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Original Research

Experimental Infection of New Zealand White Rabbits (*Oryctolagus cuniculi*) with *Leporid herpesvirus* 4

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Leporid herpesvirus* 4 (LHV4) is a novel alphaherpesvirus recently identified in domestic rabbits (*Oryctolagus cuniculi*). Little is known about the pathogenesis or time course of disease induced by this virus. We therefore intranasally inoculated 22 female New Zealand white rabbits with 8.4×10^4 $CCID_{50}$ of a clinical viral isolate. Rabbits were monitored for clinical signs, viral shedding in oculonasal secretions, and development and persistence of serum antibodies. Rabbits were euthanized at 3, 5, 7, 14, and 22 d postinfection (dpi) to evaluate gross and microscopic changes. Clinical signs were apparent between 3 to 8 dpi, and included oculonasal discharge, respiratory distress, and reduced appetite, and viral shedding occurred between 2 and 8 dpi. Seroconversion was seen at 11 dpi and persisted to the end of the study (day 22). Severe necrohemorrhagic bronchopneumonia and marked pulmonary edema were noted by 5 dpi and were most severe at 7 dpi. Pulmonary changes largely resolved by 22 dpi. In addition, multifocal splenic necrosis was present at 5 dpi and progressed to submassive necrosis by 7 dpi. Eosinophilic herpesviral intranuclear inclusion bodies were detected in the nasal mucosa, skin, spleen, and lung between 3 to 14 dpi. LHV4 is a pathogen that should be considered for rabbits that present with acute respiratory disease. LHV4 infection can be diagnosed based on characteristic microscopic changes in the lungs and spleen and by virus isolation. Serum antibody levels may be used to monitor viral prevalence in colonies.

Abbreviations: $CCID_{50}$, 50% cell culture infectious dose; CRFK, Crandall feline kidney; dpi, days post infection; LHV4, *Leporid herpesvirus* 4; RHDV, rabbit hemorrhagic disease virus; S:P, sample:positive.

Herpesviridae is a large family of enveloped, double-stranded DNA viruses within the order *Herpesvirales*. Viruses within this order are morphologically similar, possessing large genomes ranging from 125 to 290 kb. The linear DNA is packaged within an icosahedral capsid that is surrounded by a proteinaceous tegument and enclosed in a lipid envelope.[10] The family *Herpesviridae* comprises more than 100 different virus species that infect mammals, birds, and reptiles. The family is divided into 3 subfamilies—alphaherpesviruses, betaherpesviruses, and gammaherpesviruses—according to distinguishing biologic properties of the viruses. Alphaherpesviruses demonstrate rapid lytic responses in cell culture, whereas betaherpesviruses are slow-growing, often producing giant cells in tissue, and gammaherpesviruses typically infect lymphoid tissue, leading to oncogenesis. Division into subfamilies on the basis of biologic behavior is also consistent with phylogenetic analysis.[29] Phylogenetic trees of the viral species are used to further subclassify viruses into genera, which typically closely follow the evolution of the host species.[23]

There are 4 known herpesviruses of rabbits. *Leporid herpesvirus* 1 (cottontail herpesvirus) and *Leporid herpesvirus 3* (*Herpesvirus sylvilagus*) are gammaherpesviruses that have been isolated from wild cottontail rabbits (*Sylvilagus floridanus*). Both LHV1 and LHV3 were isolated incidentally from primary kidney cell cultures during searches for papillomaviruses[8] and other viruses.[13] The minor differences in immunoreactivity between LHV1 and LHV3 suggest that they are unique viruses, but complete genetic analyses are unavailable.[6] LHV3 infection can induce lymphoproliferative disease and neoplasia in cottontail rabbits,[12] but the virus is unable to establish productive infection in domestic rabbits, such as New Zealand white rabbits (*Oryctolagus cuniculi*).[14] No infection trials with LHV1 have been reported. *Leporid herpesvirus* 2, also known as virus 3 and *Herpesvirus cuniculi*, is a gammaherpesvirus that was first isolated in domestic laboratory rabbits during the search for the causative agent of chickenpox.[31] LHV2 was later isolated as a slow-growing contaminant of a primary rabbit kidney cell culture.[25] Early infection studies with LHV2 demonstrated induction of neurologic signs, including nonsuppurative encephalitis with classic herpetic intranuclear inclusion bodies, after intracerebral inoculation.[31] Recent studies suggest the histologic evidence of a mild, subclinical encephalitis after infection of New Zealand white rabbits.[37] Natural infections of *Human herpesvirus* 1 (herpes simplex 1) have been reported in rabbits, resulting in fatal encephalitis.[24,32,36]

Leporid herpesvirus 4 (LHV4) is a novel herpesvirus that was independently diagnosed and isolated from commercial rabbits

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in Alaska[19] and a pet rabbit in northern Ontario.[5] In 1990, cases of rabbit disease with similar clinical signs were reported among commercial meat rabbits in Alberta and British Columbia; the etiologic agent in these 2 cases was identified as a herpesvirus, but further genetic analyses were not performed.[27,33] Affected rabbits show variable clinical signs including lethargy, anorexia, conjunctivitis, fever, and abortion. Predominant pathologic findings include hemorrhagic dermatitis, splenic necrosis, hepatic necrosis, and multifocal pulmonary hemorrhage and edema. Distinctive glassy eosinophilic herpetic intranuclear inclusion bodies were observed in the skin and mesenchymal cells of the spleen and lung.[5,18] Postinfection morbidity and mortality have been reported to be 50% and 20%, respectively. However, the clinical disease and time course have not been studied previously, and only anecdotal reports from veterinary clients have been described. On the basis of its rapid growth and cytopathic effect in cell culture, LHV4 is classified as an alphaherpesvirus. Phylogenetic analysis of multiple genes has indicated that LHV4 segregates to the genus *Simplexvirus*,[2,18] which is unusual because this genus consists primarily of primate herpesviruses. The only other nonprimate species in this family are *Bovine herpesvirus* 2, *Macropodid herpesvirus* 1, and *Macropodid herpesvirus* 2,[10] suggesting that these viral species may have migrated from primates, such as human caregivers, to these other species.[22]

With the emergence of a newly recognized infectious disease of rabbits, it is important to consider its effect on all domestic rabbit populations, including those used in research. In addition to welfare concerns, infectious disease can introduce unacceptable variability in research data.[21] Guidelines for the care and use of laboratory animals from the Canadian Council on Animal Care and National Academy of Sciences indicate a need for the surveillance and eradication of known pathogens.[7,15] Furthermore, many studies use rabbits to answer research questions about other alphaherpesviruses. The rabbit is a popular model for the ocular keratitis induced by *Herpes simplex virus* 1 [9] and for the study of *Bovid herpesvirus* 1 and *Bovid herpesvirus* 5.[34] In addition, rabbits have been used in investigations of *Macacine herpesvirus* 1 (B virus) infections.[4] As illustrated with the discovery of LHV2, rabbit cell cultures are often used to isolate viruses.[25,31] The presence of either active or latent LHV4 infection could seriously affect the interpretation of findings from these studies. It is imperative that laboratory animal veterinarians be aware of LHV4 and be able to identify and diagnose infections in rabbits.

