounding the virion. Open arrow: loose (broken) envelope. Solid arrow: tight envelope. Scale bar=0.1 µm.

**Fig. 5.** The effect of detergent on growth of LHV-4, HHV-1, and BAdV-3. LHV-4, HHV-1, and BAdV-3 stocks were treated with 0.1% Triton X-100 at room temperature for 5 min. Both treated and untreated viruses in triplicate, at 0.2 MOI, were exposed to cells seeded in 12-well plates. Infected cells were subjected to two freeze–thaw cycles at the indicated time points. The quantity of virus in the total cell lysate at each time point was titrated in 96-well microplates by standard (TCID₅₀/ml) assays.
In order to determine whether LHV-4 is capable of causing infection in domestic rabbits, five 10-week-old, female New Zealand white rabbits were exposed to the virus via inoculation of both cornea and both nostrils. In two of the rabbits, the left cornea was abraded before exposure. Four of the five infected rabbits showed severe signs of disease by 4 dpi, especially one that had been subjected to corneal abrasion. The symptoms included conjunctivitis, hemorrhagic dermatitis around the infected eye, nasal discharge, and fever. To monitor virus replication and shedding from the eye tissue, tear swabs (suspended in 0.5 ml of tissue culture medium) were collected at 1, 3, and 5 dpi in all rabbits and at 7 and 10 dpi in four rabbits (because of the severity of the infection, one rabbit was euthanized at 5 dpi), and the virus was titered on RS cells. The amount of virus present in the swab media was considered to be representative of virus shed in the eye, and the increase in PFU as indicative of the progress of the viral infection. LHV-4 shedding in the non-abraded eyes peaked at about 1×10^3 PFU/eye at 3–7 dpi and was not detectable after 10 dpi (Fig. 7). The virus shed from the abraded eyes peaked at 3 dpi and was about 2 to 3 fold greater in amount than that in non-abraded eyes. To determine whether the virus was shed in the nasal tract, nasal swabs from all the infected rabbits were tested for the presence of virus at 1, 3, 5, 7, and 10 dpi. This was accomplished by exposing RS cells to the nasal swab media and then examining the cells for CPE. LHV-4 shedding was detected in the nasal swabs from 5 and 7 dpi, but not at other time points (data not shown). No virus was detected from the uninfected animal, and it remained healthy. These results indicate that LHV-4 can be shed in tears and into nasal tracts. Microscopic examination of tissues from the rabbit that was euthanized at 5 dpi due to the severity of the infection revealed severe necrosis of the spleen and the lymph nodes (Figs. 8B and C). This histopathology is similar to that observed in tissues from rabbits collected during the original outbreak in Alaska [Jin et al., 2008].

PCR amplification of LHV-4 sequences in tissues from infected rabbits

In order to determine the extent of LHV-4 infection, DNA was extracted from selected tissues of experimentally infected rabbits that were euthanized at 5 or 14 dpi and subjected to PCR amplification using primers designed for specific sequences in the LHV-4 RR1 gene. PCR products of the predicted size (214 bp) were observed from all samples tested (Fig. 9A), with the exception of the heart and thymus (lanes 8 and 10). Although a product was generated from heart tissue (lane 8), it was aberrant in size and was therefore interpreted as non-specific. Tissues from an uninfected control animal were also examined, and no evidence of the predicted PCR product was detected (Fig. 9B). At 14 dpi, only samples from the trigeminal ganglia (TG), eye, and tonsil were positive by PCR (Fig. 9C and data not shown). These results indicate that the virus isolated from the outbreak in Alaska is able to cause systemic infections in domestic rabbits under experimental conditions. In addition, one of the eye tissue samples collected from a second outbreak at the farm in Alaska was also positive for LHV-4 DNA (Fig. 9C, lane AK). This suggests that this outbreak could have been caused by reactivation of LHV-4.

