Investigation of Leporid herpesvirus 4, an Emerging Pathogen of Rabbits: Infection and Prevalence Studies

by

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ABSTRACT

INVESTIGATION OF LEPORID HERPESVIRUS 4, AN EMERGING PATHOGEN OF RABBITS: INFECTION AND PREVALENCE STUDIES

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Leporid herpesvirus 4 (LeHV-4) is a recently identified alphaherpesvirus that causes lethal respiratory disease in rabbits. Diagnosis has been dependent on the observation of distinctive intranuclear inclusion bodies in affected tissues. The objectives of this body of work were to describe the course of infection in laboratory rabbits, develop a serological test for the detection of antibodies to LeHV-4, and survey Ontario commercial meat rabbits and pet rabbits for LeHV-4 antibody prevalence. Based on the results of an initial dose-range finding pilot study, 22 New Zealand white rabbits were inoculated intranasally with LeHV-4 and monitored for 22 days post-infection (dpi). Clinical signs of infection, including dyspnea, serous oculonasal discharge, pyrexia and weight loss, were evident from 2 to 7 dpi. LeHV-4 was isolated from nasal secretions between 2 and 10 dpi. Gross and microscopic pathology was evaluated and suppurative necrohemorrhagic pneumonia and splenic necrosis were the major findings at peak infection (5 to 7 dpi), at which time eosinophilic herpetic inclusions were present in nasal mucosa, skin, spleen, and lung.

Virus neutralization (VN) assay demonstrated serum antibodies starting at 11 dpi and persisting until the study end (22 dpi). Polyclonal antibodies generated to inactivated virus in laboratory rabbits were neutralizing with low titres of 1:64 or less. Serum samples were obtained from 225 commercial meat rabbits and 24 pet rabbits and all were negative for LeHV-4 antibodies by VN assay. To increase the safety of the assay while expediting rabbit screening for antibody to LeHV-4, an indirect enzyme-linked immunosorbant assay (ELISA) was developed using glycoprotein G (gG) as target antigen. Recombinant LeHV-4 gG was generated using a baculovirus expression system. The presence of gG in infected Spodoptera frugiperda (Sf-21) cells was confirmed by Western blot analysis. With this infected cell lysate as target antigen, the
indirect ELISA had 100% sensitivity and 99% specificity, and can be used to screen rabbits for exposure to LeHV-4. In addition to characterizing the clinicopathological course of the disease in rabbits, this body of work has demonstrated that LeHV-4 is an uncommon disease, and a latent reservoir is unlikely in Ontario domestic rabbits.
Dedication

This thesis is dedicated to my son, Kazuo Alden Neilson. For each thesis, I also have one child. These two are both essential distractions and motivators in this process.
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The last few years of my life have been so fulfilling and I have learned a lot from many different individuals. None of this would have been possible without the support of my husband, Shane Neilson, who always encourages me to follow my dreams. Along with my children, Zdenka and Kazuo, he provides me with a rich family life to return to after a long day in the lab.

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Declaration of Work Performed

All the work presented in this thesis was performed by me, except for the following:

- Selection of primers and optimization of LeHV-4 PCR was done by Angie Darbyson.

- Plates of photos and photomicrographs in Chapter 2 were prepared by Gianni Chiappetta.

- Preparation and purification of LeHV-4 virus for polyclonal antibody production in rabbits was performed by Dr. Éva Nagy.

- Serum samples of the commercial meat rabbits were collected by Peggy Chiappetta and Brianne Davis. The VN assay of the pet and commercial meat rabbits was performed by the Animal Health Laboratory (AHL), University of Guelph using the LeHV-4 virus originally isolated by the AHL from an infected rabbit presented for postmortem examination to the AHL.

- Cutting and H&E staining of formalin-fixed tissues was also performed by the AHL.

- DNA sequencing was performed by the Laboratory Services Division, University of Guelph, Guelph, ON.

- Transformation of AcΔCC-bearing E. coli with pFastBac-gG was done by James Ackford, and many ELISA plates were run by David Leishman and Emily Shantz.

- Case material from the 1991 case of herpesvirus in a rabbit was provided by Helene Philibert, University of Saskatchewan, Saskatchewan, ON.
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Abbreviations

AcMNPV  Autographa californica multicapsid nucleopolyhedrosis virus
AcΔCC  AcMNPV with deleted chitinase and cathepsin
AcΔCC-gG  AcΔCC carrying LeHV-4 glycoprotein G (US4)
BEVS  baculovirus expression vector system
CCID$_{50}$  cell culture infective dose 50%
CRFK  Crandall feline kidney cells
DNA  deoxyribonucleic acid
dpi  days post-infection
E. coli  Escherichia coli
ELISA  enzyme-linked immunosorbent assay
ELVIS  enzyme-linked virus-inducible system
EMEM  Eagles minimum essential medium
FBS  fetal bovine serum
gG  glycoprotein G
H&E  hematoxylin and eosin stain
hpi  hours post-infection
IMDM  Iscove’s modified Dulbecco’s medium
IR  internal repeat
L-15  Leibowitz-15 Medium
LAT  latency associated transcript
mRNA  messenger RNA
miRNA  micro RNA
MOI  multiplicity of infection
ON1  Ontario isolate 1
PBS  phosphate-buffered saline
PCR  polymerase chain reaction
pfu  plaque forming unit
qPCR  quantitative PCR
RHDV  Rabbit hemorrhagic disease virus
RK-13  Rabbit kidney-13 cell line
RNA  ribonucleic acid
RR1 ribonucleotide reductase large subunit gene
S:P sample to positive ratio
Sf-21 Spodoptera frugiperda cell line 21
TBS Tris-buffered saline
TG-ROC two graph receiver operating characteristic
TR terminal repeat
UL unique long
US unique short
VN virus neutralization

Herpesviruses

BoHV-1 Bovine herpesvirus 1
BoHV-2 Bovine herpesvirus 2
BoHV-4 Bovine herpesvirus 4
BoHV-5 Bovine herpesvirus 5
EBV Epstein Barr virus (Human herpesvirus 4)
EHV-1 Equid herpesvirus 1
HCMV Human cytomegalovirus (Human herpesvirus 5)
HSV-1 Herpes simplex virus 1 (Human herpesvirus 1)
HSV-2 Herpes simplex virus 2 (Human herpesvirus 2)
LeHV-1 Leporid herpesvirus 1
LeHV-2 Leporid herpesvirus 2
LeHV-3 Leporid herpesvirus 3
LeHV-4, LHV4 Leporid herpesvirus 4
VZV Varicella zoster virus (Human herpesvirus 3)
Chapter 1: Literature Review

1.1 The family *Herpesviridae*

1.1.1 Properties of herpesviruses

*Herpesviridae* is a large family of enveloped, double-stranded DNA viruses within the order *Herpesvirales* (Table 1.1). Viruses within this order are morphologically similar with large (120-290 kbp) linear genomes, enclosed in a 100-125 nm icosahedral capsid with a triangulation number of 16 (T=16). The capsid is uniquely surrounded by an irregular tegument composed of proteins and enveloped in a lipid membrane with glycoprotein spikes. Total virion size can be as large as 260 nm (Pellett and Roizman, 2007; Davison et al., 2009). Two smaller families in this order include a collection of fish and amphibian viruses within the family *Alloherpesviridae* and a single oyster virus in the family *Malacoherpesviridae*. The majority of known herpesviruses include the hundreds of closely related viruses in the family *Herpesviridae* that infect mammals, birds, and reptiles (International Committee on Taxonomy of Viruses, 2013).

The basic genomic structure of herpesviruses is well-conserved and composed of one to two unique coding segments flanked by terminal repeats that are involved in circularization of the genome within the cell. The genome of Herpes simplex virus 1 (HSV-1; *Human herpesvirus 1; HHV-1*) is divided into two major genomic regions, the “unique long” (UL) and “unique short” (US) segments (Figure 1.1). These segments are flanked at one end by a terminal repeat sequence (TR) that is inverted at the opposite end as the internal repeat sequence (IR). The long and short segments may join together in any orientation, connected by redundant DNA called “a” sequences, resulting in 4 different isomers (McGeoch et al., 1988). Herpesviruses have between 70-200 genes, and a large proportion of the genome is dedicated to proteins and enzymes needed for viral nucleic acid synthesis. Many genomes have been fully sequenced allowing for comparative analysis with newly discovered viruses.

There are approximately 40 conserved genes in the genome of every virus within the family *Herpesviridae*, most of which are essential for the lytic replication cycle (McGeoch et al., 2006). This cycle begins with fusion of cell membrane and virion envelope, which is mediated
by glycoproteins B, H, and L. Following membrane fusion, the nucleocapsid plus tegument coat are released into the cytoplasm and transported to the nucleus. Some tegument proteins are released into the cytoplasm upon fusion and become immediately functional, shutting down cellular functions and promoting transcription of viral products (Cardone et al., 2012). Transcription is executed by cellular RNA polymerase II and begins with the early viral α- or immediate early proteins, which share a common promoter sequence, followed by β-proteins that are required for replication of viral DNA. The DNA is replicated as a rolling circle by viral DNA polymerase. Finally, structural γ-proteins are produced (Honess and Roizman, 1974; Weir, 2001; Roizman et al., 2007). DNA synthesis and capsid assembly occur in the nucleus and a primary envelope is added upon egress from the nucleus. Further proteins are produced in the cytoplasm and layered onto the tegument or added upon final enveloping when the virus is released from the cell (Mettenleiter et al., 2009).

There are two possible outcomes for a cell following infection: lytic infection or establishment of latency. Lytic infection is caused by subversion of cellular energy for virus production (Dubovi et al., 2011). Viral transcription factors, such as VP16, promote transcription of early viral genes (Weir, 2001; Roizman et al., 2007). Translation of viral proteins is favoured through degradation of cellular mRNA by a viral ribonuclease encoded by UL41 and ICP27, a tegument protein that inhibits mRNA splicing required for many cellular proteins (Taddeo and Roizman, 2006; Nojima et al., 2009). Other viral proteins are involved in destabilization of the cytoskeleton by depolymerization of actin (Dubovi et al., 2011). Pathology is observed when lytic infection causes enough damage to relevant cells that host functions are impaired.

Herpesviruses are distinguished by their ability to establish latency post-infection. This is a quiescent state in which viral DNA is present, but there is no production of infectious virus. The viral genome is maintained as a circular episome within the host cell nucleus. A few genes are transcribed to maintain latency, specifically, latency associated transcripts or LATs. LATs are present within the internal repeats and are associated with euchromatin during latency, while the rest of the genome is associated with heterochromatin (Roizman, 2011). Recently, microRNAs (miRNAs) have been found that are derived from the LAT exons (Umbach et al., 2009). These miRNAs are short nucleotide sequences that inhibit the production of the immediate early protein transcription factors, thereby preventing productive replication (Umbach et al., 2008).
1.1.2 Pathology associated with subfamilies of *Herpesviridae*

The family *Herpesviridae* is divided into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae*, which differ in the pattern of disease and lytic cycle that each induces (Pellett and Roizman, 2007). Alphaherpesviruses are distinguished by a short replicative cycle, efficient cell lysis, and propensity for latency within sensory ganglia. Betaherpesviruses, such as the cytomegaloviruses, are slow to replicate and often induce formation of large syncytia in infected tissues. Gammaherpesviruses are unique in the ability to establish latency in lymphoblastoid tissue. Traditionally, herpesviruses were classified based on host range, pathogenesis, or epidemiology. Technological advances have allowed for classifications based on other virus properties, including ultrastructural characteristics and antigenic properties by virus neutralization (VN) or enzyme-linked immunosorbent assays (ELISA). Phylogenetic analysis is the most current method of classifying viruses and is consistent with most of the prior classification systems (Davison et al., 2009). With a few exceptions, phylogenetic trees of viruses closely follow the evolution of the host species (McGeoch et al., 2006).

1.1.2.1. Alphaherpesviruses

There are four genera within the subfamily *Alphaherpesvirinae* (Davison et al., 2009). Viruses belonging to the genera *Iltovirus* and *Mardivirus* are restricted to avian species and will not be discussed further. The other two genera, consisting of *Simplexviruses* and *Varicelloviruses*, infect mammals. Most viral species within these genera initially infect epithelial cells, and are disseminated to sensory ganglia by neuronal transport where they establish latency.

HSV-1 is the causative agent of herpes labialis and the prototype *Simplexvirus* (International Committee on Taxonomy of Viruses, 2013). Most of the species in this genus are adapted to primates, including humans (HSV-1, HSV-2), Old World monkeys (*Macacine herpesvirus 1, Cercopethicine herpesvirus 2, Papio herpesvirus 1*) and New World monkeys (*Ateline herpesvirus 1, Saimiriine herpesvirus 1*). In their adapted host, simplexviruses typically cause self-limiting infections. HSV-1 in humans induces formation of vesicles on the lips and
oral mucosa, and establishes latency within the trigeminal ganglia. In aberrant hosts, infection often results in fatal encephalitis. Fatal encephalitis due to HSV-1 has been reported frequently in nonhuman primates including marmosets and gibbons (Landolfi et al., 2005; Sekulin et al., 2010). Rabbits are the most common non-primate species that develop fatal herpesviral encephalitis following HSV-1 infection (Muller et al., 2009; Sekulin et al., 2010; Sekulin et al., 2010). *Macacine herpesvirus 1*, also known as herpes B virus, and which is adapted to macaques, is well known as a zoonotic agent causing fatal encephalitis in humans (Huff and Barry, 2003). Encephalitis induced by simplexviruses is unusual in adapted hosts and is most often associated with an abnormal immune response such as with neonates (Conrady et al., 2010). The viral protein UL34.5 appears to be essential for neuroinvasion (Bower et al., 1999; Verpooten et al., 2009).

