PATHOLOGY AND DIAGNOSIS OF LEPORID HERPESVIRUS-4

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ABSTRACT

Leporid herpesvirus-4 is a novel alphaherpesvirus that has been identified as the cause of mortality in commercial and pet rabbits in North America. Experimental intranasal inoculation of laboratory rabbits has been conducted to determine the clinical course of infection, pathologic findings, virus shedding and development of serum antibodies. Rabbits show peak clinical signs between 3-7 days post infection (dpi) and include respiratory distress, serous nasal and ocular discharge and mild weight loss. Virus can be isolated from nasal secretions during this period and polymerase chain reaction (PCR) can be used to identify the virus. The main histopathologic findings are suppurative, ulcerative rhinitis (3 dpi), multifocal-to-coalescing splenic necrosis (5 dpi) and fibrinosuppurative bronchopneumonia (7 dpi). Characteristic herpesviral intranuclear inclusion bodies are present in these tissues at 3 and 5 dpi. Rabbits begin to recover at 8 dpi, and the presence of neutralizing antibodies can be demonstrated at 11 dpi. In the acute phase of the disease, LHV-4 can be diagnosed based on histopathologic findings or virus isolation from nasal secretions. Serology is useful for diagnosis in convalescent animals.

Key words: Herpesvirus, *Oryctolagus cuniculi*, histopathology, nasal shedding, pneumonia

INTRODUCTION

A new herpesvirus has been identified as the cause of mortality in commercial (Jin *et al.*, 2008a; Jin *et al.*, 2008b) and pet rabbits (Brash *et al.*, 2010) in North America. Characterization of this virus was conducted on an isolate from a commercial meat operation in which there was 50% morbidity and 20% mortality in a herd of mini-Rex and cross-breed rabbits in Alaska (Jin *et al.*, 2008b). Based on sequence information from the ribosome reductase gene, it was found that this virus belonged to the alphaherpesvirus subfamily and was closely related to human and bovine herpesviruses. A virus with similar sequence was also isolated from a pet rabbit in northern Ontario (Brash *et al.*, 2010). Prior cases of fatal herpesviruses in which LHV-4 is the most likely etiological agent have been reported in commercial and pet rabbits in northern Canada (Swan *et al*, 1991; Onderka *et al*., 1992, Wojnarowicz, 2009).

For veterinarians to effectively diagnosis and treat this disease, more information on the clinical course of infection is required. The primary goal of this study is to determine the course of disease in laboratory rabbits and identify opportunities for ante mortem and post mortem diagnosis. The prevalence of this disease in Ontario is also investigated.

MATERIALS AND METHODS

Animals and experimental design

Young female adult New Zealand white rabbits were obtained from Charles River Canada and housed in groups of 3 to 4 in a CCAC-accredited facility. The rabbits were sedated with acepromazine and butorphanol and inoculated in each nostril with 4.2 x 10^4 $CCID_{50}$ in 200 µL of culture media. Two

rabbits were used as sham-inoculated controls and two others were not experimentally manipulated. Rabbits were observed for up to 22 days post infection and treated with dietary support or subcutaneous fluids as necessary. Nasal swabs were collected daily for virus isolation and blood was collected periodically to test for virus neutralization. Three rabbits were serially euthanatized at 3, 5, 7, 14 and 22 days post infection (dpi) with an intravenous barbiturate overdose and full gross necropsy was performed with collection of tissues for histologic examination.

Cell culture and virus isolation

Leporid herpesvirus-4 was isolated from skin samples of a pet rabbit by the Animal Health Laboratory as previously described (Brash et al, 2010), passaged twice in Crandall Feline Kidney (CRFK) cells and stored at -80°C. Nasal swabs were stored in virus transport media (VTM) at -80°C until analysis. At time of analysis, the VTM was centrifuged and duplicate 100 µL aliquots were added to a monolayer of CRFK and observed for up to 5 days for cytopathic effect (CPE).

Virus neutralization assay

Serial serum dilations were incubated with 100 $CCID_{50}$ virus particles for 1 hour at 37°C in a 96 well plate. CRFK cells (2.5×10^4) were added to each well and plates monitored for CPE. Positive control antibody was generated in rabbits using inactivated virus and adjuvant. Serum samples were also obtained from 200 healthy meat rabbits at time of slaughter and 20 sick pet rabbits referred to the Avian and Exotic clinic at the Ontario Veterinary College (Guelph, ON) and tested in a similar manner by the Animal Health Laboratory, University of Guelph (Guelph, ON).