Discussion

In this report, we describe the characterization of a herpesvirus associated with a fatal infection of domestic rabbits. Several other rabbit herpesviruses have been described (Foulon and Cebrián, 1989;...
Hesselton et al., 1988; Hinze, 1971a,b; Roizman and Pellett, 2001) and have been classified tentatively as gammaherpesviruses. HHV-1 can cause experimental infections in rabbits and produce eye lesions similar to those reported here for LHV-4. Depending on the dose, such infections can cause inflammation of the conjunctiva, cornea, retina, brain, lung, kidney, and spleen. Severely infected rabbits may suffer from central nervous system infections by HHV-1 that can result in up to 50% mortality within 6 to 12 dpi (Grest et al., 2002; Weissenbock et al., 1997). Although it was initially considered that the rabbit infection in the Alaska outbreak could have resulted from HHV-1, naturally occurring HHV-1 infections of rabbits are uncommon and limited to only a few case reports (Grest et al., 2002; Weissenbock et al., 1997). We eliminated the possibility of HHV-1 infection by phylogenetic analyses of sequenced DNA.

LHV-4 has a rapid replication cycle of less than 24 h in RS cells and produces giant syncytia in RS and Vero cells (Fig. 1). The syncytia are similar to those produced by alphaherpesviruses, such as HHV-1 and pseudorabies virus (Roizman and Pellett, 2001). Nuclear inclusion bodies were observed in the skin lesions of a rabbit from the Alaskan outbreak (Jin et al., 2008), which is characteristic of herpesvirus infection. However, the plaque size of LHV-4 in RS cells is 2–3 times larger than that of HHV-1, suggesting that LHV-4 is spread between cells more efficiently (Fig. 2). This may also explain the lower titers produced by LHV-4 in RS cells, as most of the cells were dead by 3 dpi. Under EM examination, the virion has a similar size to HHV-1 and exhibits the characteristic nucleocapsid and envelope structure. In addition, the loss of infectivity caused by detergent treatment supports the presence of an envelope. Another characteristic of alphaherpesviruses is that they have a relatively broad tissue and host range, a short replication cycle, and produce multinucleated giant cells with subsequent cell death. LHV-4 appears to have a broad tissue range; we detected it in a wide range of tissues (Fig. 9A). Further, alphaherpesviruses are capable of establishing

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Fig. 8. Histopathology of a spleen from an experimentally LHV-4-infected rabbit. (A) Normal spleen section, with normal lymphocyte staining; (B) infected spleen section with necrosis; (C) infected spleen section with necrosis and red blood cells infiltration. The tissues were collected at 5 dpi. The arrows demarcate an area of necrosis (B) and hemorrhage (C), where cellular degradation and red blood cell staining are apparent. Staining was with hematoxylin and eosin.

Fig. 9. Agarose gel electrophoresis of PCR reactions specific for the LHV-4 RR1 gene (primers RR1sp-F and RR1sp-R). The predicted product is 214 bp in size. (A) PCR of total DNA extracted from tissue samples from a LHV-4-infected rabbit at 5 dpi. (B) PCR of total DNA extracted from tissue samples from an uninfected rabbit. Template DNA was as follows: P, LHV-4 genome; 1, appendix; 2, sciatic nerve; 3, kidney; 4, adrenal gland; 5, spleen; 6, liver; 7, lung; 8, heart; 9, lymph node; 10, thymus; 11, salivary gland; 12, tonsil; 13, brain stem; 14, frontal lobe; 15, trigeminal ganglion; 16, eye. MW, 1 kb-plus DNA ladder. (C) PCR of total DNA extracted from tissue samples from LHV-4-infected rabbit at 14 dpi and tissue from a rabbit from the second outbreak in Alaska. Template DNA examined from different tissue was as follows: AK, eye tissue from the infected rabbit the from second outbreak in Alaska; Ton, tonsil; E, eye; TG, trigeminal ganglion. MW, 1 kb-plus DNA ladder; P, LHV-4 DNA. Rabbits 1 and 2 were infected without corneal abrasion. Rabbit 3 was infected with corneal abrasion in of the left eye.
latent infections, primarily in sensory neurons. We detected LHV-4 DNA in the TG from experimentally infected rabbits at 5 and 14 dpi and from rabbits involved in a subsequent outbreak on the farm in Alaska (Fig. 9C), suggesting that the virus might become latent in the sensory nervous system.