There are only a few animals other than primates that are known to host simplexviruses, including cattle (*Bovine herpesvirus 2*, BoHV-2), wallabies (*Macropodid herpesvirus 1* and *2*), and domestic rabbits (*Leporid herpesvirus 4*, LeHV-4). BoHV-2 fits the paradigm of a host-adapted species causing self-limiting but severe ulcerative skin lesions on the mammary glands and teats of cattle. Latency is established in sensory ganglia of the spinal cord. Similar infections can be reproduced in guinea pigs and sheep, with intranasal inoculation leading eventually to viral latency in the trigeminal ganglia (Torres et al., 2009; Torres et al., 2010). However, severe systemic illness resulting from infection of wallabies (Callinan and Kefford, 1981) and rabbits (Jin et al., 2008a; Brash et al., 2010) with their respective simplexviruses suggests that they are not the adapted host. Although, most herpesviruses are believed to coevolve with their hosts, the phylogeny of these viruses suggests that they may have jumped from another species (Lee and Smith, 1999; Mahony et al., 1999; Babra et al., 2012).

The majority of alphaherpesviruses belong to the genus *Varicellovirus*, which infect a wide range of mammals, including humans. Like the simplexviruses, these viruses also induce primary infection in epithelial cells, but many also undergo a primary viremic stage in which the virus is distributed to internal organs. Bovine herpesvirus 1 (BoHV-1) infects either respiratory or reproductive epithelium depending on the mode of transmission, but viremia can occur leading to abortion in naïve cows and enteric disease in calves (Engels and Ackermann, 1996). Neurovirulence of herpesviruses occurs by different mechanisms. Bovine herpesvirus 5 (BoHV-5) is neuroinvasive in young cattle and likely mediated by US9, a virion protein with an
undetermined mechanism (Belknap et al., 1994; Chowdhury et al., 2006). Equine herpesvirus 1 (EHV-1) subtypes cause respiratory disease, which is mild in adult horses, but is more severe and sometimes fatal in young, naïve horses (Wilsterman et al., 2011). Viremia occurs following infection of lymphocytes. Subsequent lytic infection of endothelium can result in abortion or neurologic disease (Walker et al., 1999; Pusterla et al., 2009). Varicella zoster virus (VZV, Human herpesvirus 3), the causative agent of chicken pox in humans, is the prototype virus for this genus. VZV has a complicated life cycle with a primary and secondary viremia, and part of its pathology results from an induced vasculitis. Tonsillar infection leads to subsequent infection of CD4+ cells and primary viremia (Ku et al., 2005). Spread of the virus to internal organs, including liver and spleen, leads to a secondary viremia, which later carries virus to the skin. Viral latency is established in sensory ganglia by neuronal transport following this secondary infection of the skin (Valyi-Nagy et al., 2007). Although vasculitis has primarily been associated with varicelloviruses, such as EHV-1 and VZV, infection of corneal endothelium associated with HSV-1 is also seen sporadically (Zheng et al., 2000).

1.1.2.2 Betaherpesviruses

Members of the subfamily Betaherpesvirinae have the largest genomes among herpesviruses, which may have been the result of evolutionary gene duplication or gene capture (Davison and Bhella, 2007). These viruses tend to have longer DNA replication times, which may be the result of their large genome (Rajcani and Durmanova, 2001). The majority of the betaherpesviruses belong to the genera Cytomegalovirus and Roseolavirus, all of which infect primates. The genus Muromegalovirus contains three viruses that infect rodents and the genus Proboscivirus contains a single elephant betaherpesvirus. These viruses are highly host specific, requiring host cells for virus isolation (Rajcani and Durmanova, 2001). Most betaherpesviruses do not cause significant pathology in immunocompetent individuals. Recently mouse cytomegalovirus 2 and bat betaherpesvirus 2 were discovered after speculation and specific searches in mice and bats, respectively (Teterina et al., 2009; Watanabe et al., 2010). Full characterization of these viruses has not yet been conducted and official names have not been assigned.
Human cytomegalovirus (HCMV, *Human herpesvirus 5*) was initially associated with neonatal mortality after investigation of associated lesions of the salivary gland. Infants thought to have died from congenital syphilis were instead found to have distinctive enlarged ductular epithelial cells in the salivary glands, indicative of viral infection (Farber and Wolbach, 1932). The nuclei of these cells had dense, basophilic inclusions surrounded by a clear halo, a characteristic of cytomegaloviruses. HCMV has the largest genome of all the herpesviruses encoding more than 200 proteins. Approximately 30 of these genes are conserved within betaherpesviruses and are important in regulation of replication and cell tropism (Mocarski Jr., 2007). UL24 appears to be necessary for growth in endothelial cells, but not epithelial cells or fibroblasts. UL23, known as a temperance gene, encodes a protein that limits viral replication in fibroblasts, but not epithelial or endothelial cells (Dunn et al., 2003). The molecular mechanism by which this cell-specific regulation occurs is unknown but is likely an important viral survival mechanism.

Disease from HCMV occurs in neonates and immunocompromised individuals. Neuroinvasion and subsequent accumulation of cytomegalovirus occurs in neonates, likely because of their poorly developed blood-brain barrier. Initial infection occurs in acinar and ductular epithelia of secretory glands, especially the salivary gland. Persistent low level replication may be maintained in epithelial cells, and viral shedding occurs in secretions, including saliva, ocular secretions, and milk. The highest risk in adults is seen following immunosuppression associated with solid organ transplants, which can directly result in acute hepatitis and pneumonitis or indirectly lead to graft rejection (Emery, 2012).

There are a few unique properties of betaherpesviruses that deserve to be mentioned. G-protein coupled receptor-like genes are present in the genome and act as chemokine sinks, preventing chemotaxis of neutrophils and instead, attracting carrier cells, such as monocytes. Additionally, infection of the endothelium with resultant inflammation is thought to contribute to the development of atherosclerosis in humans and has been implicated in the pathogenesis of coronary artery disease (Levi, 2001). Effects on infection of monocytes may be related to early onset of immune senescence (Almanzar et al., 2005). The mechanism and significance of these properties are still being explored.
1.1.2.3 Gammaherpesviruses

The strategy of viruses belonging to the subfamily Gammaherpesvirinae is to establish persistent viral infection and latency within the host. Two of the genera specifically target lymphocytes, lymphocryptoviruses that infect primates and rhadinoviruses that infect other mammals. Epstein Barr virus (EBV; Human herpesvirus 4), a type of lymphocryptovirus, infects B cells through binding of viral glycoprotein gp350 to complement receptor 2 (CR2) on the cell surface. A second viral glycoprotein, gp42, interacts with cellular major histocompatibility complex (MHC) class II molecules to trigger fusion of the cell and viral membrane for virus entry into the cell (Hutt-Fletcher, 2007). Latency is the result rather than lytic infection (Ackermann, 2006). The pathology associated with lymphocryptoviruses and rhadinoviruses is a nonspecific response to viral infection leading to lymphocyte proliferation, from which EBV infection and associated pathology in humans gets the name mononucleosis. These viruses have a subset of genes that alter other gene expression, leading to transformation of cells (Ackermann, 2006). Gammaherpesviruses, especially lymphocryptoviruses, are able to transform lymphocytes and almost all Burkitt’s lymphomas in humans are associated with EBV infection (Barton et al., 2011). The three previously identified gammaherpesviruses of rabbits, Leporid herpesvirus 1, 2 and 3 (LeHV-1, LeHV-2, and LeHV-3) most likely belong to the genus Rhadinovirus. Their status as unique viruses has not yet been confirmed and they have not been assigned official names. These viruses are discussed in detail in section 2.3.

The causative agents of malignant catarrhal fever, Alcephaline herpesvirus 1 and Ovine herpesvirus 2, are also gammaherpesviruses and they belong to the genus Macavirus. Unlike the other genera, the primary pathology results from immune system activation in aberrant species. In sheep and wildebeest, these viral infections have minimal impact. However, in cattle and cervids, these viruses cause severe pathology. Recruitment of cytotoxic T cells causes necrosis of infected epithelial cells, resulting in inflammation, ulceration and exudation of the oral and nasal cavities (Ackermann, 2006; Russell et al., 2009). They are similar to simplexviruses in that aberrant hosts are much more susceptible to developing overt disease following infection compared to the adapted host.
1.2. LeHV-4 in commercial and pet rabbits

1.2.1 Discovery of LeHV-4

Identification of LeHV-4 was made from a spontaneous case of infected animals originating from a commercial meat rabbit farm in Alaska (Jin et al., 2008a; Jin et al., 2008b). Affected rabbits initially developed conjunctivitis, which was followed by systemic illness, including depression and anorexia, within 24 hours. Further clinical signs developed during the course of infection, including weakness, respiratory distress, torticollis, weight loss, diarrhea, ulcerative skin lesions, abortions, and ataxia. Infected animals succumbed to the disease in 3 to 7 days and there was 50% morbidity and 29% mortality, although it is unknown whether infected rabbits hosted other concurrent infections, such as Pasteurella multocida. There were 55 rabbits on the index farm and 16 died or were euthanized as a result of the infection. In the following year, conjunctivitis, cutaneous lesions, systemic signs, and abortions recurred in several rabbits on the farm. This recurrence suggested recrudescence from latency, and the rabbitry was depopulated.

The case description focused on post mortem findings in a 6-week old rabbit from this outbreak (Jin et al., 2008a). Gross lesions occurred on the skin in several locations including the mucocutaneous junction of the anal and ocular regions and skin of the dorsum. Multiple hemorrhagic lesions were observed in the heart, spleen, lung and skin. Microscopic evaluation demonstrated eosinophilic intranuclear inclusion bodies characteristic of herpesviruses within respiratory epithelium, epidermis, endothelium, and unidentified dermal mesenchymal cells, which may represent endothelium. Lysates of epithelial cells produced rapid syncytial formation and cell lysis when plated onto cultures of rabbit kidney-13 (RK-13) cells, consistent with an alphaherpesvirus. Further characterization by transmission electron microscopy confirmed the presence of enveloped nucleocapsids with a capsid diameter of 100 nm and total diameter of 120 nm (Jin et al., 2008a). Initially this virus was differentiated from EHV-1 and HSV-1 based on restriction digest pattern with BamH1 (Jin et al., 2008b). This was followed by phylogenetic analysis of the nucleotide sequence of the ribonucleotide reductase large subunit gene (RR1), the most conserved gene among herpesviruses. There were no matches from previously sequenced genomes (Jin et al., 2008a).
A year later, a similar diagnosis was made in a pet rabbit from Northern Ontario (Brash et al., 2010). The rabbit was one of six in the household. Although one rabbit was reportedly blind following the outbreak, the other 5 rabbits were nursed back to health following signs of systemic illness. Similar herpesvirus intranuclear inclusion bodies were noted within epithelial and endothelial cells from the pet animal that died. The virus isolated from this Canadian case was genetically similar to the one identified in Alaskan commercial meat rabbits based on the RR1 gene, and had the same morphology by transmission electron microscopy. Clinical signs included conjunctivitis, facial swelling, and systemic signs of illness. A post mortem investigation revealed necrotizing hemorrhagic pneumonia, generalized endothelial damage, and epithelial infection.

1.2.2 Initial investigations of LeHV-4 pathology

In an infection study conducted by Jin et al. (2008a), combined corneal and intranasal inoculations with a total of $6.4 \times 10^4$ plaque-forming units (PFU) was reported to result in overt disease in four of five domestic rabbits, with one mortality at day five (Jin et al., 2008b). At 4 dpi, viral shedding was at a peak and LeHV-4 DNA was detected in multiple tissues, including spleen, brain, and eye. However, by 14 dpi, LeHV-4 DNA was predominantly found in the trigeminal ganglia. This is strongly suggestive of the development of latency. Specific tests were not conducted by these researchers to differentiate between latency and lytic infection.

Based on the histopathology findings from the reported natural and experimental cases in rabbits from the U.S.A. and Canada, the infection cycle of LeHV-4 appears most closely related to EHV-1. Initial infection by LeHV-4 has been shown within anal, ocular, and nasal epithelium (Jin et al., 2008b). Rapid local spread of virus to deeper tissues likely results in the observed severe and often fatal bronchopneumonia. Although not yet demonstrated, a viremic phase would be necessary for secondary infection of the spleen, skin, placenta, and endothelium. Latency is suspected to occur within neuronal ganglia. It is unusual that no signs of encephalitis have been observed in association with LeHV-4. Absence of encephalitis may be useful in differentiating LeHV-4 from HSV-1 infections in rabbits, since the microscopic appearance of alphaherpesviral inclusions are the same regardless of species. Alternatively, encephalitis may be more sporadic, as in the case of HSV-1 in humans, and has just not been observed to date.
Cases with similar clinical signs and microscopic lesions have been reported previously in commercial meat rabbits in Alberta and British Columbia (Swan et al., 1991; Onderka et al., 1992) in the late 1980’s and early 1990’s. In both cases, there were 3 adult rabbit mortalities noted on each farm. The case in Alberta involved an additional 17 kit mortalities. In these rabbits, epidermal and conjunctival lesions were observed along with systemic signs of infection including anorexia and lethargy. Gross and microscopic evaluations revealed similar findings, as per the northern Ontario case, with syncytia formation, inflammation and necrosis of cells within the skin, spleen, and lungs of affected animals, as well as glassy eosinophilic intranuclear inclusion bodies, which are typical of herpetic infections. Electron microscopy confirmed the presence of herpesvirus particles. Because of the lack of molecular technology at the time, more complete characterization of these viral agents could not be conducted. It is intriguing to consider that these rabbits were also infected with LeHV-4, suggesting much earlier infections in Canadian rabbits. In fact, an even earlier investigation into the cause of respiratory diseases in rabbits led to the isolation of a DNA virus from nasal swabs, which was suspected to be a novel herpesvirus. Electron microscopy revealed 100 nm enveloped virions, consistent with the size of herpesviruses (Renquist and Soave, 1972). Further investigations were not pursued at the time.