A preliminary investigation of a suspected herpesvirus infection in 2 domestic New Zealand white rabbits demonstrated splenic and hepatic necrosis, pulmonary congestion and edema, and necrosis at the site of inoculation.[27] After the initial discovery of LHV4, a few young New Zealand white rabbits were experimentally inoculated both intranasally and intracorneally with very large doses of virus and developed conjunctivitis and systemic illness.[18] Pathology was conducted only on one animal at the peak of infection, and revealed splenic and lymph node necrosis.[18] Whether rabbits can recover from the disease, how long they shed virus after infection, and if and when protective antibody titers develop were not evaluated.

In the current study, we characterized the progression of clinical signs and the gross and microscopic changes after the intranasal inoculation of adult female New Zealand white rabbits with a sublethal dose of LHV4. We further evaluated viral shedding, neutralizing antibody production, and the recovery of rabbits from infection. Although bacterial infection may contribute to the progression and severity of disease in pet or commercial rabbit settings, we used SPF rabbits to isolate the direct pathologic effects of LHV4. This study provides essential information to veterinarians for the diagnosis of LHV4 in rabbits during various stages of active infection and convalescence.

Materials and Methods

Animals. Female adult New Zealand white rabbits (2.8 to 3.1 kg; $n = 22$) were obtained from Charles River Canada (St Constant, Canada). Serologic information provided by the supplier indicated that rabbits were free of reovirus type 3, lymphocytic choriomeningitis virus, parainfluenzavirus 1, parainfluenzavirus 2, rotavirus, rabbit hemorrhagic disease virus, *Bordetella bronchiseptica*, cilia-associated respiratory bacillus, *Helicobacter* spp., *Lawsonia* spp., *Pasteurella multocida*, *Pasteurella aeruginosa*, *Salmonella* spp., *Treponema cuniculi*, and *Clostridium piliforme*. In addition, rabbits were tested for and noted to be free of LHV4 virus and antibodies prior to study initiation. Rabbits were housed in groups of 4 to 6 on kiln-dried, autoclaved pine shavings (Harlan Laboratories, Mississauga, Ontario, Canada) in floor pens, received water and pellets (Teklad Global High Fiber Rabbit Diet, Harlan Teklad, Madison, WI) ad libitum, and were provided with autoclaved timothy hay ad libitum and with less than 50 g daily of fruits, vegetables, and toasted oat cereal as foraging treats. Rabbits were maintained on a 12:12-h light:dark cycle and at constant temperature (20 ± 4 °C) and relative humidity (30% to 70%). Rabbits were acclimated to the facility and to individual handling and grooming for 5 d prior to experimental manipulation. The facilities and procedures involving animals are in compliance with the Animals for Research Act of Ontario[28] and the guidelines of the Canadian Council on Animal Care.[7] The University of Guelph Animal Care Committee approved the study protocol.

Infection trials. A pilot study was conducted on 2 rabbits to estimate the challenge dose for the full-scale study and to develop endpoint-scoring protocols for clinical monitoring. Rabbits were challenged with a total dose of 1.7×10^5 cell culture infectious dose 50% ($CCID_{50}$) via intranasal inoculation. Rabbits were monitored 4 to 6 times daily for changes in behavior and signs of illness, such as dyspnea. Rectal temperatures were collected daily.

Rabbits were sedated with acepromazine (0.3 mg/kg SC; Atravet, Wyeth, Guelph, Ontario, Canada) and butorphanol (0.3 mg/kg SC; Torbugesic, Wyeth). Local anesthetic (100 µL 2% lidocaine, AstraZeneca, Wilmington, DE) was instilled into each nostril.[34] For the full-scale study, 16 rabbits were inoculated in each nostril with 4.2×10^4 $CCID_{50}$ in 200 µL culture medium (composed of equal parts of IMDM, DMEM, and Leibowitz 15 solution [Gibco, Grand Island, NY] supplemented with penicillin, streptomycin [Animal Health Laboratory, Guelph, Ontario, Canada], and 5% FBS [Sigma, St. Louis, MO]). Two control rabbits were sedated and inoculated with 200 µL of culture media and monitored for 22 d prior to euthanasia. Two additional control rabbits were purchased and maintained in a separate room but not experimentally manipulated. Rabbits were observed 4 to 6 times daily for as long as 22 d post infection (dpi) and treated with dietary support (hay, fresh fruit and vegetables, toasted oat cereal) or subcutaneous fluids, as necessary. Body weights and nasal swabs for virus isolation were collected daily. To test for serum antibodies, blood was collected at 0, 3, 5, 7, 11, 14, 17 and 22 dpi. Randomly assigned rabbits ($n = 3$) underwent topical anesthesia of the lateral

ear vein with anesthetic cream (EMLA, APP Pharmaceuticals, Schaumberg, IL) and euthanized by using intravenous barbiturate overdose (Pentobarbital, Schering-Plough, Kirkland, Canada) at 3, 5, 7, 14, and 22 dpi, and full necropsies were conducted.

Histopathology and scoring. Tissue samples including nasal sections, perinasal skin, conjunctiva, thyroid, esophagus, trachea, salivary gland, lymph node, lung, heart, thymus, liver, spleen, pancreas, kidney, adrenal gland, gastrointestinal tract, reproductive tract, cerebrum, and cerebellum were collected and fixed in 10% neutral-buffered formalin for at least 24 h and routinely processed for histology including paraffin embedding, sectioning, and hematoxylin and eosin staining. Nasal turbinates were decalcified (Cal-Ex II, Fisher Scientific, Rochester, NY) for an additional 24 h prior to processing.

A semiquantitative scoring system for the lung sections was developed and was based on the relative severity and distribution of edema, hemorrhage, necrosis, and inflammatory cell infiltrate. Each component was assigned a score of 0 to 4 (0, normal; 1, minimal [focal lesion]; 2, mild [less than 10% of section affected]; 3, moderate [10% to 25% of section affected]; and 4, marked [more than 25% of section affected]), with a maximal score of 16 for the most severely affected tissue. Slides were randomized, scored, and peer-reviewed for consistency.

Cell culture and virus isolation. Crandall feline kidney (CRFK) cells were obtained from the University of Guelph Animal Health Laboratory (Guelph, Ontario, Canada) and maintained in media composed of equal parts of IMDM, DMEM, and Leibowitz 15 solution (Gibco) supplemented with 10% FBS (Sigma), penicillin, and streptomycin (Animal Health Laboratory). Concentrated virus was prepared as previously described.[26] Infected cell monolayers were harvested at maximal cytopathic effect, pooled, and clarified by centrifugation at 4000 × *g* for 20 min. Virus was pelleted through a 30% sucrose cushion at 28,000 × *g* for 2 h and resuspended in 10 mM Tris, 1 mM EDTA (pH 8.0).