The data presented in this report indicate that LHV-4 is a novel alphaherpesvirus. It is most closely related to BoHV-2, which was originally isolated in 1957 in cattle in Africa and has subsequently been detected with seropositivity rates of up to 80% in some herds (Imai et al., 2005). BoHV-2 has been detected in a variety of ruminant species, including domestic and wild sheep (mouflon) and several species of deer, with PCR positivity rates of 3–50% (Kalman and Eyged, 2005). BoHV-2 is unusual because it has a close evolutionary relationship to the primate alphaherpesvirus lineage that includes HHV-1, HHV-2, CeHV-2, and CeHV-16. It has been suggested that this relationship reflects an ancient cross-infection of a primate virus into an ungulate population (Ehlers et al., 1999; Lotthammer and Ehlers, 1990). Based on the identification of a member of the BoHV-2 lineage in rabbits, it is possible that cross-infection may have originally occurred in an animal other than an ungulate.

It is unclear why LHV-4 has not been reported more frequently. Two outbreaks of a herpes-like virus in rabbit colonies in Alberta and British Columbia, Canada, were documented from the early 1990s (Onderka et al., 1992; Swan et al., 1991). The symptoms described in these reports were similar to those that we report here. It is possible that LHV-4 is present in an animal reservoir found in northwestern North America, in which it causes asymptomatic infections and is infrequently comes in contact with domestic rabbits. It is well documented that some herpesviruses can cause a more severe infection in species other than their natural host, which may explain the lack of a previous description of this virus in its native host. It is also possible that LHV-4 is present in wild rabbit populations but is not often transmitted to domestic rabbits (Schmidt et al., 1992).

In the late spring of 2007, the year following the initial report of the LHV-4 outbreak in Alaska, the virus reappeared on the same farm. It is unclear whether the virus established a latent infection in rabbits on the farm and was reactivated by environmental factors or whether the rabbit population on the farm was re-infected from a reservoir in another species.

Materials and methods

Cell culture and virus isolation

RS cells, RK cells (RK-13B, American Type Culture Collection, Rockville, MD), and Vero cells were cultured at 37 °C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Sigma-Aldrich) with 5% CO2 in a humidified incubator. LHV-4 was isolated from frozen skin samples with hemorrhagic lesions from an affected rabbit from the 2006 outbreak in Wasilla, Alaska (Jin et al., 2008).

Virus growth curves

RS, RK, and Vero cell monolayers were infected with HHV-1 (McKrae strain) (Hill et al., 1982) or LHV-4 at a multiplicity of infection (MOI) of 0.01 (200 μl of 5 × 10^4 PFU/ml virus was added to wells seeded with 1 × 10^6 cells). Cell monolayers were absorbed with the virus for 1 h and washed once with phosphate-buffered saline (PBS; 3.2 mM Na2HPO4, 0.5 mM KH2PO4, 1.3 mM KCl, 135 mM NaCl, pH 7.4). Infected cells were maintained in DMEM supplemented with 5% FBS and antibiotics as described above. At the indicated time points, infected cells in 1 ml DMEM were subjected to three freeze–thaw cycles. After brief centrifugation to remove large cellular debris, the total amount of infectious virus was determined by standard plaque assays (Harmenberg et al., 1980) on RS cells. The quantity of virus in the cell lysate at each time point was determined in triplicate samples. In addition, the inocula were back titered by standard plaque assays to ensure that the input virus titer was in the expected range.