1.2.3 Other natural infections with herpesviruses

Prior to identification of LeHV-4, three rabbit-specific herpesviruses had been described; which all belonged to the subfamily Gammaherpesvirinae. LeHV-1 and LeHV-3 are similar gammaherpesviruses that infect wild cottontail rabbits (Sylvilagus floridanus). These two viruses were found independently by researchers trying to isolate other viruses in rabbits. LeHV-1 (Cottontail herpesvirus) was discovered when investigators were searching for lapine specific papillomaviruses (Cebrian et al, 1989). Further research on LeHV-1 has been limited because of the apparent minimal biological impact of the virus on domestic rabbits. LeHV-3 (Herpesvirus sylvilagus) was discovered inadvertently in a primary rabbit kidney cell culture while efforts were being made to isolate a novel rabbit poxvirus (Hinze, 1971), and it is the best studied of the rabbit gammaherpesviruses in terms of biology. LeHV-3 causes lymphoproliferative disease only in cottontail rabbits. Serologic comparison of a few major proteins suggests they are unique viruses (Cajean-Feroldi and Laithier, 1995). Whether they truly are different viruses from each
other remains to be determined (Brabb and Di Giacomo, 2012). Although the definition has been debated through the years, currently it is believed that separate viral species should have several different defining properties and occupy a unique ecological niche (Van Regenmortel et al., 2013). Unfortunately, neither of these viruses are currently available for further study. LeHV-3 infection can induce lymphoproliferative disease and neoplasia in cottontail rabbits (Hesselton et al., 1988), but the virus is unable to establish productive infection in domestic rabbits such as New Zealand white rabbits (*Oryctolagus cuniculi*) (Hinze and Wegner, 1973). No infection trials with LeHV-1 have been reported.

LeHV-2, also known as virus 3 or Herpesvirus cuniculi is a gammaherpesvirus that was first isolated in domestic laboratory rabbits while researchers were investigating the causative agent of chicken pox (Rivers and Tillett, 1923; Rivers and Tillett, 1924a). It was later isolated as a slow growing contaminant of primary rabbit kidney cell cultures (Nesburn, 1969). This virus was initially passaged in rabbits via intratesticular injection, and intranuclear inclusion bodies were reported in the endothelium of the testicle. Initial studies showed that intracerebral inoculation with LeHV-2 resulted in neurologic signs including tremor, ataxia and salivation. Microscopic analysis revealed a nonsuppurative encephalitis with intranuclear inclusion bodies (Rivers and Stewart, 1928). In later investigations, small foci of nonsuppurative encephalitis were observed following intracerebral inoculation, but no neurologic clinical signs were observed (Zygraich et al., 1972). In both studies, erythema was observed at the site of injection from days 4 to 7. LeHV-2 appears to remain at the site of inoculation with no further systemic dissemination. No studies have reported using any intranasal inoculation or oral dosing, but the pathology is significantly different from that of LeHV-4.

HSV-1 has been inadvertently transmitted as a zooanthroponotic infection from human caregivers to pet rabbits (Weissenbock et al., 1997; Muller et al., 2009; Sekulin et al., 2010). Because it is an alphaherpesvirus, HSV-1 is able to establish a productive infection in a broad range of hosts. In all reported cases in rabbits, death has been due to development of severe acute nonsuppurative encephalitis with clinical neurologic signs in evidence prior to death. HSV-1 is an important differential diagnosis for herpesvirus infections in domestic rabbits, but the pathology and clinical signs are very different from those seen with LeHV-4 infection. It is possible that LeHV-4 may be transmitted to humans or other animals, although this has yet to be explored.
1.2.4 Evolutionary origin of LeHV-4

The sporadic and isolated nature of spontaneous LeHV-4 cases is puzzling and the origin of this novel leporid herpesvirus is unknown. Further investigation of the genome and disease pathogenesis may provide additional clues regarding its evolution. Alphaherpesviruses are known to infect multiple species, and it may be that LeHV-4 represents an aberrant infection in the rabbit from another species. However, there are no known herpesviruses reported to date with similar RR1 genes as for LeHV-4. LeHV-4 is also unlikely to be a variant of pre-existing rabbit herpesviruses, such as LeHV-1, LeHV-2, or LeHV-3, as these are gammaherpesviruses with very different biological behaviour. Rapid host adaptation is rare in herpesviruses, as is conversion between subfamilies of viruses, as the double-stranded DNA is replicated with a high degree of accuracy (Cleaveland et al., 2001). Nuclear entry, such as that observed for herpesvirus, was shown to be a barrier for interspecies transmission (Pulliam and Dushoff, 2009; Luis et al., 2013).

Based on the RR1 sequence, LeHV-4 was found to be most similar to BoHV-2 and HSV-1 (Jin et al., 2008b). Following complete genome sequencing, investigation of the phylogeny of the more variable glycoprotein B gene has indicated similar evolutionary relatedness as for the RR1 gene. BoHV-2 is most closely related to LeHV-4 and the two viruses branch together with the Catarrhini (old world primate) simplexviruses and macropodid herpesviruses, supporting the idea that the viruses may have jumped host species in the distant past (Babra et al., 2012). Of 25 genes from BoHV-2 that were analyzed, 24 were more similar to LeHV-4 compared with those from Macacine herpesvirus 1 or HSV-2, although still very distinctive from LeHV-4.

1.2.5 Properties of emerging viruses

One of the ways in which new viruses emerge is from cross-species transmission or spillover (Childs et al., 2007a; Childs et al., 2007b). This requires a common or similar receptor on cells from both species. In the secondary host, the pathology noted post-infection may be very different than that seen in the virus-adapted species, as has been described for the alphaherpesviruses. Virus spillover necessarily requires close contact between the carrier species
and the new host. To be considered unique, sustained transmission must occur within the new host as well as genetic modifications that adapt the virus to the new host.

Pathogen and disease emergence in the human population has been described through the years related to the changes in how humans exist (Cleaveland et al., 2007). For human viral zoonoses, some infections seem to require repeated reintroduction while other viruses adapt to humans quickly and are maintained within the population. Measles and human immunodeficiency viruses are believed to originate from zoonoses, but have adapted well to humans. In contrast, obligate zoonoses such as Q-fever and brucellosis require repeated introduction (Cleaveland et al., 2007). Given the sporadic nature of the reported cases of LeHV-4 infection, it may be that the virus is being repeatedly introduced from an unaffected primary host. Alternatively, because commercial rabbits are infrequently submitted to diagnostic laboratories for pathologic evaluation, it may be that previous cases have been missed, being instead attributed to infections with other microorganisms. Epidemiologic investigations should be conducted to determine whether there is a reservoir of infected rabbits within the commercial meat, laboratory, or pet populations.

Wild rabbits (cottontails) may have been the source of the virus. Although neither the disease nor the virus have been demonstrated in wild rabbits, a case of necrotizing bronchiolitis and interstitial alveolitis with syncytia and intranuclear inclusion bodies was reported in Michigan in a cottontail rabbit in the early 1990’s. The pathology is similar to the LeHV-4 infections, but with no genetic identification of the causative herpesvirus (Schmidt et al., 1992). Bats and wild rodents are other potential sources of novel viruses, although these species tend to transmit RNA viruses (Smith and Wang, 2013). Bats have been shown to be a reservoir for adenovirus, and canine adenovirus 1 and 2 may have emerged from bat adenovirus 2 (Kohl et al., 2012).

Traditionally, it has been believed that RNA mutability is a major property associated with cross-species of viruses, and thus, RNA viruses are more likely to transmit across species than DNA viruses (Cleaveland et al., 2001). This has been demonstrated in a study that showed viruses that replicate in the cytoplasm, such as RNA viruses, are more likely to jump species (Pulliam and Dushoff, 2009). Some recent studies have also indicated that host factors may be more important than virus properties in determining likelihood of cross-species transmission.
Sympatry, multiple species sharing the same living space, and physiologic similarity are highly associated with viral richness in bats and therefore increased likelihood of cross-species transmission (Streicker et al., 2010; Luis et al., 2013). It will be of great interest to determine the origin of LeHV-4.

1.3. Differential diagnosis of LeHV-4 infection

1.3.1 Respiratory diseases of rabbits

The most common etiology of respiratory disease in pet and commercial domestic rabbits is bacterial infection. *Pasteurella multocida* and *Bordetella bronchiseptica* both colonize the upper respiratory tract and are able to cause severe respiratory disease in rabbits (Glavits and Magyar, 1990). *Pasteurella multocida* is the most frequent bacterial pathogen of commercial and pet rabbits and can also be the cause of otitis media, sinusitis, metritis, subcutaneous and internal abscesses, and septicemia (Percy and Barthold, 2007). However, in a recent study of 121 pet rabbits in Europe, *Bordetella bronchiseptica* was found in 52% of pet rabbits with clinical signs of respiratory disease (Rougier et al., 2006). These results contradict earlier studies that suggested *Bordetella bronchiseptica* required *Pasteurella* coinfection to be pathogenic (Deeb et al., 1990), but a direct correlation between colonization and disease was not made. In experimental settings, *Pasteurella multocida* causes fibrinopurulent pleuropneumonia with systemic involvement, including hepatitis and lymphoid atrophy, while rabbits infected with *Bordetella bronchiseptica* had fibrinopurulent pneumonia with only mild pleuritis and no systemic effects (Glavits and Magyar, 1990). Other bacterial agents involved in pneumonia include *Staphylococcus* spp., *Pseudomonas* sp and *E. coli* (Rougier et al., 2006).

Secondary bacterial infections may complicate viral infections and vice versa. Bovine respiratory disease complex is a good example in which viral infections, including bovine herpesvirus 1, bovine parainfluenza-3 virus, and bovine viral diarrhea virus, and stress predispose cattle to developing bacterial pneumonias with agents such as *Pasteurella multocida*, *Mannheimia hemolytica* or *Histophilus somni* (Horwood and Mahony, 2011; Portis et al., 2012). The mechanisms by which viruses may act to increase susceptibility to bacterial infection include destruction of the epithelial barrier via direct lysis or immune system activation (i.e.,
bystander effect), modulation of the immune response, or upregulation of bacterial receptors (Peltola and McCullers, 2004). Secondary bacterial infections may also mask the presence of a viral agent, leaving viral infections undetected. The impact of LeHV-4 in combination with other agents is likely significant. Indeed, early studies into respiratory disease in rabbits demonstrated the presence of a herpesvirus in a colony of rabbits (Renquist and Soave, 1972). At this time, laboratory rabbits were not specific pathogen free. *Pasteurella multocida* was a common problem and it is likely that these rabbits also harboured subclinical bacterial infections.

Young rabbits may host respiratory fungal infections, but these tend to be subclinical and transient. *Aspergillus* spp. granulomas can occur in young rabbits between 2-4 weeks of age, but resolve by 5 weeks with only patchy areas of increased pulmonary macrophages remaining. Resolution is likely related to maturation of the immune system (Matsui et al., 1985). *Pneumocystis carinii* sp. *orvcolagi* is also able to establish infections in young rabbits (Tamburrini et al., 1999; Dei-Cas et al., 2006). Typically, there are no indications of respiratory disease in either of these fungal infections.

In rabbits older than 4 to 5 years, pulmonary neoplasia may underlie clinical signs of respiratory disease. Uterine adenocarcinoma is the most common neoplasia in older, intact female rabbits, occurring in up to 80% of older intact does (Greene, 1959; Ingalls et al., 1964). There is a high rate of metastasis to abdominal organs and the lungs that often results in death (Greene and Saxton, 1938; Walter et al., 2010). Pulmonary metastases from hepatic hemangiosarcoma (Guzman et al., 2000) and osteoblastic osteosarcoma (Ishikawa et al., 2012) have been reported with no clinical signs to indicate respiratory compromise. In contrast, a malignant mixed Müllerian tube tumour with pulmonary metastases was diagnosed in a rabbit with respiratory difficulty (Zadravec et al., 2012). Primary pulmonary neoplasia is rare in domestic rabbits, but a primary pulmonary histiocytic sarcoma has been recently reported (Leissinger et al., 2013). Mediastinal tumours such as thymomas and thymic lymphomas can also contribute to respiratory compromise (Kunzel et al., 2012).