Leporid herpesvirus 4 had previously been isolated by the Animal Health Laboratory from skin samples from a pet rabbit,[5] and frozen stocks were used for the current study. The virus was passaged twice in CRFK cells and stored at −80 °C. The $CCID_{50}$ was determined by endpoint dilution assay. Nasal swabs were stored in virus transport media (equal parts IMDM, DMEM, and Leibowitz 15 solution supplemented with 5% FBS, penicillin, and streptomycin) at −80 °C until analysis. At time of analysis, the viral transport medium was centrifuged to remove cellular debris, and duplicate 100-µL aliquots were added to a monolayer of CRFK in 24-well plates and observed for as long as 5 d for cytopathic effect. Samples were determined to be negative after 3 blind passages.

Virus neutralization assay. Positive-control polyclonal antibodies were generated in rabbits by using inactivated virus and adjuvant (Pacific Immunology, Ramona, CA). Serial serum dilutions were incubated with 100 $CCID_{50}$ viral particles for 1 h at 37 °C in a 96-well plate. CRFK cells (2.5×10^4) were added to each well, and plates were monitored daily for cytopathic effect through 5 dpi.

ELISA. A serial 'checkerboard' assay was conducted to determine the optimal amount of coating antigen, serum dilution, and secondary antibody concentration. Unless otherwise stated, all incubations were done at 37 °C, and plates were washed 4 times between steps by using 250 µL wash buffer (0.05% Tween 20 in PBS) for each wash. The protein concentration of the viral concentrate was determined by using a Bradford assay (BioRad, Hercules, CA), and viral concentrate was lysed in 0.05% sarcosine. Immulon 2B 96-well plates (Thermo-Scientific, Rochester, NY) were coated overnight with viral lysate containing 100 ng viral protein in 100 µL calcium carbonate buffer (pH 9.6) at 4 °C. The plates were blocked with 100 µL 5% FBS in PBS for 1 h. Duplicate 200-µL aliquots of 1:50-diluted samples were added to wells and incubated at 37 °C for 1 h. Goat antirabbit IgG complexed with alkaline phosphatase (dilution, 1:5000; KPL, Gaithersburg, MD) was added in 100-µL aliquots and incubated for 1 h. Paranitrophenyl phosphate substrate (Sigma) was added and incubated at room temperature for 5 to 10 min. The reaction was stopped by using 3 N NaOH and read at 520 nm. Sera from a rabbit with neutralizing titer of 1:1024 was used as a positive control on each plate, thereby enabling the calculation of sample:positive (S:P) ratios:

$$\text{S:P ratio} = \frac{\text{Mean } OD_{520} \text{ of sample} - \text{mean } OD_{520} \text{ of negative control}}{\text{Mean } OD_{520} \text{ of positive control} - \text{mean } OD_{520} \text{ of negative control}}$$

PCR. Total DNA was isolated from cell culture supernatant, frozen tissues, or scrolls of paraffin-embedded tissues by using DNA Mini Kits (Qiagen, Gaithersburg, MD). Specific primers (LHV4f-wd, 5′ CCA CCA ACG TCT CCG CCG TGT T 3′; LHV4rev, 5′ AGC TTT GGC GCG GTG CAG AAG C 3′) were selected to amplify a 138-bp sequence within the ribosome reductase 1 gene of LHV4; primer sequences are not present in *Herpes simplex virus* 1, *Herpes simplex virus* 2, *Bovid herpesvirus* 2, *Oryctolagus cuniculi* or *Felis cattus* genomes. PCR amplification was conducted in a 25-µL reaction containing a maximum of 100 ng template DNA, 1.0 U Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA), PCR buffer solution, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.4 µM each forward and reverse primers. Reactions underwent initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s. Samples underwent a final incubation at 72 °C for 10 min and were maintained at 4 °C. PCR products were run on a 1% agarose gel containing 1% SybrSafe reagent (Invitrogen, Grand Island, NY) and visualized on a ChemiDoc system (BioRad).

Statistical analyses. Where applicable, reported values represent mean ± SE. Between-day histopathology scores were analyzed by using one-way ANOVA (Excel, Microsoft Corporation, Redmond, WA), followed by a manual post hoc Student–Newman–Keuls testing. An α level of less than 0.05 was accepted for significance.

Results

Clinical signs. The rabbits in the pilot study showed decreased activity at 3 dpi compared with before inoculation and developed serous oculonasal discharge by 4 dpi. By 5 dpi, rabbits were dull and lethargic, had serosanguinous nasal discharge, and moderate to marked dyspnea, as evidenced by open-mouthed breathing with extension of the head and neck. Both rabbits were mildly dehydrated at 5 dpi and were supplemented with subcutaneous fluids. Over the next 12 h, there was no improvement in their respiratory condition, and both rabbits were euthanized at 5.5 dpi. Throughout the course of infection, both rabbits had decreased food intake, with as much as 15% total body weight loss by the end of the study.

In light of these findings, we reduced the dose for the full-scale trial by 50% to avoid marked dyspnea at 5 dpi and to ensure that rabbits recovered from acute infection. After intranasal inoculation,

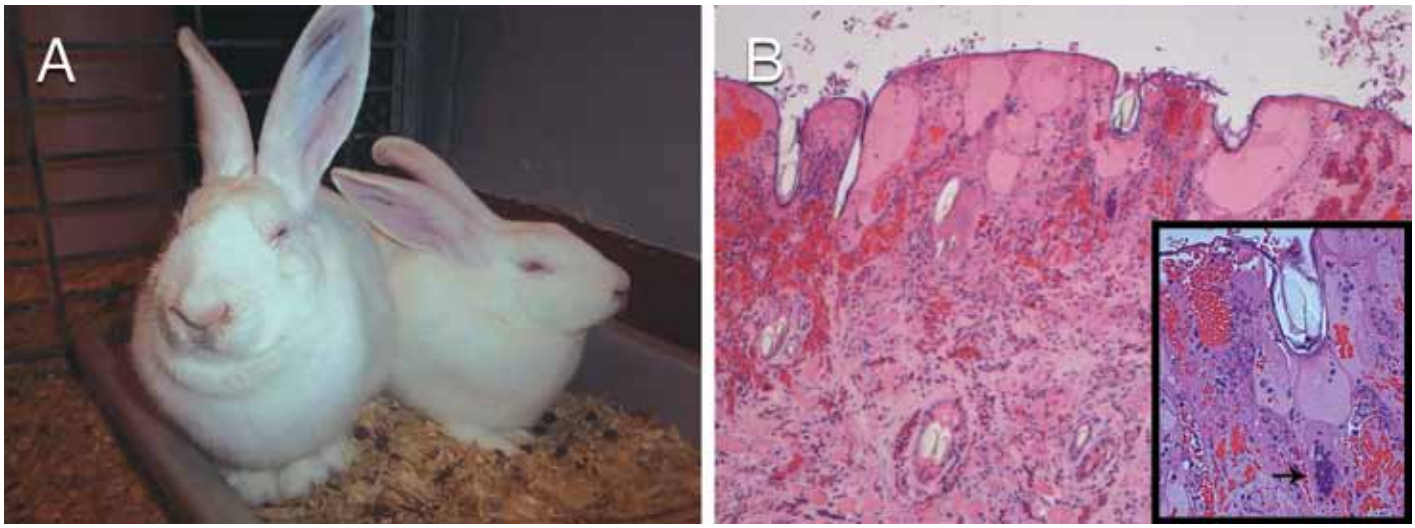

Figure 1. Clinical and cutaneous microscopic findings in LHV4-infected rabbits; 95% of rabbits infected with LHV4 exhibited serous to serosanguinous oculonasal discharge at peak disease. (A) Rabbits with focal alopecia on the skin overlying the nose, serous oculonasal discharge, and periocular swelling. (B) Photomicrograph of affected skin from rabbit, demonstrating epidermal necrosis, dermal edema, and hemorrhage, with multinucleated syncytial cells containing intranuclear inclusion bodies (inset, arrow; magnification, 200×); hematoxylin and eosin stain; magnification, 40×.