Polymerase chain reaction (PCR)

Total DNA was isolated from cell culture supernatant or scrolls of paraffin-embedded tissues using DNA mini kits (Qiagen). Specific primers were selected that amplify a 138 bp sequence within the ribosome reductase 1 gene of LHV-4 and are not present in HSV-1, HSV-2, BoHV-2, *Oryctolagus cuniculi* or *Felis cattus* genomes. PCR amplification was performed in a 25 uL solution containing up to 100 ng of template DNA, 1.0U of Platinum Taq polymerase (Invitrogen), PCR Buffer solution (Invitrogen), 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.4 µM forward and reverse primers. This solution was subjected to 94°C for 2 minutes followed by 35 cycles of 94°C for 30 seconds, 63°C for 30 seconds, and 72°C for 30 seconds. A final incubation at 72°C for 10 minutes and maintained at 4°C.

RESULTS AND DISCUSSION

Rabbits inoculated with the virus develop clinical signs starting at 3 dpi and resolving by 10 dpi (Table 1). Initial signs at 3 dpi are sneezing or serous nasal discharge. All except one rabbit developed respiratory signs by 5 dpi which included serous nasal discharge, sneezing and laboured breathing. In its most severe form, rabbits would lift their heads to breathe and were cyanotic. Overall, rabbits were quieter during the acute phase of the disease and weight loss was noted during this period.

Table 1: Progression of clinical signs (number affected/total).

Days post infection	Predominant respiratory signs	Average Weight (kg)
0	No clinical signs	2.77±0.13
3	Sneezing/nasal discharge (6/16)	2.75±0.12
5	Laboured breathing (7/13)	2.64±0.15
7	Serosanguinous nasal discharge (8/10)	2.51±0.21
10	Purulent nasal discharge (2/7)	2.68±0.20
15	Upper respiratory tract stridor (1/7)	2.89±0.27

Significant histopathologic findings were identified in the nasal turbinates, lung and spleen. Focal epithelial ulceration in the nasal mucosa and distinctive herpetic intranuclear inclusion bodies within syncytial cells were the first evidence of disease. This progressed to a more extensive suppurative

rhinitis with ostelytic lesions of the turbinates. Lesions in the lung appeared as multifocal hemorrhage, progressing to an acute fibrinosuppurative, necrotizing bronchopneumonia affecting approximately 90% of the lung by 7 dpi. This is characterized by a marked neutrophilic infiltrate within bronchioles and effacement of pulmonary and is accompanied by marked fibrin deposition, blood and edema fluid. Within the spleen multifocal necrosis of red pulp is seen at 5 dpi, and progresses to affect more than 75% of the red pulp by 7 dpi. By 14 dpi, the splenic lesions are absent and the spleen has recovered.

Table 2: Histopathology of infected rabbits.

Days post infection	Nasal mucosa	Lung	Spleen
3	Focal epithelial ulceration with syncytia and intranuclear inclusion bodies	Focal hemorrhage	-
5	Suppurative rhinitis with epithelial necrosis and intranuclear inclusion bodies	Multifocal hemorrhage, edema	Multifocal necrosis of red pulp
7	Epithelial necrosis, submucosal edema and fibrin deposition	Acute fibrinosuppurative necrotizing pneumonia	Coalescing splenic necrosis (75-100% of red pulp)
10	Suppurative rhinitis	Edema	-
14	Nasal turbinate atrophy	Focal hemorrhage	-

Prior to development of clinical signs, virus was isolated from nasal secretions (Figure 1). Approximately 60% of rabbits were shedding virus at 2 dpi, and all except one of the infected rabbits was shedding by 3 dpi. Shedding continued until 10 dpi. Cytopathic effects were typically observed by 36 hours post infection (hpi). PCR amplification and sequencing of the virus isolated from the nasal swabs confirmed its identity as LHV-4 fulfilling Koch's postulates. Virus isolation and followed by confirmation by PCR could be used for ante mortem diagnosis, but requires at least 48 hours for diagnosis.

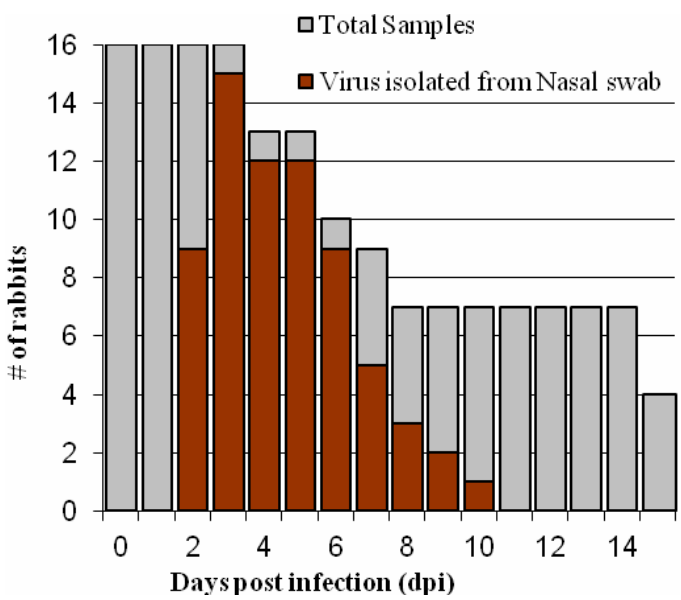

Figure 1: Shedding of virus in nasal secretions

Animals were supported through the acute phase of the disease by providing highly palatable foods and subcutaneous fluids. Neutralizing antibodies were first detected in the serum of infected animals at 11 dpi and were present for the remainder of the study (Figure 2). Animals that are suspected to have recovered from infection with LHV-4 should show a neutralizing titre, providing an additional ante mortem test for the virus. None of the serum samples obtained from healthy commercial rabbits or the sick pet rabbits showed a neutralizing titre. Although the sample size was small, these results indicate that LHV-4 is a rare virus in Ontario.