Although rabbits can develop allergic airway disease (Keir and Page, 2008), most reports are only anecdotal in nature and there is no supporting literature regarding this condition.
1.3.2 Viral diseases of rabbits

There are relatively few known viral diseases of rabbits (Brabb and Di Giacomo, 2012; Kerr and Donnelly, 2013). The majority are poxviruses and papillomaviruses that result in proliferative skin lesions. Rabbit (Shope) fibroma virus (Poxviridae), Cottontail rabbit (Shope) papillomavirus and oral papilloma virus all produce proliferative lesions in rabbits that are more severe in domestic rabbits (Oryctolagus cuniculus) than in wild rabbits (Sylvilagus spp.). These viruses are of minor significance to the health of rabbits except for myxomavirus, a Leporipoxvirus. In addition to myxomatous proliferative cutaneous lesions, this virus rapidly leads to death in domestic rabbits, likely through depletion of T-cells and secondary infections. An amyxomatous strain of myxomavirus was associated with hemorrhagic pneumonia (Marlier et al., 2000), but this pathology was likely dependent on exacerbation of concurrent subclinical infections (Marlier et al., 1999; Marlier et al., 2000).

Rabbit hemorrhagic disease virus (RHDV), a calicivirus, can also cause hemorrhagic pneumonia. RHDV primarily infects hepatocytes causing apoptosis and resulting in hemorrhagic hepatitis and disseminated intravascular coagulation (Abrantes et al., 2012). Endothelial and tissue macrophages are also infected with virus, and activation may lead to endothelial damage and disseminated intravascular coagulation, resulting in hemorrhage in multiple organs, especially the lung (Alonso et al., 1998; Ramiro-Ibanez et al., 1999). Hemorrhagic pneumonia can have a similar appearance to field infections with LeHV-4 (Embury-Hyatt et al., 2012). Other nonpathogenic strains of RHDV are known to infect rabbits, but only appear to provide partial protection to more pathogenic strains of RHDV (Strive et al., 2013).

A few other viruses are known to infect rabbits. Rotavirus, coronavirus, adenovirus, and astrovirus are believed to contribute to enteritis complex (Percy et al., 1993; Kerr and Donnelly, 2013). Additionally, viruses with a wide host range including rabies virus and Borna disease virus are minor contributors to neurologic disease in rabbits (Kerr and Donnelly, 2013). Other viruses such as polyomavirus have been found incidentally and do not cause pathology (Percy and Barthold, 2007).

In addition to natural infections, laboratory rabbits have been shown to be susceptible to experimental infections of herpesviruses from humans and cattle, and have been used as experimental models (Zhou et al., 2012). Rabbits have been used extensively for the study of
HSV-1 including mechanisms of latency (Flores et al., 2013), development of corneal keratitis (Morris et al., 2012; Webre et al., 2012) and more recently in the development of HSV-1 based cancer therapies (Zhou et al., 2012). Rabbits have also been used as models of herpes B virus infection (Bennett et al., 1999). As a smaller animal model, rabbits have been used to replace cattle in the study of the neuropathogenesis of BoHV-1 and BoHV-5 encephalitis (Chowdhury et al., 2006; Silva et al., 2010). Most of the research is done on the less host-specific alphaherpesviruses, but rabbits are also a successful model for malignant catarrhal fever (Li et al., 2011). Rabbits have been used unsuccessfully as models for BoHV-4 abortion (Naeem et al., 1990) and the study of chorioretinitis due to HCMV (Dunkel et al., 1993). The susceptibility of rabbits to these experimental infections may complicate diagnosis in the laboratory setting.

1.4. Diagnosis of viral infections

1.4.1 Types of virology tests

There are two main ways of diagnosing viral infections. Direct tests demonstrate the presence of the virus by virus isolation, direct visualization, detection of viral proteins or nucleic acid, or by the biological properties of the virus. Indirect tests demonstrate the presence of antibodies to the virus subsequent to infection. A complete discussion of all diagnostic tests is beyond the scope of this thesis, and is available in reference texts (Storch, 2007; Dubovi et al., 2011). The following sections will focus on the theory relating to tests used in the diagnosis of herpesviral infections.

1.4.1.1. Virus isolation

Virus isolation is the gold standard for direct viral testing. Originally, viruses were propagated in laboratory animals including rhesus macaques (poliovirus) and neonatal mice (Coxsackie viruses) or embryonated eggs (influenza viruses) (Hsiung, 1984). For some viruses such as RHDV, viruses cannot be propagated in cell culture and whole animals are still required for propagation (Abrantes et al., 2012). Starting in the early 20th century, the ability to culture cells decreased the cost and usage of animals for virus propagation, but substantial increases in the study of viruses really began following the isolation of poliovirus in non-neuronal cell cultures in the 1950’s. With the ability to offer cryopreserved cell lines commercially, diagnostic
virology using virus isolation became a common practice in the 1970’s (Leland and Ginocchio, 2007), and herpesvirus isolation became one of the most frequently conducted tests in human diagnostic virology (Hsiung, 1984). Virus isolation is preferred as the isolates can be used to further characterize the virus, however the assay only detects viable virus.

The use of different cell lines and the cytopathic effect caused by the virus isolates can be informative when characterizing a new virus. Differences in cell tropism have been exploited and used to differentiate between poliovirus and other enteroviruses by comparing their infectivity in panels of human cell lines (Hsiung, 1984). Differences in cytopathic effect, such as rounding of cells, cell clumping, and formation of syncytia, can also be used to initiate diagnosis of specific viruses (Leland and Ginocchio, 2007). The discovery of increased susceptibilities of certain cell lines to specific viruses has improved the speed of virus isolation and diagnosis (Hsiung, 1984). As described in Section 1.1.2, the differences in susceptible cell type and time to cytopathic effect form the basis for the classification of subfamilies of the family *Herpesviridae*.

Some viruses will not propagate in cell cultures and other assays must be used for virus characterization. Hemagglutination detects viral cross-linking of red blood cell surface proteins and can be useful for the initial identification of influenza viruses and coronaviruses, which both express hemagglutinin (Storch, 2007). Immunofluorescence, the detection of viruses by fluorescence-labelled immunoglobulins, can be used to detect virus-infected cells directly in a clinical sample. Although less sensitive than cell culture, this method has been useful for detecting cytomegalovirus in peripheral blood, which can otherwise take many days to diagnose by virus isolation (Leland and Ginocchio, 2007). The equipment and expertise to read fluorescence tests has been an impediment in human community hospitals, but this is less of a concern in veterinary medicine, in which virology testing is centralized to animal health laboratories.

Traditional cell culture is limited by both time to diagnosis and specificity. Decreased time to cytopathic effect has been observed with shell vials that are centrifuged following virus inoculation and viruses can be identified with the use of antibody-based staining methods (Leland and Ginocchio, 2007). The use of multiple fluorochromes and several different cell cultures on a single cover slip allows for identification of multiple viruses from a single sample and this technique has been applied to the isolation of adenovirus, HCMV, HSV-1 and HSV-2
using two cell lines (MRC-5 and A549 cells) (Brumback and Wade, 1994). Bioengineered cell culture systems, such as ELVIS (enzyme-linked virus-inducible system), significantly improve time to diagnosis and specificity (Leland and Ginocchio, 2007). In an ELVIS system designed for detection and differentiation of HSV-1 and HSV-2, a reporter gene, such as lacZ, is placed under the control of a promoter that is activated by VP16, which is released upon viral entry into the cell. A visible colorimetric reaction is observed by 24 hours and immunocytochemistry is used subsequently to differentiate between the two viral species (Crist et al., 2004).

1.4.1.2 Detection of viral nucleic acid

Direct detection of virus using molecular methods is rapidly replacing virus isolation as a gold standard (Leland and Ginocchio, 2007). After the initial description of polymerase chain reaction (PCR) in 1985, there was an explosion in the use of this diagnostic tool. In particular, PCR is especially recommended for the detection of viruses with long latencies, such as pseudorabies virus, viruses that only propagate in difficult to maintain cell lines, such as bovine leukosis virus in lymphocytes (Belak and Ballagi-Pordany, 1993) or virus detection in samples that may be toxic to cell culture, such as semen (OIE, 2009).

The greatest advantages of PCR are the increased sensitivity and rapid test results. In the late 1990's, the gold standard for HSV-1 and HSV-2 diagnosis was virus isolation by shell vial culture. In one study, the limit of detection of conventional PCR was 0.3 pfu, more sensitive than virus isolation (Slomka et al., 1998). Sensitivity was further increased with the development of quantitative PCR (qPCR) in which amplification of DNA is detected as the reaction proceeds. With qPCR, sensitivity of virus isolation was found to be between 25% and 78% depending on the study (Espy et al., 2000; Wald et al., 2003). Sensitivity was found to be directly proportional to the number of genome copies with qPCR able to detect as low as 10 genome copies compared to \(10^4\) genome copies necessary for virus isolation. DNA was also found to be more stable than live virus, yielding fewer false negatives as a result of sample preservation (Wald et al., 2003). The versatility and sensitivity of PCR allows for the testing of low titre samples obtained from less invasive sites, including ocular and nasal secretions, milk and feces.

Cross-contamination of samples and lack of specificity was initially a major concern with PCR, but this can be minimized by implementing appropriate laboratory practices (Belak, 2007). Conventional PCR required multiple steps including isolation of DNA from a clinical sample,
amplification of DNA and examining the products by gel electrophoresis. The development of qPCR, in which amplification and detection take place simultaneously, greatly reduces the risk of cross-contamination. The amplification of hydrolysis probes ligated to 20-mer target specific sequences, which emit a detectable signal, adds an additional level of specificity, and is the industry standard in diagnostic PCR (Gunson et al., 2006). With these technological advances, PCR assays have become much more reliable.

The additional power of PCR is its ability to yield more information on the virus. Genetic analysis of isolates can help determine viral epidemiology, which is very important in global monitoring of disease (Belak, 2007). The reaction is flexible allowing for identification of a range of viruses. For example, this multiplex technique can be used for amplification of multiple species of herpesviruses, such as HSV-1, HSV-2 and VZV in a single reaction (Baron et al., 1996), or assays designed to be very specific for a particular viral strain (Druce et al., 2002). As the specificity increases, the risk of missing viruses due to evolving genomes increases, particularly for RNA viruses (Belak, 2007), but this can be overcome by choosing primers in conserved regions of the genome (Gunson et al., 2006). With the appropriate measures in place, PCR is an excellent rapid, sensitive and specific tool for diagnosis of viral infections.

1.4.1.3. Indirect Assay – Antibody detection

Indirect assays detect the response to virus infection by the formation of serum antibodies, which signifies viral exposure (Storch, 2007). The longevity of the humoral antibody response varies among different herpesviral species and can be short-lived or lifelong. For example, infection with HSV-1 and HSV-2 results in lifelong production of serum antibodies (Douglas and Couch, 1970; Zweerink and Stanton, 1981). In contrast, the longevity of serum antibodies formed following BoHV-1 infection is variable, lasting anywhere from 7-21 days following infection with the V155 strain and up to 3 years after vaccination (Biswas et al., 2013). As a simplexvirus, antibodies to LeHV-4 would be expected to last a lifetime and provide a good indication of disease prevalence.

A classical method of detecting serum antibodies is by analysing their effect on propagation of virus in cell culture. Inhibition of virus propagation by serum antibodies is tested by virus neutralization (VN) assay. The hemagglutination-inhibition assay works in a similar way, in which serum antibodies block the hemagglutination of erythrocytes by virus. The VN
assay gives important biological information and is considered the gold standard against which new serologic assays are evaluated (OIE, 1996).

Serum antibodies can also be assayed using molecular techniques. Western blots of infected cell lysates have been used to differentiate between HSV-1 and HSV-2 infections and are used as a confirmatory test in positive cases (Golden et al., 2005). ELISAs are based on a specific antigen-antibody interaction, and the methodology was developed in the 1970’s to replace radioimmunoassays (Lequin, 2005). ELISAs have become a versatile means of quantitative detection of many types of molecules. The simplest model for viral antibody testing involves coating 96-well plates with inactivated virus. Serum is added to the wells and if antibodies are present they bind to the virus. The presence of bound antibody is detected with a secondary antibody linked to an enzyme that catalyzes a reaction involving a colourimetric change. Change in colour is determined by absorbance of light and can be quantified. This method is effective, but requires propagation of virus for coating of the wells as target antigen, which may result in human safety concerns.

1.4.2 Development of a subunit ELISA for the detection of LeHV-4 antibodies

1.4.2.1 Alphaherpesvirus glycoproteins

There are 11 known glycoproteins in the viral envelope of HSV-1 (Table 1.3) and 10 of these are predicted in the genome of LeHV-4 (Babra et al., 2012). Glycoproteins B, H and L are important for cell attachment and entry and are likely to result in highly sensitive tests (Akhtar and Shukla, 2009). However these glycoproteins, along with gM and gN, are conserved across all three herpesvirus subfamilies and assays developed for assessing these proteins are not likely to be specific to LeHV-4. Antibodies directed against glycoprotein D are capable of neutralizing HSV-1 (Koelle and Corey, 2003) and EHV-1 (Foote et al., 2005). Glycoprotein D is present in alpha- and betaherpesviruses and is thought to be associated with specific cell entry. The other glycoproteins are unique to viruses belonging to the subfamily Alphaherpesvirinae.

There have been numerous studies examining the immunogenicity of herpesvirus proteins with a major focus on differentiating between HSV-1 and HSV-2. Fatal encephalitis can develop in newborns that are infected with HSV-2 during the birthing process, and identifying mothers
with latent infections is critical in patient management (Ratnam et al., 2007). These studies have identified glycoprotein G as the best target for distinguishing between HSV-1 and HSV-2 infections (Arvin et al., 1983; Lee et al., 1986). Glycoprotein G is involved in herpesvirus immune evasion by binding to chemokines and preventing migration of leukocytes (Van de Walle et al., 2007). Glycoprotein C can also be used to differentiate between HSV-1 and HSV-2 (Scheper et al., 2010), and is another potential target for the development of a subunit ELISA assay for LeHV-4.