95% (15 of 16) of rabbits developed clinical signs of infection, ranging from serous nasal discharge to severe respiratory distress. The remaining rabbit (euthanized at 22 dpi) never evinced respiratory or other clinical signs and may have been misdosed or resistant to infection. This animal was cohoused with other rabbits that demonstrated significant respiratory disease and did not develop neutralizing antibodies or shed virus throughout the trial. This rabbit was determined to be an outlier and was excluded from further analyses.

At 2 dpi, several rabbits began sneezing. Nasal discharge was apparent in most rabbits, starting at 3 dpi and persisting to 14 dpi. Early in the infection, the discharge was serous, becoming serosanguinous and then more turbid by 7 dpi (Figure 1). Clinical signs were noted as early as 2 dpi but were most significant between 5 to 7 dpi. During this time, some animals exhibited dyspnea, characterized by open-mouthed breathing and neck and head extension. Rabbits were treated with subcutaneous fluids as needed and given additional food supplements to encourage eating. At 7 dpi, 2 rabbits appeared mildly cyanotic. Mild periocular swelling was present in another 2 rabbits at 8 dpi. Clinical condition improved starting at 8 dpi, and all rabbits were clinically normal when euthanized at 22 dpi. Core body temperature increased to 41.0 °C (range, 39.7 to 42.2 °C) until peak infection, after which it returned to normal (Figure 2). Food refusal with weight loss and body weight gain suppression occurred in inoculated rabbits until 7 dpi (Figure 2). No clinical changes were observed in control rabbits throughout the course of the study.

Viral shedding. Viral isolation demonstrated nasal shedding of virus particles from 1 to 10 dpi (Figure 3). There was considerable animal-to-animal variation in the amount of virus shed. Most swabs yielded a titer of less than 100 $CCID_{50}$/mL, with peak titer reaching 1.46×10^4 $CCID_{50}$/mL. The animal with the highest titer demonstrated signs of respiratory difficulty at peak infection but survived to the end of the study. Two rabbits that consistently had titers of less than 1000 $CCID_{50}$/mL recovered and were euthanized in the final group at 22 dpi. Otherwise, there did not appear to be a correlation between clinical signs and the amount of virus

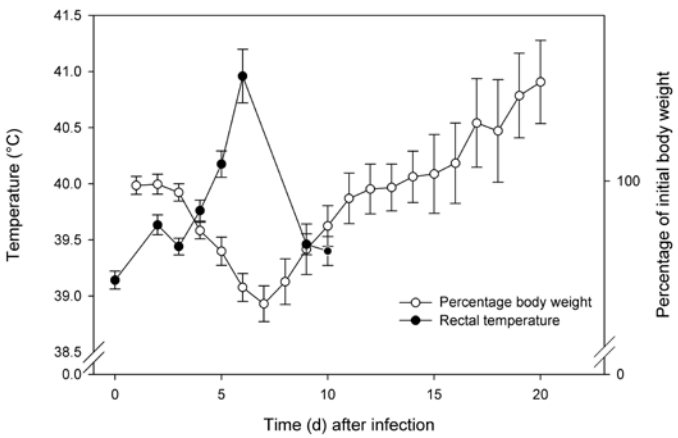

Figure 2. Core body temperature is elevated at peak infection and body weight decreases until peak infection. (A) Rectal temperature of all rabbits was collected daily except at 7 and 8 dpi, to minimize clinical stress. (B) Daily body weight was collected from each animal throughout the study and compared with prestudy body weight to determine percentage initial body weight. Animals were still in a growth phase, such that both loss of body weight and suppression of weight gain were evident in infected rabbits, beginning 3 dpi.

shed. PCR amplification and Sanger sequencing demonstrated that the virus isolated from our inoculated rabbits was 100% identical to the original isolate (data not shown). In addition to nasal secretions, paraffin-embedded lung from the index case and frozen lung and spleen—but not liver or kidney samples—from the pilot rabbits were virus-positive by PCR analysis.

Antibody response. Virus neutralization assays demonstrated the presence of neutralizing antibodies to LHV4 in all 15 infected rabbits starting at 11 dpi, after resolution of the main clinical signs. Neutralizing antibodies were not present in control animals. These results were confirmed by using an indirect ELISA directed toward whole-virus lysate (Figure 4); an S:P ratio greater than 0.200 was consistent with a positive reaction. There

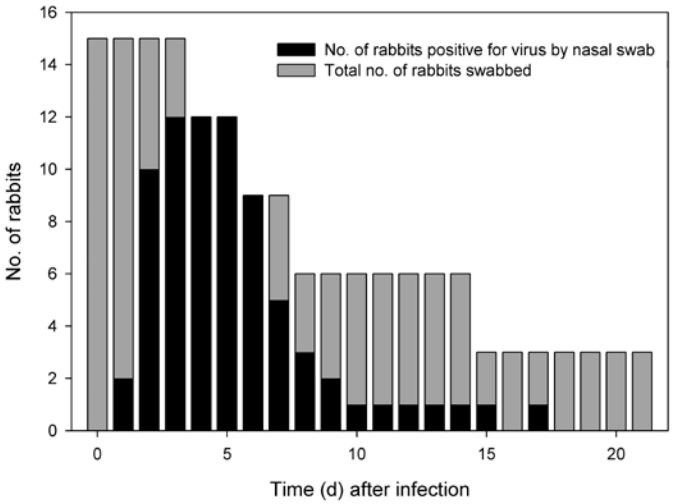

Figure 3. Virus was isolated from nasal secretions of infected rabbits from 1 to 10 dpi, with a peak titer of 1.46×10^4 $CCID_{50}$/mL at 5 dpi. Virus isolation was conducted by using CRFK cells in 24-well plates. Duplicate 100-µL aliquots were added to cell monolayers and monitored for 5 dpi. Samples were determined to be negative after 3 blind passages.