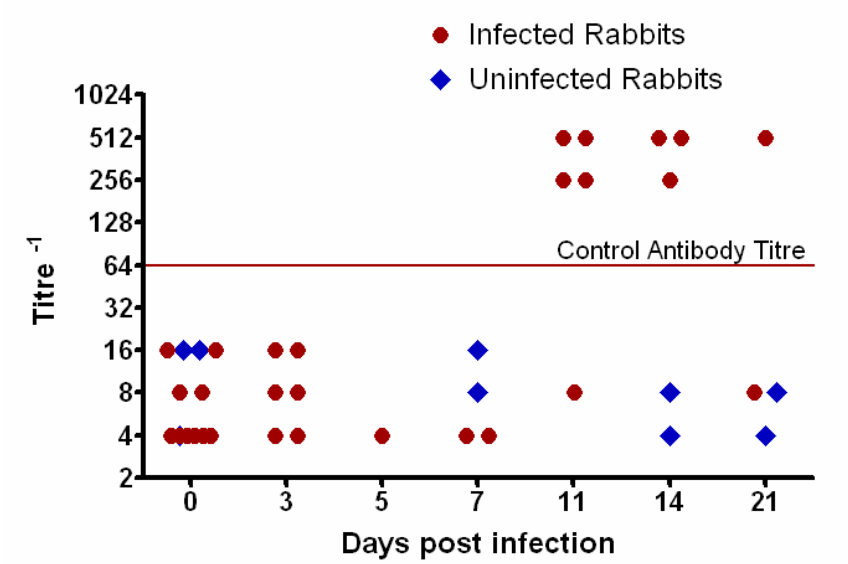

Figure 2: Titres of neutralizing antibody in infected and uninfected rabbits

CONCLUSIONS

This study showed that in specific-pathogen free rabbits, intranasal inoculation of *leporid herpesvirus-4* causes acute suppurative rhinitis, necrosuppurative bronchopneumonia and splenic necrosis, which is manifested clinically by nasal discharge, respiratory distress and mild weight loss. In SPF rabbits, supportive care can ameliorate the disease allowing animals to recover. The contribution of concurrent infectious agents has not been investigated in this study and may have contributed to high mortality rates previously reported. Our preliminary data suggests that this is a rare disease in Canada reflecting the low incidence reported in the literature. The cases reported thus far were in northern regions and outdoor housing may be a contributing factor. Further investigations are being conducted to determine viral sequence information which may reveal further clues as to its origin. Currently, virus isolation and virus neutralization assays can be used to diagnosis the presence of this virus. Conventional PCR can be used to identify virus from fluids and paraffin embedded tissue. Other rapid and sensitive tests that do not require cell culture are also being developed.

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REFERENCES

Brash M.L., Nagy E., Pei Y., Carman S., Emery S., Smith A.E., Turner P.V. 2010. Acute hemorrhagic and necrotizing pneumonia, splenitis and dermatitis in a pet rabbit caused by a novel herpesvirus (*leporid herpesvirus-4*). *Can Vet J. 51: 1383-1386.*

Jin L., Valentine B.A., Baker R.J., Löhr C.V., Gerlach R.F., Bildfell R.J., Moerdyk-Schauwecker M. 2008a. An Outbreak of a Fatal Herpesvirus infection in Domestic Rabbits in Alaska. *Vet Pathol. 45: 369-374.*

Jin L., Löhr C.V., Vanarsall A.L., Baker R.J., Moerdyk-Schauwecker M., Levine C., Gerlach R.F., Cohen S.A., Alvarado D.E., Rohrmann G.F. 2008b. Characterization of a novel alphaherpesvirus associated with fatal infections of domestic rabbits. *Virology. 378: 13-20.*

Onderka D.F., Papp-Vid G., Perry A.W. 1992. Fatal herpesvirus infection in commercial rabbits. *Can Vet J., 33, 539-543.*

Swan C., Perry A., Papp-Vid G. 1991. Alberta. Herpesvirus-like viral infection in a rabbit. *Can Vet J., 32: 627-628.*

Wojnarowicz C. 2009. First Saskatchewan outbreak of a new, fatal herpesvirus infection of rabbits. *Animal Health Perspectives. 5: 3-4.*