### 1.4.2.2. Glycoprotein expression using baculovirus expression system

There are a number of methods that have been used to produce herpesvirus glycoproteins for use in serologic assays. Originally glycoprotein C was purified from virus-infected cell lysates on columns using monoclonal antibodies (Arvin et al., 1983). Glycoprotein G proteins from EHV-1 and EHV-4 have been expressed as GST-fusion proteins in *Escherichia coli* (Crabb et al., 1995). Both of these methods resulted in proteins that were effective in diagnostic tests, but each has a disadvantage. Purification of viral antigens requires mass production of virus and creates potential laboratory biosafety concerns. Further, glycosylation is a post-translational modification that occurs in eukaryotic cells and the protein produced in bacteria may not have the same antigenicity. Glycoprotein D from *Macacine herpesvirus 1* expressed in *E. coli* appears only as a single band (Liao et al., 2011), likely reflecting a lack of glycosylation. Although these proteins have performed well in diagnostic tests, a system that is better able to mimic glycosylation patterns of the mammalian hosts would be desired for consistent results.

The use of insect cells infected with recombinant baculovirus allows for production of glycosylated proteins, though the pattern may be slightly different than mammalian cells (Harrison and Jarvis, 2006). The glycosylation can be improved through transient expression in mammalian cells, but the protein yield is not as high (Condreay et al., 1999). Although baculovirus proteins can be produced in mammalian cells, virus production does not occur (Volkman and Goldsmith, 1983), which significantly decreases the biosafety risk.

Baculovirus expression systems have been designed by modification of *Autographa californica* multicapsid nucleopolyhedrosis virus (AcMNPV), an insect virus. The gene for the
protein of interest is cloned and replaces the gene for polyhedrin which has a unique promoter that drives synthesis of large amounts of protein that package virus into occlusion bodies just before cell lysis (Hitchman et al., 2009). Commercial kits are available that enable easy genetic manipulation of the viral genome which is maintained as a plasmid or ‘bacmid’ in bacteria. A smaller plasmid vector contains a multiple cloning site and transposon 7 sites for site-specific integration into the bacmid. Baculovirus is reconstructed following transfection into insect cells. Insect cells infected with baculovirus are used subsequently to express the protein of interest (Kato et al., 2010).

Baculovirus vectors have been used for in vitro production of herpesvirus glycoproteins and used as the target antigen for herpesvirus serology. Glycosylation of glycoprotein D and G were the first HSV glycoproteins to be produced in a baculovirus vector (Krishna et al., 1989; Sanchez-Martinez and Pellett, 1991). Higher molecular weight bands were observed when produced in the absence of tunicamycin suggesting effective glycosylation. In the case of HSV-2 gG production, cleavage into a secreted and membrane-associated portion did not appear to occur in insect cells (Sanchez-Martinez and Pellett, 1991). When used in immunoblot assays, the two gG homologues effectively discriminated between HSV-1 and HSV-2 antibodies (Sanchez-Martinez et al., 1991). Full length gG from HSV-2 has been difficult to produce, and the C-terminal fragment, engineered to be secreted from infected insect cells, has been used as a target antigen in an indirect ELISA with moderate sensitivity and specificity (Ikoma et al., 2002). Similar results have been observed with the glycoprotein G from Macacine herpesvirus 1 that effectively discriminated from HSV-1 infected sera in an indirect ELISA (Perelygina et al., 2005). Expression of glycoprotein G in baculovirus vectors appears to be an efficient method of generating immunogenic proteins that are specific to the virus of interest.

### 1.4.3 Validation and implementation of tests in the diagnostic laboratory

There are standards published for validation of diagnostic tests for both human and veterinary medicine (Burd, 2010; OIE, 2012). The guidelines for each are similar, but veterinary diagnostic tests should also include validation for the species of interest. Guidelines for specific types of tests including nucleic acid testing (OIE, 2009; Burd, 2010) and ELISA based assays (OIE, 1996) are also available.
The OIE emphasizes evaluation of the validity of a test to ensure its fitness for the intended purpose (OIE, 2012). The first step in development and validation of a diagnostic test is identifying the goal of the test. In the case of infectious disease testing, the purpose is usually to either confirm presence or absence of a pathogen. There are five main stages to assay development. The first two stages are considered development stages in which the use of the assay is assessed for fitness with its purpose, following which it is optimized and standardized. These stages can usually occur in a research setting. The third stage determines the test characteristics, including analytical sensitivity and specificity, accuracy, and precision. The test should be repeatable between runs and reproducible between laboratories. The last two stages involve ongoing monitoring and evaluation of the assay to ensure ongoing consistency (OIE, 2012).

For optimization and standardization of an indirect ELISA (OIE, 1996), it is recommended to have at least 5 sera samples ranging from low to high concentration collected ideally from 5 different subjects. These samples should be run in triplicate to quadruplicate on five different plates on five different days. The goal for standardization is to have the coefficient of variance between runs and replicates within 20%. Analytical sensitivity is determined based on end-point dilution of the serum; and analytical specificity is assessed by testing sera from animals with similar infections.

To evaluate the performance characteristics of an indirect ELISA, it is necessary to establish a cut-off point above which a test is positive and below which it is considered negative (OIE, 1996). There are multiple ways of establishing these cut-points that may result in high sensitivity or high specificity. Two separate cut-off points may also be chosen with an intervening range of equivocal values. Based on the chosen cut-off point, diagnostic sensitivity and diagnostic specificity can be determined. Large numbers of known samples are required for this stage, with a recommendation of 1000 positive samples and 5000 negative samples. Reproducibility between labs testing the same samples requires the cooperation of other laboratories, with a recommendation of testing 20 samples in three different labs. To determine accuracy, virus neutralization is considered a gold standard, but newer technology may perform better than existing gold standards (Belak, 2007).
### Table 1.1. Taxonomy of select herpesviruses.

<table>
<thead>
<tr>
<th>Order</th>
<th>Family</th>
<th>Subfamily</th>
<th>Genus</th>
<th>Species</th>
</tr>
</thead>
</table>
| Herpesvirales | Herpesviridae | Alphaherpesvirinae | Simplexvirus | Herpes simplex virus 1 *  
|             |              |                 |            | Herpes simplex virus 2  
|             |              |                 |            | Macacine herpesvirus 1  
|             |              |                 |            | Macropodid herpesvirus 1  
|             |              |                 |            | Macropodid herpesvirus 2  
|             |              |                 |            | Bovine herpesvirus 2  
|             |              |                 |            | Leporid herpesvirus 4  |
|             |              | Varicellovirus |            | Varicello zoster virus *  
|             |              |                 |            | Bovine herpesvirus 1  
|             |              |                 |            | Bovine herpesvirus 5  
|             |              |                 |            | Equid herpesvirus 1  |
|             |              | Iltoirus        |            | Gallid herpesvirus 1 *  |
|             |              |                 |            | Gallid herpesvirus 2 *  |
|             |              | Betaherpesvirinae | Cytomegalovirus | Human cytomegalovirus *  |
|             |              |                 |            | Muromegalovirus  
|             |              |                 |            | Murid herpesvirus 1 *  
|             |              |                 |            | Murid herpesvirus 2  |
|             |              |                 |            | Lymphocryptovirus  
|             |              |                 |            | Epstein Barr virus *  
|             |              |                 |            | Kaposi sarcoma herpesvirus  
|             |              |                 |            | Rhadinovirus  
|             |              |                 |            | Saimiriine herpesvirus 2 *  
|             |              |                 |            | Murid herpesvirus 4  
|             |              |                 |            | Macavrus  
|             |              |                 |            | Alcelaphine herpesvirus 1 *  
|             |              |                 |            | Ovine herpesvirus 2  
|             |              |                 |            | Percavirus  
|             |              |                 |            | Equid herpesvirus 2 *  |
|             |              | Gammaherpesvirinae | Unclassified | Leporid herpesvirus 1  
|             |              |                 |            | Leporid herpesvirus 2  
|             |              |                 |            | Leporid herpesvirus 3  |
|             | Malacoherpesviridae |                 |            |                          |
|             | Alloherpesviridae |                 |            |                          |

* indicates type species
Figure 1.1. Basic structure of HSV-1 genome showing terminal repeats (TR), internal repeats (IR) and unique (U) portions of the long (L) and short (S) segments. Also indicated are the areas of terminal redundancy a, a’. Adapted from McGeoch et al., 1988.

Table 1.2. Herpesvirus studies that use rabbits as experimental models

<table>
<thead>
<tr>
<th>Virus</th>
<th>Model</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSV-1</td>
<td>Latency and expression of miRNAs</td>
<td>(Flores et al., 2013)</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Herpetic stromal keratitis - model for reactivation from latency</td>
<td>(Morris et al., 2012; Webre et al., 2012)</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Anticancer therapy with thymidine kinase deleted HSV-1</td>
<td>(Zhou et al., 2012)</td>
</tr>
<tr>
<td>Macacine herpesvirus 1</td>
<td>Infection studies for development of a vaccine</td>
<td>(Bennett et al., 1999)</td>
</tr>
<tr>
<td>EBV</td>
<td>Infection studies</td>
<td>(Sano et al., 2013)</td>
</tr>
<tr>
<td>Ovine herpesvirus 2</td>
<td>Malignant catarrhal fever infection studies</td>
<td>(Li et al., 2011)</td>
</tr>
<tr>
<td>BoHV-1 BoHV-5</td>
<td>Neuropathogenicity studies</td>
<td>(Belknap et al., 1994; Chowdhury et al., 2006)</td>
</tr>
</tbody>
</table>
Table 1.3. Alphaherpesvirus glycoproteins that are predicted in the LeHV-4 genome.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Number of amino acids *</th>
<th>Predicted molecular mass (kDa)*</th>
<th>% Similarity between LeHV-4 and B0HV-2 *</th>
<th>Conserved in all three subfamilies</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL27</td>
<td>Glycoprotein B</td>
<td>878</td>
<td>96.6</td>
<td>66.7</td>
<td>yes</td>
<td>binds to heparan sulphate on cell surface (Cardone et al., 2012)</td>
</tr>
<tr>
<td>UL44</td>
<td>Glycoprotein C</td>
<td>429</td>
<td>47.2</td>
<td>40</td>
<td></td>
<td>facilitates binding of gB to heparan sulfate; blocks binding of C3b to C5 evading the innate immune system (Kostavasili et al., 1997)</td>
</tr>
<tr>
<td>US6</td>
<td>Glycoprotein D</td>
<td>403</td>
<td>44.3</td>
<td>63</td>
<td></td>
<td>binds to nectin-1 or HVEM inducing fusion (Cardone et al., 2012)</td>
</tr>
<tr>
<td>UL22</td>
<td>Glycoprotein H</td>
<td>845</td>
<td>93.0</td>
<td>42</td>
<td>yes</td>
<td>component of fusogen complex (Cardone et al., 2012)</td>
</tr>
<tr>
<td>UL1</td>
<td>Glycoprotein L</td>
<td>156</td>
<td>17.2</td>
<td>54</td>
<td>yes</td>
<td>component of fusogen complex (Cardone et al., 2012)</td>
</tr>
<tr>
<td>US8</td>
<td>Glycoprotein E</td>
<td>504</td>
<td>55.4</td>
<td>38</td>
<td></td>
<td>with gE, important in cell-to-cell spread and formation of syncytia; forms an Fc receptor and binds IgG (Van de Walle et al., 2003)</td>
</tr>
<tr>
<td>US7</td>
<td>Glycoprotein I</td>
<td>335</td>
<td>36.9</td>
<td>35</td>
<td></td>
<td>with gE, important in cell-to-cell spread and formation of syncytia; forms an Fc receptor and binds IgG</td>
</tr>
<tr>
<td>US4</td>
<td>Glycoprotein G</td>
<td>808</td>
<td>88.9</td>
<td>33</td>
<td></td>
<td>secreted portion may bind chemokines (Bryant et al., 2003; Van de Walle et al., 2007)</td>
</tr>
<tr>
<td>UL10</td>
<td>Glycoprotein M</td>
<td>410</td>
<td>45.1</td>
<td>51</td>
<td>yes</td>
<td>involved in export of virions and docking at inner cell membrane (Baines et al., 2007)</td>
</tr>
<tr>
<td>UL49A</td>
<td>Glycoprotein N</td>
<td>97</td>
<td>10.7</td>
<td>33.3</td>
<td>yes</td>
<td>involved in export of virions and docking at inner cell membrane (Crump et al., 2004)</td>
</tr>
<tr>
<td>UL53</td>
<td>Glycoprotein K</td>
<td>334</td>
<td>36.7</td>
<td>56</td>
<td></td>
<td>involved in formation of syncytia with UL20 (Chowdhury et al., 2013)</td>
</tr>
<tr>
<td>US5</td>
<td>Glycoprotein J</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td></td>
<td>antiapoptotic function through granzyme (Jerome et al., 2001)</td>
</tr>
</tbody>
</table>

* Data from Babra et al., 2012.
Research Goals and Objectives

Information on the pathogenesis of LeHV-4 in rabbits is limited to case reports (Jin et al., 2008a; Brash et al., 2010) and a small infection trial designed to fulfill Koch's postulates (Jin et al., 2008b). Details on the route of infection, progression of disease, and prevalence in rabbit populations are essential to assist veterinarians in managing clinical cases. The objectives of this study are as follows:

1. To document the clinical time course of disease, including any associated pathology, following intranasal inoculation of rabbits with LeHV-4 (Chapter 2). The isolate from the pet rabbit case in Northern Ontario in 2007 made an infection trial possible (Brash et al., 2010). Based on the previous reports (Jin et al., 2008a; Jin et al., 2008b; Brash et al., 2010), development of fever, oculonasal discharge and viral shedding were expected. Although pulmonary pathology was not reported in one experimental infection using a high challenge dose of live virus (Jin et al., 2008b), it was hypothesized that LeHV-4 infection can act as the sole agent causing hemorrhagic pneumonia in rabbits. The infection trial also was used to determine if rabbits can survive infection and recover, as was suggested by anecdotal information supplied by a referring veterinarian of the index case (Brash et al., 2010) and recurrence of clinical signs in the Alaskan outbreak (Jin et al., 2008a).