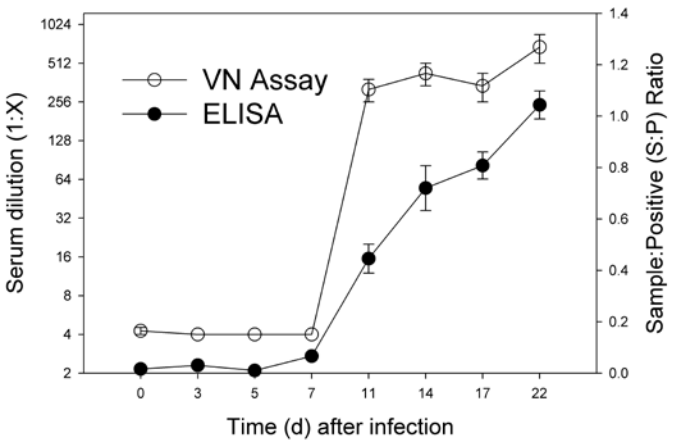

Figure 4. Infected rabbits seroconverted to LHV4 by 11 dpi. Both the virus neutralization (VN) assay (closed circles) and whole-virus ELISA (open circles) demonstrated serum antibodies specific to LHV4 at 11 dpi that persisted until the end of the study (22 dpi).

was 100% agreement between the virus neutralization assay and indirect-antigen ELISA. Antibody titers persisted until 22 dpi, the last date of collection.

Pathology. Gross findings were limited to the facial skin, nasal cavity, lungs, spleen, and liver and were most obvious in the lungs. At 3 dpi, there was moderate hemorrhage and consolidation of the right cranial lung lobe, with acute, random multifocal hemorrhages scattered throughout the parenchyma (Figure 5). The pulmonary pathology progressed in severity at 7 dpi to involve almost 80% of the lung parenchyma, with marked consolidation, hemorrhage, and edema bilaterally and marked serosanguinous exudation on cut section. By 14 dpi, the lungs were still moderately consolidated and edematous, but fewer hemorrhages were noted, and some animals demonstrated normal parenchyma in dorsocaudal lung regions. Grossly, by 22 dpi, the lungs appeared near normal but retained a somewhat rubbery consistency.

Microscopically within the lungs at 3 dpi, there were areas of acute multifocal to coalescing inflammation, alveolar septa necrosis, and type II pneumocyte hyperplasia centered around small and large bronchioles, with moderate mixed leukocytic infiltrates (neutrophils, alveolar macrophages, and lymphocytes) admixed with karyorrhectic debris (Figure 5). Occasional syncytial cells were present, in addition to mild perivascular and alveolar edema. Bronchiolar epithelium was hyperplastic, nonciliated, and necrotic, and small airways were either collapsed or occluded by numerous mature and degenerate neutrophils, alveolar macrophages, and cellular debris. By 5 dpi, lesions were similar but in addition included marked, patchy alveolar flooding with edema fluid, hemorrhage, and fibrin deposition. Prominent multifocal peribronchiolar lymphoid aggregates were noted also. Severe necrohemorrhagic bronchopneumonia with marked pulmonary edema and bronchiectasis was seen by 7 dpi. Multifocally, dense aggregates of coccoid bacteria were present along the denuded and attenuated epithelium in larger airways. At 14 dpi, there was reepithelialization of larger airways and reduced hemorrhage. However, moderate patchy alveolar necrosis with mixed inflammatory infiltrates and pulmonary flooding with edema fluid were still evident in some lobes. Marked resolution of inflammation was noted by 22 dpi, with patchy edema, prominent peribronchiolar lymphoid aggregates, and locally extensive peribronchiolar fibrosis and type II pneumocyte hyperplasia with mild, mixed infiltrates of lymphocytes, plasma cells, macrophages, and occasional neutrophils. Margination of nuclear chromatin and glassy, pale eosinophilic intranuclear inclusion bodies (Cowdry type A inclusions) were noted within epithelial cells, macrophages, and lymphocytes between 3 to 14 dpi. Scoring of microscopic sections of lung yielded a similar trend, with scores (mean ± SE) of 2.3 ± 0.5, 4.0 ± 0.5, 11.0 ± 3.3, 14.0 ± 0.9, 12.7 ± 0.3, and 4.2 ± 0.8, at 0, 3, 5, 7, 14, and 22 dpi, respectively. Histopathology scores at 5, 7, and 14 dpi were significantly ($P < 0.05$) higher than those at other times.

Mild splenomegaly with multifocal necrosis was grossly apparent at 5 dpi, progressing to moderate splenomegaly with coalescing areas of necrosis affecting as much as 90% of the parenchyma by 7 dpi (Figure 6). Spleens appeared grossly normal at subsequent times.

Microscopically, there was moderate, multifocal to coalescing splenic necrosis at 5 dpi, with marked hemorrhage and fibrin exudation (Figure 6). Numerous multinucleated syncytial cells with pale eosinophilic intranuclear inclusion bodies were scattered throughout the necrotic debris. By 7 dpi, there was submassive splenic necrosis. Remarkably, splenic lesions were largely resolved by 14 dpi, with scattered focal areas of fibrosis and fibroblast aggregation in 2 of the 3 rabbits in this group. Mild, multifocal splenic fibrosis was seen at 22 dpi.

Focal alopecia and crusting was noted on the nasal dorsum of one rabbit at 5 dpi and another rabbit at 7 dpi. Microscopically, this finding corresponded to locally extensive epidermal ulceration and necrosis with focal dermal edema, congestion, hemorrhage, and mild mixed, predominantly neutrophilic infiltrates (Figure 1). Numerous pale eosinophilic glassy intranuclear inclusion bodies and multinucleated syncytial cells were present subtending the ulcerated and necrotic epithelium.

By 14 to 22 dpi, nasal turbinate loss was evident grossly (Figure 7). There was moderate congestion and edema of nasal mucosa at 3 dpi, with scattered intraepithelial multinucleate syncytial cells