Detergent treatment of virus

Aliquots (180 μl) of LHV-4 or HHV-1 (McKrae strain) or bovine adenovirus type 3 (BADV-3) (Mattson et al., 1987) at 1 × 10^6 PFU/ml were mixed with 20 μl of 1% Triton X-100 (final concentration 0.1%) at room temperature for 5 min, respectively. The treated virus and a separate aliquot of untreated virus were diluted to 1 × 10^5 PFU/ml in PBS before infection. The 12-well plates (seeded with about 5 × 10^5 cells/well) were then infected with 100 ml (0.2 MOI) of treated or untreated virus at 1 × 10^5 PFU/ml in triplicate. Following absorption, the infected cells were maintained in equal volumes of DMEM supplemented with 5% FBS and antibiotics, as described above. At the indicated time points, infected cells in 1 ml DMEM were subjected to two freeze–thaw cycles. After brief centrifugation to remove large cellular debris, the total amount of infectious virus was determined by standard (TCID50 assay). The quantity of virus in the total cell lysate at each time point was determined three times in the same manner.

Preparation of virions

The isolated virus was propagated in RK cells maintained in DMEM supplemented with 5% calf serum and antibiotics as described above. Confluent cell monolayers were infected with plaque-purified virus at an MOI of 0.1. Virus was harvested when more than 90% of the cells showed CPE. The infected flasks were subjected to two freeze–thaw cycles. The medium harvested from infected RK cells was cleared of cells and cell debris by centrifugation at 9,000 × g for 30 min. The virions were then centrifuged through a 60% sucrose cushion in a Beckman Model XL-70 ultracentrifuge at 25,000 rpm for 1 h in an SW28 rotor. The pellets were suspended in PBS and frozen at −70 °C until required. HHV-1 (McKrae strain) was grown in RS cells and purified as described above.

Electron microscopy of virions

Virus particles purified by ultracentrifugation of infected cell suspensions, as described above, were adsorbed to formvar-coated carbon-stabilized copper grids by floating grids on −20 μl drops of the sample spotted on parafilm. The grids were then blotted dry with Whatman filter paper and immediately floated on −20 μl drops of 2% phosphotungstic acid (PTA) (pH 6.9) in water for 30 s. Excess PTA was removed by side-blotting and the grids were allowed to air dry. Images were obtained with a Phillips EM 300 electron microscope.

Purification of viral DNA

Viral DNA was extracted either from virions purified, as described above, or from purified intracellular nucleocapsids. For nucleocapsid purification, infected cells were harvested when 70 to 90% of the monolayer of a 75 ml tissue culture flask showed CPE and processed as described previously (Jin et al., 2000). Briefly, the cells were washed once with PBS, scraped off the flask, resuspended in 25 ml PBS and pelleted at 5000 rpm at 4 °C for 20 min. The cell pellet was then resuspended in 18 ml hypotonic buffer (10 mM Tris–HCl, 10 mM KCl, 5 mM EDTA, pH 8.0) and 2 ml Triton X-100 and incubated on ice for 10 min. The cell debris was removed by low speed centrifugation at 5000 rpm at 4 °C for 5 min. The viral nucleocapsids were then centrifuged through a 60% sucrose cushion, as described above, for virion purification. Purified virions or nucleocapsids were digested overnight at 50 °C in 10 mM Tris–HCl (pH 8.0), 100 mM EDTA, 1% N-lauroyl sarcosine, and 200 μg/ml protease K. The viral DNA was extracted twice, with an equal volume of phenol:chloroform (1:1),...
and then precipitated with 2 volumes of ethanol and 1/10 volume of 3.0 M sodium acetate. The precipitate was washed once in 70% ethanol and dissolved in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