2. To validate and standardize a virus neutralization assay for LeHV-4 antibodies (Chapter 2, 3). Other herpesviruses such as HSV-1 result in the production of serum antibodies that persist throughout life (Douglas and Couch, 1970; Zweerink and Stanton, 1981; Ho et al., 1993). Rabbits that recover from LeHV-4 infection are likely to develop serum antibodies that can be assayed by VN assay. To validate this assay, positive control antibodies were generated in rabbits against an inactivated virus preparation. The polyclonal antibodies were expected to perform similar to serum obtained from rabbits that developed LeHV-4 antibodies subsequent to infection, such as those obtained in the experimental infection.
3. To develop an indirect subunit ELISA assay that will allow for high throughput screening of commercial meat and laboratory rabbits for antibody to LeHV-4 (Chapter 3). Glycoprotein G is used to differentiate serum antibodies of HSV-1 and HSV-2 (Arvin et al., 1983; Lee et al., 1986), and was predicted to be a useful target antigen for the diagnosis of LeHV-4. Glycoprotein G was cloned into a baculovirus vector and expressed in insect cells. Cell lysates were used as coating antigen in indirect ELISA and all serum samples were tested using the gG-indirect ELISA. VN assay was used as a gold standard from which to establish a cut-off point and determine the diagnostic sensitivity and diagnostic specificity of the gG-indirect ELISA.

4. To investigate seroprevalence of LeHV-4 in Ontario rabbits (Chapter 3). LeHV-4 has only rarely been reported. It is thought to be rare within rabbits, but a reservoir may exist in domestic rabbits. To test this hypothesis, serum samples from commercial meat rabbits, laboratory rabbits and pet rabbits were tested by VN assay and indirect subunit ELISA.
Chapter 2: Experimental Infection of New Zealand White Rabbits
(Oryctolagus cuniculi) with Leporid herpesvirus 4

This chapter has been published as:


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 $8.4 \times 10^4$ 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 days post infection (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.

2.1 Introduction

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 (Davison et al., 2009). 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 (Pellett and Roizman, 2007). Phylogenetic trees of the viral species are used to further subclassify viruses into genera, which typically closely follow the evolution of the host species (McGeoch et al., 2006).

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 and other viruses (Hinze, 1971; Cebrian et al., 1989). Minor differences in immunoreactivity between LHV1 and LHV3 have been demonstrated suggesting that they are unique viruses, but complete genetic analyses are lacking (Cajean-Feroldi and Laithier, 1995). LHV3 infection can induce lymphoproliferative disease and neoplasia in cottontail rabbits (Hesselton et al., 1988), but the virus is unable to establish productive infection in domestic rabbits, such as New Zealand white rabbits (*Oryctolagus cuniculi*) (Hinze and Wegner, 1973). 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 (Rivers and Tillett, 1924a; Rivers and Tillett, 1924b). LHV2 was later isolated as a slow-growing contaminant of a primary rabbit kidney cell culture (Nesburn, 1969). Early infection studies with LHV2 demonstrated induction of neurologic signs, including nonsuppurative encephalitis with classic herpetic intranuclear inclusion bodies, after intracerebral inoculation (Rivers and Stewart, 1928). Recent studies suggest the histologic evidence of mild, subclinical encephalitis after infection of New Zealand white rabbits (Zygraich et al., 1972). Natural infections of *Human herpesvirus* 1 (Herpes simplex virus 1) have
been reported in rabbits, resulting in fatal encephalitis (Weissenbock et al., 1997; Muller et al.,
2009; Sekulin et al., 2010).

*Leporid herpesvirus* 4 (LHV4) is a novel herpesvirus that was independently diagnosed and
isolated from commercial rabbits in Alaska and a pet rabbit in northern Ontario (Jin et al., 2008a;
Brash et al., 2010). 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 (Swan et
al., 1991; Onderka et al., 1992). LHV4 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 (Jin et al., 2008b; Brash et al., 2010).
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* (Jin et
al., 2008b; Babra et al., 2012), 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 (Davison et al., 2009), suggesting that
these viral species may have migrated from primates, such as human caregivers, to these other
species (Mahony et al., 1999).

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 (Lipman and Perkins, 2002). 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 (Canadian Council on Animal Care,
1993; Institute for Laboratory Animal Research, 2011). 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 (Dasgupta and BenMohamed, 2011) and for
the study of *Bovine herpesvirus* 1 and *Bovine herpesvirus* 5 (Valera et al., 2008). In addition, rabbits have been used in investigations of *Macacine herpesvirus* 1 (B virus) infections (Bennett et al., 1999). As illustrated with the discovery of LHV2, rabbit cell cultures are often used to isolate viruses (Rivers and Tillett, 1924a). 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 (Onderka et al., 1992). 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 (Jin et al., 2008b). Pathology was conducted only on one animal at the peak of infection, and revealed splenic and lymph node necrosis (Jin et al., 2008b). 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.

### 2.2 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 and the guidelines of the Canadian Council on Animal Care (Canadian Council on Animal Care, 1993; Institute for Laboratory Animal Research, 2011). 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 $1.7 \times 10^5$ 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 (Valera et al., 2008). For the full-scale study, 16 rabbits were inoculated in each nostril with $4.2 \times 10^4$ 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 (Ojkic and Nagy, 2001). 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 (Brash et al., 2010), 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. Protein concentration of the virus concentrate was determined by using a Bradford assay (BioRad, Hercules, CA) and viral concentrate was lysed in 0.05% sArsosine. Immulon 2B 96-well plates (Thermo-Scientific, Rochester, NY) were coated overnight with viral lysate containing 100 ng of viral protein in 200 µL of 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 from which a sample:positive ratio could be calculated.

**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 (LHV4fwd, 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 MgCl₂, 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.

2.3 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, 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 2.1). 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 2.2). Food refusal with weight loss and body weight gain suppression occurred in inoculated rabbits until 7 dpi (Figure 2.2). 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 2.3). 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 $1.46 \times 10^4$ 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\textsubscript{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 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 2.4); a sample:positive (S:P) ratio greater than 0.200 was consistent with a positive reaction. There 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 2.5). 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 2.5). 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 2.6). 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 2.6). 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 2.1). 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 2.7). There was moderate congestion and edema of nasal mucosa at 3 dpi, with scattered intraepithelial multinucleate syncytial cells 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 2.7). 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, consisting 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.

2.4 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 (Jin et al., 2008a). The virus induces a severe bronchopneumonia, 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 (Brash et al., 2010). 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 (Embury-Hyatt et al., 2012). 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 (Abrantes et al., 2012). *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 exudates (Percy and Barthold, 2007), 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* (Jaglic et al., 2008). 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 (Beasley, 2010). 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 (Jin et al., 2008a; Brash et al., 2010) 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 (Jin et al., 2008a; Brash et al., 2010). Direct contact between skin and virus may be necessary for dermal lesions, as is the case with vesicles produced by Human herpesvirus 1 (Kinchington et al., 2012). We have not attempted to address the cause of abortions reported in the Alaska infection (Jin et al., 2008a); to do so might require the infection of pregnant does or intravaginal inoculation. Abortions due to Equine herpesvirus 1 in equid and murine models are both caused by vascular insufficiency resulting from viral endothelial damage (Walker et al., 1999). 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 (Hesselton et al., 1988).

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 (Brash et al., 2010). 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 (Jarvis, 2009). 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 (Jin et al., 2008a). 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 (Mahony et al., 1999). 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.
Figure 2.1. 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 syneytial cells containing intranuclear inclusion bodies (inset, arrow; magnification, 200×); hematoxylin and eosin stain; magnification, 40×.

Figure 2.2. 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.
Figure 2.3. Virus was isolated from nasal secretions of infected rabbits from 1 to 10 dpi, with a peak titer of $1.46 \times 10^4$ 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 2.4. 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).
Figure 2.5. 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×.
Figure 2.6. 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, 100x. (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, 1000x.
Figure 2.7. 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×.
CHAPTER 3. A glycoprotein G based indirect enzyme-linked immunosorbent assay for the detection of *Leporid herpesvirus 4* antibodies in domestic rabbits (*Oryctolagus cuniculi*)

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An indirect enzyme-linked immunosorbant assay (ELISA) has been developed for *Leporid herpesvirus 4* (LeHV-4), a recently identified alphaherpesvirus that causes nonsuppurative pneumonia in domestic rabbits (*Oryctolagus cuniculi*). Glycoprotein G, encoded by the US4 gene, is able to discriminate between closely related simplexviruses. Therefore, the US4 gene from LeHV-4 was amplified from genomic DNA isolated from concentrated LeHV-4 and a hemagglutinin-epitope tag was added to the C-terminus. The amplicon was cloned into the baculovirus expression vector (AcBacΔCC) and transformed into insect cells to generate recombinant baculovirus (AcΔCC-gG). *Spodoptera frugiperda* (Sf-21) cells were infected with AcΔCC-gG and the cell lysates examined by Western blot to confirm the presence of immunoreactive proteins. Antigen was detected from 48 to 96 hours post-infection, with optimal expression at a multiplicity of infection of 4.0. The AcΔCC-gG infected insect cell lysates were subsequently used as target antigen in an indirect ELISA. No antibodies to LeHV-4 were detected by either VN assay or gG-ELISA in a serologic survey of 227 commercial, 24 pet, and 22 laboratory rabbits in Ontario. The presence of serum antibodies in experimentally infected rabbits could be demonstrated with the indirect ELISA. TG-ROC analysis was used to determine a cut-off point of 0.400, at which the gG-ELISA has excellent sensitivity (100%) and specificity (99%) as compared to virus neutralization (VN) assay. This gG-based indirect ELISA is the only serological indirect ELISA described for LeHV-4, and could be used for diagnosis in an outbreak or to screen rabbits to maintain LeHV-4 free populations.

3.1 Introduction

*Leporid herpesvirus 4* (LeHV-4) is an alphaherpesvirus that causes hemorrhagic pneumonia, splenic necrosis, and ulcerative skin lesions in domestic rabbits (Jin et al., 2008a; Brash et al., 2010) with significant morbidity and mortality. Experimental intranasal inoculation of specific pathogen free (SPF) rabbits with LeHV-4 has shown that peak clinical signs occur
between 3 to 7 days post-infection (dpi) with virus shedding occurring between 2 to 10 dpi. With supportive care, some rabbits can recover and neutralizing antibodies are present by 11 dpi (Sunohara-Neilson et al., 2013).

The origin of LeHV-4 is unknown. The virus is a novel member of the genus *Simplexvirus*, distinct from *Human herpesvirus 1* (HSV-1), and appears to be most closely related to *Bovine herpesvirus 2* (Jin et al., 2008b; Brash et al., 2010; Babra et al., 2012). An investigation into the seroprevalence of LeHV-4 may reveal important epidemiologic information. If this is a common host-adapted herpesvirus species, then it can be hypothesized that many pet and commercial domestic rabbits are likely to have been exposed and will have serum antibodies, as was discovered for the macropodid herpesviruses of kangaroos and wallabies (Wilks et al., 1981). The absence of serum antibodies in the rabbit population would indicate that it is either a rare disease, or the virus is not host-adapted to rabbits.

Currently, other than virus isolation in cell culture there are no commercial tests available for the specific diagnosis of LeHV-4. A virus neutralization assay can be adapted to test for serum antibodies, and this is facilitated by the distinct cytopathic effect in cell culture (Jin et al., 2008b; Babra et al., 2012). However, the VN assay takes several days to perform, requires technical expertise, and propagation of live virus. The assay may not be specific for LeHV-4 as cross-neutralizing antibodies have been observed for several primate alphaherpesviruses (Eberle et al., 1989; Perelygina et al., 2005). In addition, domestic rabbits are susceptible to HSV-1 infection (Gruber et al., 2009; Muller et al., 2009; Sekulin et al., 2010), such that any screening or diagnostic test developed for LeHV-4 ideally should be able to differentiate between these viruses.