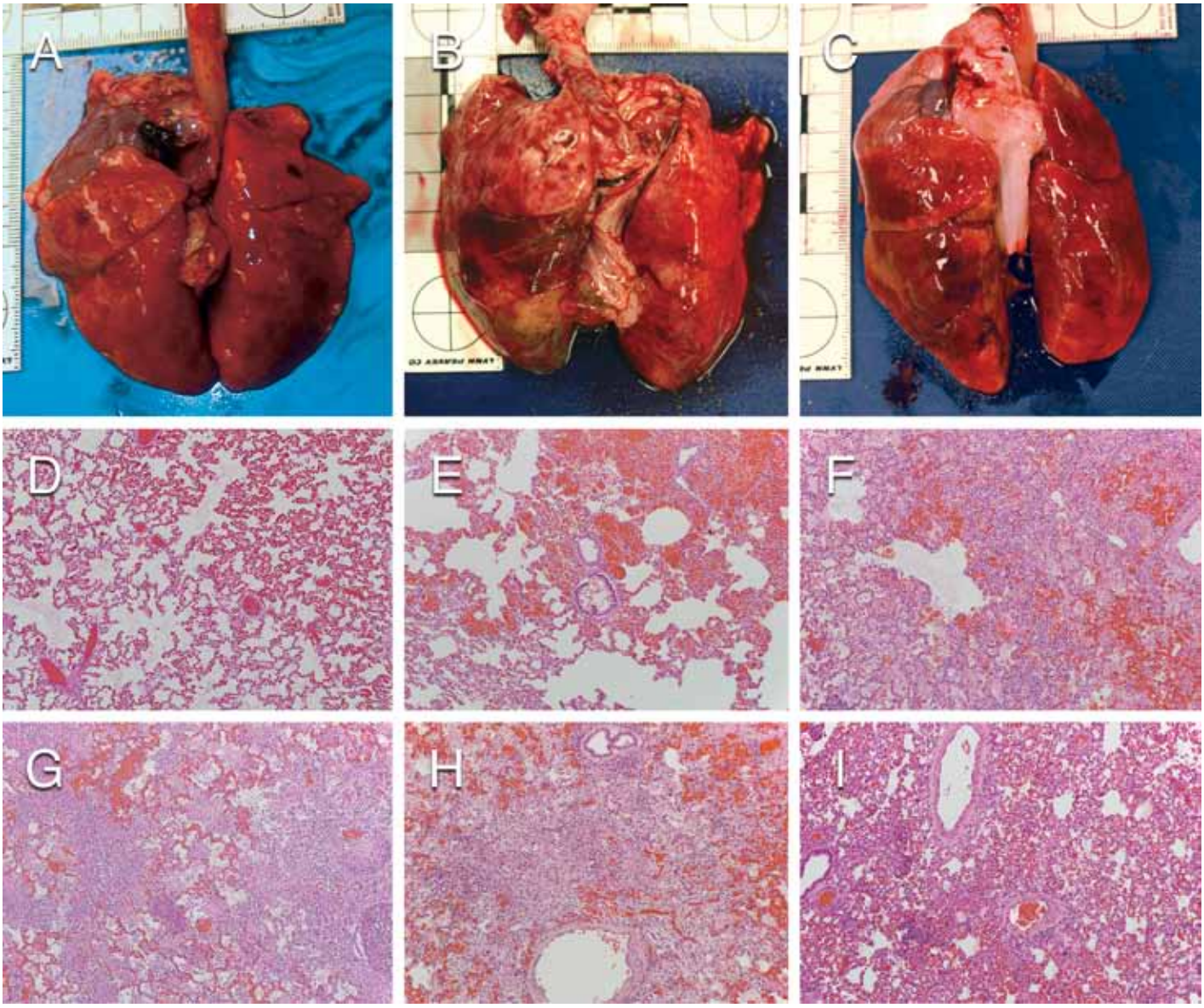

Figure 5. Lung pathology after LHV4 infection in rabbits. Representative gross appearance of lungs from rabbits euthanized at (A) 5 dpi, (B) 7 dpi, and (C) 14 dpi, demonstrating progressive hemorrhage and areas of pulmonary consolidation following infection. Photomicrographs of lung tissue from (D) unmanipulated control, (E) 3 dpi, (F) 5 dpi, (G) 7 dpi, (H) 14 dpi, and (I) 22 dpi demonstrate progressive edema, congestion, hemorrhage, mixed inflammatory cell infiltrates, and parenchymal necrosis and consolidation from 3 dpi to 14 dpi, with partial resolution of microscopic changes by 22 dpi. Hematoxylin and eosin; magnification, 100×.

containing glassy eosinophilic intranuclear inclusion bodies and loss of cilia, patchy submucosal necrosis with infiltrating neutrophils, and moderate to marked frank hemorrhage into the lumen (Figure 7). The edema and congestion within the nasal mucosa was marked by 5 dpi, with focal ulceration of the epithelium. Sections appeared similar at 7 dpi, with the addition of numerous mature and degenerate neutrophils admixed with fibrin and blood within the nasal cavity. There was marked necrosis of the nasal mucosa with extensive epithelial ulceration, submucosal edema, mixed, predominantly neutrophilic infiltrates into the submucosa, moderate to marked turbinate bone atrophy, and fibrinosuppurative exudation within the nasal cavity by 14 dpi. By 22 dpi, proliferation of woven turbinate bone was noted, with moderate osteoblast activity. Mild submucosal fibrosis and edema was present, and the overlying ciliated epithelium appeared normal.

Focal hepatic necrosis was noted grossly at 7 dpi in a single rabbit and consisted of scattered foci of hepatocellular necrosis, local neutrophilic and lymphocytic infiltrates, and intranuclear inclusion bodies within surrounding hepatocytes. There were no gross or microscopic hepatic changes at other times.

Discussion

The current study demonstrated that LHV4 causes significant clinical disease in adult New Zealand white rabbits after intranasal inoculation with a viral challenge, similar to that noted in a previous study.[18] The virus induces a severe bronchopneumo-