**Primers and PCR amplification**

Selection of primers for amplification of LHV-4 sequences was based on conserved amino acid sequence domains of RR1 located in the HHV-1 protein approximately at residues 962 (QFV/IAMLPT) and 1105 (GLKTGMYY). Degenerate primers RR1-F (CAG GAA ACA GCT ATG CGRB) and RR1-R (RTA RTA CAT ICC NTG YTT) were synthesized (Invitrogen). The M13 reverse primer sequence was included at the beginning of RR1-F to facilitate sequencing of the PCR product. The primers used for screening the presence of viral DNA in tissue samples were located in the LHV-4 RR1 gene: RR1sp-F (TGT ACC RTI GCI YTI ATG CCN CA) and RR1sp-R (RTA RTA CAT ICC NTG YTT).

PCR using the degenerate oligomers was performed in a 25-µl solution consisting of 2.5 µl 10× Pfx amplification buffer (Invitrogen), 0.5 µM MgSO4, 1.0 µl dNTPs at 10 mM each, 2 µM primers (RR1-F and RR1-R), 1.0 U Platinum Pfx DNA polymerase (Invitrogen), and 0.2 µg viral DNA. The mixture was subjected to 94 °C for 2 min, and 10 cycles of 94 °C for 30 s, 47 °C for 45 s, and 72 °C for 45 s, then 30 cycles of 94 °C for 20 s, 49 °C for 45 s, and 50 °C for 30 s, and followed by a 7-min elongation reaction at 72 °C after the final cycle. PCR amplification with LHV-4-specific primers for the detection of viral DNA in tissues was performed as follows: a 25-µl solution consisting of 2.5 µl 10× Pfx amplification buffer (Invitrogen), 2.5 µl 10× PCR Enhancer solution (Invitrogen), and 0.2 µg viral DNA. The mixture was subjected to 94 °C for 2 min, and 30 cycles of 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 45 s, followed by a 7-min elongation reaction at 72 °C after the final cycle.

**DNA sequencing**

Sequences were determined by direct sequencing of PCR products purified with a ChargeSwitch PCR Clean-Up kit (Invitrogen). Further data were generated using primers designed from the initial sequence. All sequencing was done, using viral genomic DNA as the template, and carried out by the Center for Gene Research and Biocomputing (CGRB) at Oregon State University. The CGRB used an ABI Prism®3730 Genetic Analyzer with a ChargeSwitch PCR Clean-Up kit, employing ABI Prism®3730 Data Collection Software v. 3.0 and ABI Prism® DNA Sequencing Analysis Software v. 5.2. The 3391-bp sequence containing the LHV-4 RR1 and RR2 genes has been deposited in Genbank (EU19871). Phylogenetic analysis of the concatenated amino acid sequences of RR1 and RR2 was carried out using MacVector 9.5.2 software with settings: neighbor joining; best tree; tie breaking=systematic distance; gaps distributed proportionally; bootstrap (1000 replicates).

**Infection of domestic rabbits**

Six to 10-week-old pathogen-free New Zealand white rabbits were acquired from Simonsen Laboratories, and were maintained and handled under veterinary supervision in accordance with National Institutes of Health guidelines and the Oregon State University Animal Care and Use Committee (IACUC, ACUP#3542). Five were inoculated with a ChargeSwitch PCR Clean-Up kit (Invitrogen). Further sequencing was done, using viral genomic DNA as the template, and carried out by the Center for Gene Research and Biocomputing (CGRB) at Oregon State University. The CGRB used an ABI Prism®3730 Data Collection Software v. 3.0 and ABI Prism® DNA Sequencing Analysis Software v. 5.2. The 3391-bp sequence containing the LHV-4 RR1 and RR2 genes has been deposited in Genbank (EU19871). Phylogenetic analysis of the concatenated amino acid sequences of RR1 and RR2 was carried out using MacVector 9.5.2 software with settings: neighbor joining; best tree; tie breaking=systematic distance; gaps distributed proportionally; bootstrap (1000 replicates).

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