The use of individual surface glycoproteins as target antigens has improved the specificity of assays developed to detect alphaherpesviruses. Both glycoprotein G and C have been demonstrated to differentiate between antibodies from closely related alphaherpesviruses (Arvin et al., 1983; Eberle et al., 1989; Drummer et al., 1998) and have been used for this purpose in indirect ELISAs (Ikoma et al., 2002; Katz et al., 2012). Glycoprotein G is unique to alphaherpesviruses and is thought to modulate the host immune system by acting as a chemokine binding protein, thereby preventing chemotaxis (Bryant et al., 2003; Van de Walle et al., 2007). The best characterized is glycoprotein G from HSV-2, which is expressed as a 104 kDa precursor
protein and cleaved into a 72 kDa secreted portion and 32 kDa membrane-associated portion (Balachandran et al., 1987; Su et al., 1987). Glycoprotein G is highly variable among viral species. For example, there is only 33% amino acid similarity to glycoprotein G of HSV-2 versus LeHV-4 (Babra et al., 2012), making it a good choice for LeHV-4 specific antibody detection.

The baculovirus expression vector system (BEVS) (Hitchman et al., 2009; Jarvis, 2009) has yielded high expression of glycoprotein G from several herpesviruses (Sanchez-Martinez and Pellett, 1991; Perelygina et al., 2005; Van de Walle et al., 2007), and was chosen as a vector for production of LeHV-4 glycoprotein G. The US4 gene, which is predicted to encode glycoprotein G, was cloned into a baculovirus expression vector from the Ontario LeHV-4 isolate (Brash et al., 2010). Infected insect cell lysates were subsequently used as antigen in an indirect ELISA. Serum samples from commercial, pet and laboratory rabbits were tested for antibodies and compared to results obtained from a virus neutralization assay. We showed that the glycoprotein G indirect ELISA has excellent sensitivity compared with the virus neutralization assay.

3.2 Materials and Methods

Cells and virus

Crandall feline kidney (CRFK) cells were received from the Animal Health Laboratory (Laboratory Services Division, University of Guelph) and cultured in medium containing IMDM (Invitrogen), EMEM and L-15 (Invitrogen) supplemented with 5-10% fetal bovine serum (FBS), penicillin and streptomycin as previously described (Sunohara-Neilson et al., 2013). The Ontario isolate 1 (ON1) of LeHV-4 (Brash et al., 2010) was propagated in CRFK cells.

AcBacΔCC as a bacmid in Escherichia coli (E. coli), the plasmid ΔhispFastBacB in E. coli (Elliot, 2012), and Spodoptera frugiperda (Sf)-21 cells were gratefully received from P. Krell (University of Guelph, Guelph, ON). AcBacΔCC is a mutated form of AcMNPV in which cathepsin and chitinase are not produced (Kaba et al., 2004). Sf-21 cells were maintained in Grace’s insect medium (Invitrogen) supplemented with penicillin, streptomycin, and 10% FBS.
**Generation of recombinant baculovirus**

The US4 gene, which is predicted to encode glycoprotein G (gG), was amplified from genomic DNA isolated from concentrated LeHV-4 (Brash et al., 2010) with the gG2a-fwd and gG2-rev primers (Table 3.1) using HotStar HiFidelity polymerase kit (Qiagen, Gaithersburg, MD) in a 25 uL reaction using 10 to 30 ng DNA and Q-solution, as per the manufacturer’s instructions. The PCR product (LHV4-gG2) was isolated with GeneJet Gel extraction kit (Thermo-Scientific, Ottawa, ON) following kit directions. The PCR product was subsequently amplified with a forward primer incorporating a PstI restriction enzyme site proximal to the start codon (gGPstI-fwd) and a reverse primer incorporating a hemagglutinin (HA)-epitope tag and HindIII restriction enzyme site on either side of the stop codon (gGHAHindIII-rev) to generate LHV4-gGRE. LHV4-gGRE was cloned into the multiple cloning site of ΔHispFastBac by digestion with PstI and HindIII (Fermentas, Ottawa, ON), followed by ligation with T4 ligase to produce pFastBac-gG, and transformed into Top10 E. coli to produce pFastBac-gG. Sequence and orientation of the gG insert were confirmed by PCR using gGSeq-fwd, gGSeq-rev, pFB-fwd and gG2-rev primers. Sequencing was done by the Molecular Biology Unit using the same primers (Laboratory Services Division, University of Guelph, Guelph, ON). pFastBac-gG was transformed into E. coli bearing AcBacΔCC to generate recombinant bacmid. The presence of gG in the bacmid was confirmed by PCR using M13-F(-40) and M13-R primers and then transfected into Sf-21 cells to generate recombinant baculovirus bearing glycoprotein G (AcΔCC-gG).

**Preparation of gG antigen**

Recombinant baculovirus (AcΔCC-gG) was infected into Sf-21 cells with a multiplicity of infection (MOI) from 0 to 10. Cells and media were harvested at 24, 48, 72, and 96 hpi and the cells recovered by low-speed centrifugation. The cell pellet was washed three times in phosphate buffered saline (PBS, pH 6.2) and resuspended in PBS (pH 7.4) supplemented with protease inhibitors (Roche, Mississauga, ON). An aliquot containing 300,000 cells was pelleted by low-speed centrifugation and retained for Western blot analysis. The remaining suspension was sonicated on ice at 100% amplitude for 2 min at 10 s intervals (Sonic Dismembrator, Thermo-Fisher-Scientific, Rochester, NY). Protein concentration was determined by Bradford assay (Bio-Rad, Mississauga, ON).
Polyacrylamide gel electrophoresis and Western blot analysis

Infected Sf-21 cells were lysed in electrophoretic sample buffer (0.06 M Tris, pH 8.0, 10% glycerol, 2% β-mercaptoethanol, 2% sodium dodecyl sulfate, 0.01% bromophenol blue) and separated by sodium dodecyl sulfate 10% polyacrylamide gel electrophoresis (SDS-PAGE). Western blotting onto nitrocellulose membrane (Bio Rad, Mississauga, ON) was conducted as described (Towbin et al., 1992). Blots were blocked in 1% bovine serum albumin (BSA; Fisher-Scientific, Fair Lawn, NJS) in Tris-buffered saline (TBS) for 1 hour at room temperature and overnight at 4°C. Blots were incubated with sera from infected rabbits (1:500) or mouse anti-HA monoclonal antibody (1:10000 dilution) in 1% BSA in TBS-Tween (0.05% Tween 20 in TBS) for 1 h at room temperature. Blots were washed 3 times in TBS-Tween and incubated with secondary antibody, goat-anti-rabbit horse radish peroxidase (Dako, Glostrup, Denmark; 1:5000) or rabbit-anti-mouse horse radish peroxidase (Invitrogen, Mississauga, ON; 1:10000) for 1 h at room temperature. Blots were washed again with TBS-Tween, incubated with Amersham ECL Western Blotting Reagents (GE Life Sciences, Baie d’Urfe, PQ) for 1 min, and visualized with a ChemiDoc imaging system (Bio-Rad, Mississauga, ON).

Enzyme-linked immunosorbent assay

AcΔCC-gG-infected Sf-21 cell lysates and concentrated LeHV-4 were used as primary antigens for the ELISA. Serial checkerboard assays were done to optimize blocking buffer, antigen, and serum dilution. Cells were infected at an MOI of 4 and harvested at 72 hpi and prepared as described above. Immulon-2B ELISA plates (Thermo-Scientific, Rochester, NY) were coated with 200 ng of protein per well in 200 µL of 0.5 M carbonate-bicarbonate buffer (pH 9.6) overnight at 4°C and were blocked with 0.5% fish skin gelatin (Sigma, St. Louis, MO) in PBS-Tween (0.05% Tween 20 in PBS) for at least 1.5 h at 37°C. Plates were incubated with rabbit serum diluted 1:100 in PBS-Tween for 1 h at 37°C, followed by incubation with goat-anti-rabbit antibody conjugated to alkaline phosphatase for 1.25 h at 37°C. Plates were washed 4 times with PBS-Tween after incubation with coating buffer, primary antibody, and secondary antibody. Plates were developed with para-nitrophenyl phosphate substrate (Sigma-fast p-NPP tablets; Sigma, Oakville, ON) and the OD۴۰۵ was determined. Sera from a rabbit with neutralizing titre of 1:1024 was used as a positive control on each plate from which a sample:positive ratio was calculated.
Serum samples and virus neutralisation (VN) assay

Serum was obtained at slaughter from 250 commercial meat rabbits originating from 50 farms across southern Ontario. With the owner’s consent, sera were also collected from 24 pet rabbits presenting to the Ontario Veterinary College for various reasons. Serum samples were collected from 22 New Zealand white rabbits (Charles River Canada, Montreal, PQ) used in an LeHV-4 infection trial before infection and at 3, 5, 7, 14, and 22 dpi (Sunohara-Neilson et al., 2013). Polyclonal antibodies to inactivated virus were generated in rabbits by Pacific Immunology (Ramona, CA) and referred to as hyperimmune serum. Sera from commercial and pet rabbits were analysed by VN assay for LeHV-4 antibodies by the Animal Health Laboratory (AHL, Guelph, ON). Rabbits with a neutralizing titre greater than 1:16 were considered positive. The indirect ELISA cut-off point using Sample:Positive ratio was determined using two graph receiver operating characteristic (TG-ROC) curves as described (Greiner et al., 2000).

Statistics

Results are presented as mean ± standard error. A one-way ANOVA was performed followed by Student-Newman Keuls (SNK) post hoc analysis to determine differences between groups. Linear regression was performed using SigmaPlot 10.0 (Systat software, Chicago, IL).

3.3 Results

Glycoprotein G from LeHV-4

The full sequence of the amplicon that was produced by PCR and cloned into ΔHispFastBacB is 2547 nt, and predicts a protein of 849 aa (Appendix A; Figures A1, A2). This sequence is 94% homologous to the US4 sequence (Mega5.2; (Tamura et al., 2011)) published for the Alaska isolate (Babra et al., 2012). The ON1 isolate also contains an additional 38 amino acid insert (366-404) not present in the published sequence. The nearest matches with other proteins include BoHV-2 (46%) and HSV-2 (25%). A highly conserved transmembrane region is present in all four glycoprotein G homologues. The sequence is being submitted to GenBank, Accession number pending.
Immunoreactivity of proteins based on Western blot analysis

A consistent banding pattern is observed from Western blot analysis of LeHV-4 proteins using virus lysates when probed with serum from infected rabbits at 14 and 22 dpi (Figure 3.1). Two major bands at 135 and 28/30 kDa are observed and less prominent bands are observed at 52, 70, and 80 kDa. There are minor differences in band intensity between rabbit sera. In contrast, hyperimmune sera directed at inactivated LeHV-4s demonstrate a distinctively different banding pattern with five major bands at 24, 46, 80, 92, and 127 kDa. Some of these bands may be nonspecific as the 46 and 92 kDa proteins are also present in uninfected CRFK cell lysates (Figure 3.2). No bands were detected with sera from control rabbits before infection or in hyperimmune sera directed at an unrelated target.

Abundant immunoreactive protein was present in cell lysates from Sf-21 cells infected with the recombinant baculovirus AcΔCC-gG, but not in lysates from uninfected or AcBacΔCC-infected Sf-21 cells. Numerous bands are detected when probed with sera from both infected and hyperimmune rabbits (Figures 3.2, 3.3). The protein is present from 48 hpi to 96 hpi (Figure 3.2), and at all MOI’s tested (Figure 3.3). The infected rabbit serum most strongly detects bands at 45, 54, 50, and 65 kDa, while the hyperimmune serum most strongly detects bands of molecular mass 82, 100 and 110 kDa. The HA-epitope tag detects the bands most strongly at 37, 45, 82, and 100 kDa which have the same mobility of proteins detected by both the hyperimmune and infected rabbit sera. No bands were detected for sera collected from naïve rabbits (data not shown). A corresponding glycoprotein G band (between 83 and 120 kDa) in the LeHV-4 concentrates was not detected.

ELISA and Seroprevalence

An indirect ELISA using AcΔCC-gG infected Sf-21 cell lysates as a target antigen was optimized using serial checkerboard assays. The optimum results are achieved using 200 ng of protein/well from Sf-21 infected cell lysates as target antigen, 0.05% fish skin gelatin as blocking buffer, 1:100 serum dilutions, and 1:5000 dilution of secondary antibody. A cut-off point was determined by two methods. One convention is to take the mean plus two standard deviations of the negative samples. All of the serum samples from commercial and pet rabbits had neutralizing titres less than 1:6 and were considered negative by VN assay. The specificity using this method is 95.7% (Table 3.2). The crossing point of TG-ROC curves is a preferred method that...
maximizes sensitivity and specificity. Rabbits experimentally inoculated intranasally with live virus (Sunohara-Neilson et al., 2013) produced neutralizing antibodies at titres ranging from 1:128 to 1:1024 and generated 15 positive samples for the TG-ROC analysis. Using TG-ROC, maximum sensitivity (100%), and specificity (99.6%) were obtained at a cut-off point of 0.400 (Figure 3.4). An indeterminant range (IR) between 97.5% specificity and 97.5% sensitivity occurs between S:P ratios of 0.25 and 0.41. Negative samples falling within this range were repeated and were all less than 0.25 (data not shown). The unexposed laboratory rabbits had significantly lower S:P ratios compared to conventional rabbits (commercial and pet rabbits) (Figure 3.5). There was a positive correlation between the S:P ratio obtained by gG indirect ELISA and neutralizing titres obtained by virus neutralization assay (Figure 3.6a). A similar trend is observed between values obtained by the gG and whole virus indirect ELISA (Figure 3.6b). Two rabbits that received a subcutaneous injection of inactivated virus with adjuvant had neutralizing titres of only 1:16 and 1:32, but extremely high S:P ratios by indirect ELISA.