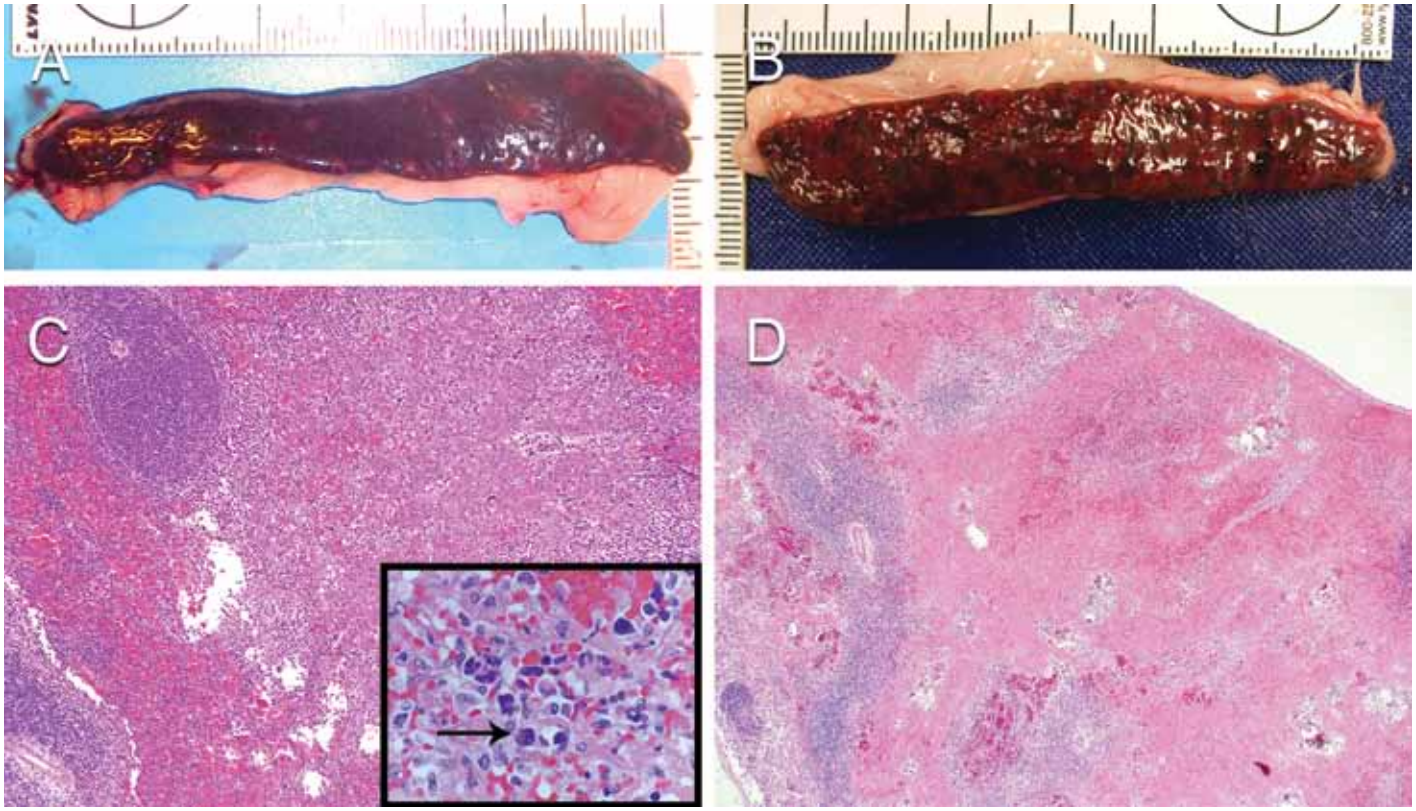

Figure 6. Multifocal splenic necrosis was present between 5 and 7 dpi. Gross photographs of spleens from rabbits euthanized at (A) 5 dpi and (B) 7 dpi demonstrate multifocal areas of acute hemorrhage and necrosis. Photomicrographs of the same tissue from (C) 5 dpi and (D) 7 dpi demonstrate multifocal to coalescing areas of hemorrhage and necrosis primarily affecting the red pulp. Hematoxylin and eosin stain; magnification, 100×. (C) A rabbit at 5 dpi with LHV4 (inset) demonstrates multinucleated syncytia (arrow), some of which contain glassy, eosinophilic intranuclear inclusion bodies typical of herpetic infections. Hematoxylin and eosin stain; magnification, 1000×.

nia, with clinical signs of infection predominantly involving the upper and lower respiratory tract. We provided nonpharmacologic supportive care (fluids and food supplements) through the early course of the disease. However, rabbits maintained beyond 6 to 8 dpi likely require no further interventions, as long as no secondary opportunistic infections occur. Given that *Pasteurella multocida* infections are common in pet and commercial meat rabbit populations, LHV4-associated disease may be more severe in these groups. The pathology at peak infection was similar to that observed in the index case from which the virus was isolated.[5] Intranasal inoculation of rabbits with LHV4 caused ulcerative rhinitis locally, with acute necrotizing, hemorrhagic pneumonia and splenic necrosis developing at peak infection.

Characteristic herpetic intranuclear inclusions were noted only briefly in selected tissues. The lack of distinctive viral inclusion bodies at other time points makes the diagnosis of herpesvirus challenging microscopically, and cases may have been missed in the past. Immunohistochemical assays have not been developed and would be useful for confirming the presence of virus in tissue.

When confronted with marked pulmonary edema and hemorrhage, one of the primary differential diagnoses is rabbit hemorrhagic disease virus (RHDV) infection. The first diagnosis of RHDV in Canada was made recently.[11] In RHD, the nasal discharge is hemorrhagic, and massive hepatic necrosis is often present, potentially facilitating the differentiation of RHD from LHV4 infection. Furthermore, splenomegaly but not splenic necrosis is characteristic of RHDV infection.[1] *P. multocida* is another differential diagnosis that needs to be considered. Peracute, necrotizing *Pasteurella*-induced bronchopneumonia can occur, associated with alveolar necrosis and flooding with fibrinohemorrhagic exudate,[30] similar to that observed at 7 dpi in the current study. In addition, marked necrohemorrhagic pulmonary lesions, similar to those we noted at 5 dpi with LHV4, have been described in immunosuppressed rabbits after intranasal inoculation of *P. multocida*.[16] Hemorrhage and marked pulmonary edema reflect acute lung injury and can be due to numerous conditions, including sepsis, shock, adverse drug reactions, and immune-mediated disease.[3] The combination of pulmonary, splenic, nasal, and skin lesions with the presence of glassy eosinophilic intranuclear inclusion bodies is unique to LHV4 infection.

Other pathologic changes, such as hemorrhagic dermatitis and myocarditis, have been reported to occur in natural infections of rabbits with LHV4[5,19] but were not observed in the current study. These findings may depend on different routes of infection, sex, age, or strain or on coinfection with other agents and were not explored in the current study. Perhaps the most striking difference was the size and extent of skin lesions that have been reported to occur. In the current study, alopecia and ulcerative dermatitis were focal and limited to the face of a few animals, whereas reports from clinical cases describe multiple, 0.3- to 1.5-cm lesions on the dorsum as well as the face.[5,19] Direct contact between

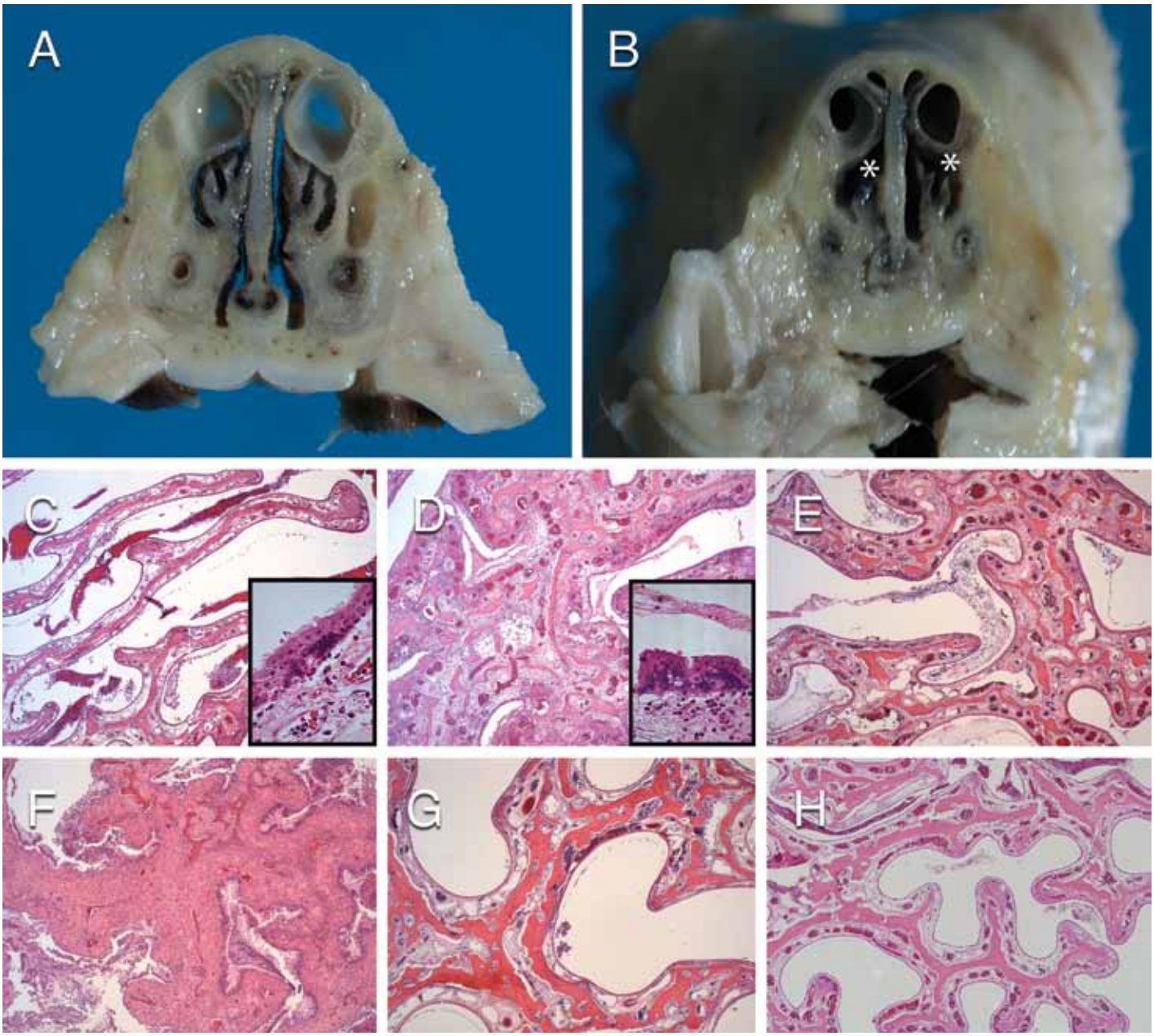

Figure 7. Progressive mucosal ulceration, mucosal, and submucosal edema, hemorrhage, and bony loss of nasal turbinates were seen between 3 and 14 dpi with LHV4 in rabbits. Cross sections of nasal sinuses from (A) a control rabbit and (B) a rabbit euthanized 14 dpi with LHV4 demonstrate moderate bilateral loss of bony turbinates (*) in the infected animal. Photomicrographs of sections of nasal turbinates from rabbits infected intranasally with LHV4 and euthanized at (C) 3 dpi, (D) 5 dpi, (E) 7 dpi, (F) 14 dpi, and (G) 22 dpi demonstrate increasing mucosal and submucosal edema, congestion, and hemorrhage, with loss of underlying bone by 14 dpi. Bony regeneration is present at 22 dpi. Frank blood, degenerate inflammatory cells and sloughed and necrotic epithelial cells are present within the nasal lumen from 3 to 14 dpi. Hematoxylin and eosin stain; magnification, 200×. At 3 dpi (panel C, inset), focal piling of nasal mucosal epithelial cells with underlying syncitia containing intranuclear inclusion bodies are present. Hematoxylin and eosin stain; magnification, 400×. At 5 dpi (panel D, inset), locally extensive ulceration of nasal epithelium was present. The defect is covered by a mat of fibrin and syncytial cells, many with intranuclear inclusion bodies. Fibrin strands are present in the lumen, and the mucosal defect is subtended by moderate submucosal edema and a mixed inflammatory cell infiltrate. Hematoxylin and eosin stain; magnification, 400×.

skin and virus may be necessary for dermal lesions, as is the case with vesicles produced by *Human herpesvirus* 1.[20] We have not attempted to address the cause of abortions reported in the Alaska infection;[19] to do so might require the infection of pregnant does or intravaginal inoculation. Abortions due to *Equine herpesvirus* 1 in equid and murine models both are caused by vascular insufficiency resulting from viral endothelial damage.[35] The myocardial lesions recorded in the LHV4 infection of Alaskan commercial rabbits may be age-related. Experimental *Herpesvirus sylvilagus* infection of juvenile cottontail rabbits leads to myocarditis, which is not observed in adult rabbits.[12]

It is interesting to note that one rabbit in the current study showed no signs of infection, despite being inoculated similarly to other study animals and despite being cohoused with other rabbits shedding infective virus until 14 dpi. We suspect that this aberrant rabbit was misdosed, despite a similar technique as for other animals, but this scenario does not explain lack of virus transmission from cohoused rabbits. Perhaps this rabbit was naturally more resistant to infection, but we did not see evidence of neutralizing or crossreacting antibodies. In the recent Canadian clinical case of natural LHV4 infection, the affected rabbit was cohoused with several other rabbits prior to death. Anecdotally, only 2 of those conspecifics later became sick and were nursed back to health.[5] This finding suggests LHV4 may not be highly contagious among rabbits or that other extenuating factors may be needed to augment the likelihood and severity of infection and clinical disease manifestation.

Clinical samples collected during the infection trial confirmed the utility of standard virologic assays for the diagnosis of LHV4 infection. Virus isolation and PCR assays can be performed on nasal secretions during active infection. PCR analysis provides rapid results with decreased biohazard risk than do virus isolation assays; and PCR amplification also is effective on frozen and formalin-fixed paraffin-embedded tissues. Our future plans include the development of a real-time PCR assay for rapid diagnosis of LHV4 and quantification of virus in tissues. Serum antibodies can be detected as early as 11 dpi by virus neutralization assays and whole-virus ELISA. The use of an ELISA for the detection of antibodies is a rapid, safe, and highly sensitive method compared with a virus neutralization assay. A subunit ELISA assay that uses a viral glycoprotein expressed in a baculovirus vector is being developed. Baculovirus expression systems exist that allow for genomic modification within bacterial carriers and, once designed, produce large quantities of protein with minimal virus.[17] In addition, subunit ELISA can be designed to offer both high specificity and sensitivity. These tests can be used for the diagnosis of active infections and for routine health surveillance.

The risk of LHV4 infection in laboratory rabbits and the source of this novel virus are unknown. The few reported spontaneous cases have arisen in rabbits with access to the outdoors. The virus may have been transferred from wildlife, possibly through insect vectors.[19] However, the location of LHV4 within the genus *Simplexvirus* suggests that the virus may have arisen as a variant of a primate virus that was passed through humans.[22] Although the virus likely has low prevalence, vigilance regarding LHV4 is warranted because the introduction of this pathogen into commercial SPF rabbit populations would be devastating.

In conclusion, intranasal infection of rabbits with LHV4 has provided information about local viral effects on the skin and nasal epithelium and systemic effects in the lung, spleen, and liver. The current study has demonstrated the range of pathology throughout the course of acute infection and into convalescence; these data likely will facilitate accurate postmortem diagnoses. The diagnostic utility of tests for virus, viral DNA, and serum antibodies at different stages of disease has been demonstrated, and the refinement of these assays is in progress. Additional investigations using different routes of infection or ages and life stages of rabbits may provide further information regarding LHV4 pathogenesis.

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