3.4 Discussion

An indirect ELISA that uses the glycoprotein G produced in recombinant baculovirus as target antigen has been developed to differentiate LeHV-4 antibody negative from positive serum samples from rabbits. LeHV-4 encodes the longest homologue of glycoprotein G within the alphaherpesviruses. The significance of this finding is unknown. When expressed in Sf-21 cells, the banding pattern of immunoreactivity by Western blot analysis is similar to other glycoprotein G homologues with clusters of glycosylated protein bands detected (Slomka et al., 1995; Bryant et al., 2003). Rabbits exposed to inactivated virus appeared to have immunoreactivity to the full length protein, with a predicted molecular mass of 86 kDa, likely with numerous glycosylated isoforms giving rise to several bands between 83 and 120 kDa. In contrast, the infected rabbits generated a humoral response directed more at a smaller product, between 45 and 65 kDa, which may represent the C-terminal cleavage product. The exact location of the cleavage site of glycoprotein G homologues is unknown, but it is predicted to occur between amino acids 260 and 437 in HSV-2 (Su et al., 1993). If the 45 kDa band represents a C-terminal fragment of the LeHV-4 glycoprotein G homologue, cleavage would be predicted to occur around amino acid
376 and would overlap the region determined for HSV-2. All of the bands appear to contain the HA-epitope tag and are likely to be membrane associated with an intact C-terminus.

The gG-based indirect ELISA has high sensitivity and specificity compared with the VN assay, and can be used as an effective screening tool when using a sample:positive cut off value of 0.400. Repetition of the test or use of VN assay as a confirmatory test is recommended for results in the indeterminate range (0.25 < S:P <0.41). We believe that this assay provides an important alternative to the VN assay for several reasons. Although it takes significant time and cost to prepare the antigen coated plates, the biosafety risk with recombinant baculovirus is much lower because no live mammalian virus is required, and more protein is produced per infected cell. ELISAs can be automated, both increasing the speed of assay set-up and reducing the element of human error in interpreting equivocal cytopathic effect (Leland and Ginocchio, 2007), which is required for the VN assay. Finally, the ELISA requires significantly less serum, only 2 µL are required to run this test in duplicate compared with 25-50 µL for the VN assay. This is important because it can be difficult to obtain sufficient sample for all needed tests in sick or dehydrated rabbits.

There are several refinements that could be made to this test prior to validation. Assay performance is affected by the ‘cleanliness’ of animals, as evidenced by higher S:P ratios for conventionally raised commercial and pet rabbits as compared to SPF rabbits raised for the laboratory. This effect may be due to increased circulating immunoglobulins in conventional rabbits. Much higher than expected S:P ratios were also found for hyperimmune sera, which also contained antibodies that detected proteins in the CRFK cells. For this reason, hyperimmune serum is not a good positive control. It is possible that the hyperimmune serum could be further purified by removal of non-specific immunoglobulins, but this remains to be evaluated. It may also be possible to further purify glycoprotein G from insect cell lysates using lectin-affinity chromatography (Olofsson et al., 1986).

Based on the lack of sequence similarity between gG from LeHV-4 and HSV-1, we suspect that there would be minimal cross-reactivity between antibodies from rabbits infected with HSV-1 versus LeHV-4. However, HSV-1 remains an important differential diagnosis for LeHV-4 in domestic rabbits. We would like to confirm that S:P ratios obtained from HSV-1 positive rabbit serum also fall within the negative range for our test.
No bands corresponding to the molecular weight of recombinant glycoprotein G (86 kDa) were detected in Western blots using whole virus lysates, which indicates that there is a low abundance of glycoprotein G in the virion. This is in agreement with studies on HSV-1 and HSV-2, in which the most abundant proteins are glycoprotein B, C, D and a nucleocapsid protein of 150 kDa (Zweerink and Stanton, 1981; Eberle et al., 1989). The production of monoclonal antibodies or isolation of glycoprotein G antibodies would be useful to confirm the presence of glycoprotein G in the virus concentrate; however, differences in glycosylation patterns in mammalian and insect cells may still affect relative migration in SDS-PAGE (Jarvis and Finn, 1996). A monoclonal antibody to glycoprotein G may also be useful for development of immunohistochemical assays.

Based on our seroprevalence data, there does not appear to be an LeHV-4 virus reservoir in the Ontario rabbits that we tested, including pet, commercial meat, and laboratory rabbits. Both ELISA and VN results were negative indicating that this is an uncommon virus of domestic rabbits in Ontario. Age of rabbits at time of testing may have influenced the results. Most commercial meat rabbits are slaughtered between 11-14 weeks of age, and the laboratory rabbits were of a similar age. In the case of HSV-2, prevalence increases with age and sexual maturity (Smith and Robinson, 2002). Our survey was also limited by the number of pet and laboratory rabbit serum samples. Testing more samples may reveal the presence of LeHV-4 in these populations.

The ELISA developed in this study detects only IgG antibodies and does not detect earlier IgM serum antibodies (Braciale et al., 2007). Following intranasal infection of BoHV-1, IgM antibodies can be detected as early as 2 dpi, whereas IgG antibodies are not detected until 11 dpi, but persist for at least 180 dpi (Spilki et al., 2012). IgM-based assays have also been useful in differentiating primary and latent infections of HSV-2 (Ho et al., 1993). The onset of clinical signs of LeHV-4 infection occurs between 2 and 8 dpi. It is possible that future development of a specific IgM-based ELISA assay could allow for detection of serum IgM antibodies in the early stages of infection and remains to be explored.

The IgG-based assay developed in this study is ideal for detection of latent infection or prior exposure. With the low prevalence determined by our seroprevalence survey, the primary role of this test would be to ensure the population remains free of LeHV-4. For this purpose, a
very sensitive test is desired to ensure detection of disease. In this type of population, false positives may result requiring adjunctive testing. The importance of keeping the population free of LeHV-4 justifies this additional expense.

Acknowledgements

The authors wish to thank the rabbit producers and clients from whom rabbit serum was collected for this study, and thank Peggy Chiappetta and Brianne Davis for collecting the serum at the abattoirs. Special thanks to James Ackford and David Leishman for technical assistance in generating the recombinant baculovirus. This project was supported by the OVC Pet Trust Fund and the OMAFRA-UG Agreement through the Animal Health Strategic Investment fund (AHSI) managed by the Animal Health Laboratory of the University of Guelph.
Table 3.1. Primers used for PCR and sequencing of LeHV-4 glycoprotein G. Stop and start codons are indicated in bold. Restriction enzyme sites are underlined. The sequence encoding the HA-epitope is double-underlined.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>gG2a-fwd</td>
<td>5’-TTT GGG CGT GGT TGC ATA AAG-3’</td>
</tr>
<tr>
<td>gG2-rev</td>
<td>5’-CGC TAG TAC AT C CGC AAT GG-3’</td>
</tr>
<tr>
<td>gGPstI-fwd</td>
<td>5’-AAA CTG CAG AA ATG CAA TCA CAGC-3’</td>
</tr>
<tr>
<td>gGHAHindIII-rev</td>
<td>5’-AAAA AAG CT TTT GTA AGC GTA ATC TGG AAC ATC GTA TGG GTA GTA CAT CCG CAA TGG AAT GTG CGC-3’</td>
</tr>
<tr>
<td>gGSeq-fwd</td>
<td>5’-GAC CAC TTT GTG TGC GTA G-3’</td>
</tr>
<tr>
<td>gGSeq-rev</td>
<td>5’-TCT GCC GTA GCA TTG GGG-3’</td>
</tr>
<tr>
<td>M13 F(-40)</td>
<td>5’-GTT TTC CCA GTC ACG AC-3’</td>
</tr>
<tr>
<td>M13 R</td>
<td>5’-CAG GAA ACA GCT ATG AC-3’</td>
</tr>
<tr>
<td>pFB-fwd</td>
<td>5’-ACAGTTTAGAACCAGAAACAGG-3’</td>
</tr>
</tbody>
</table>
Figure 3.1. Western blot analysis of LeHV-4 whole virus lysates by sera from multiple rabbits. Concentrated LeHV-4 virus was subjected to SDS-PAGE and probed with sera from rabbits inoculated with live virus (infected rabbits) or inactivated virus with adjuvant (hyperimmune sera). Control sera was obtained from rabbits prior to exposure (0 dpi, pre) and hyperimmune sera directed to an unrelated protein (2960).
Figure 3.2. Western blot analysis of Sf-21 cell lysates infected with AcΔCC-gG from 24 to 96 hpi. Sf-21 cells were infected with recombinant baculovirus (AcΔCC-gG) at a multiplicity of infection of 4.0 and harvested at 24, 48, 72 and 96 hpi. Cells were lysed in SDS loading buffer and analyzed by Western blot using A. Infected rabbit serum, B. Hyperimmune rabbit serum, C. HA monoclonal antibody. Lanes: LHV4: concentrated virus; Sf-21: uninfected cells; Bac: Sf-21 cells infected with AcΔCC; CRFK+LHV4: CRFK cells infected with LeHV-4; CRFK no LHV4: uninfected CRFK cells.
Figure 3.3. Western blot analysis of Sf-21 cell lysates infected with AcΔCC-gG at different MOIs. Sf-21 cells were infected with recombinant baculovirus (AcΔCC-gG) at a multiplicity of infection of 0.08, 0.8, 4.0 and 8.0 and harvested at 72 hpi. Cells were lysed in SDS loading buffer and analyzed by Western blot using A. Infected rabbit serum, B. Hyperimmune rabbit serum, C. HA monoclonal antibody. Lanes: LHV4: concentrated virus; Sf-21: uninfected cells; Bac: Sf-21 cells infected with AcΔCC; CRFK+LHV4: CRFK cells infected with LeHV-4; CRFK no LHV4: uninfected CRFK cells.
Table 3.2. S:P ratio cut-off point for ELISA based on standard deviation of samples testing negative by VN assay

<table>
<thead>
<tr>
<th>Negative samples</th>
<th>n=227</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean S:P ratio</td>
<td>0.066</td>
</tr>
<tr>
<td>Standard deviation (SD)</td>
<td>0.062</td>
</tr>
<tr>
<td>Cut-off criteria</td>
<td>mean + 1 SD</td>
</tr>
<tr>
<td>Cut-off point</td>
<td>0.128</td>
</tr>
<tr>
<td>True negatives</td>
<td>202</td>
</tr>
<tr>
<td>Specificity</td>
<td>89.0%</td>
</tr>
</tbody>
</table>

Figure 3.4. Determination of ELISA cut-off points using TG-ROC. Sensitivity and specificity are plotted for different S:P ratios. The crossing point indicates the point of maximum sensitivity and specificity at 0.400. The S:P ratios where sensitivity and specificity are equal to 97.5% indicate the indeterminate zone between which confirmatory testing would be recommended.
Figure 3.5. Average S:P ratios for laboratory, pet and commercial rabbits that were negative by VN assay. S:P ratios (mean ± SEM) of negative samples from laboratory, pet and commercial meat rabbits are shown. * One way ANOVA followed by post hoc SNK analysis indicated that the average S:P ratio of laboratory rabbits was significantly different than either pet or commercial rabbits.
Figure 3.6. Regression analysis to determine relationship of S:P ratios obtained by gG-ELISA to other serologic assays. Serum samples from SPF rabbits collected during an infection trial at 0, 3, 5, 7, 14, and 22 dpi were analyzed by gG-based indirect ELISA and compared to results from A. VN assay and B. Whole virus ELISA. * Neutralizing serum dilution was <1/4.
CHAPTER 4. General Discussion

LeHV-4 is an alphaherpesvirus that has recently emerged as a cause of mortality in rabbits. The virus has been characterized (Jin et al., 2008b; Babra et al., 2012), but all that is known about the disease comes from case reports (Jin et al., 2008a; Brash et al., 2010), and no diagnostic tests are commercially available, except for virus isolation. Many details remain to be investigated including the pathogenesis of LeHV-4 infection, and prevalence in the population. The objectives of this study were to describe the progression of disease, develop safe and efficient diagnostic tests, and survey Ontario rabbits for prevalence of infection. The work presented here will lead to improved diagnosis, which will help with individual case management and LeHV-4 surveillance.

Pathology associated with LeHV-4 infection

An experimental infection was conducted in New Zealand white rabbits and the pathology throughout acute infection and recovery was described (Chapter 2). As expected, clinical signs noted in experimental animals were similar to those reported previously (Jin et al., 2008a; Jin et al., 2008b; Brash et al., 2010), and can form the basis for diagnosis. Most of the clinical signs, such as weight loss and fever, are non-specific signs of illness. LeHV-4 should be high on a list of differential diagnoses when these signs are accompanied by dyspnea. Gross and histopathology findings were also similar to that reported in one clinical case, with necrohemorrhagic pneumonia, splenic necrosis, and intranuclear inclusion bodies indicative of LeHV-4 infection at the peak of infection. However, intranuclear inclusion bodies are not present at all stages, and their absence does not rule out LeHV-4 infection. It is likely that other LeHV-4 infections have remained undiagnosed in the past due to the time at which animals were evaluated in the course of the disease with a lack of herpetic inclusions and prevalence of bacterial infections.

This work provides the first evidence that LeHV-4 alone can induce the pulmonary pathology reported. Experimental infection using the Alaskan isolate was not reported to result in pneumonia (Jin et al., 2008b). Hemorrhagic pneumonia was observed in an experimental infection with an uncharacterized herpesvirus, quite possibly LeHV-4, but Pasteurella multocida
was also isolated, complicating case interpretation (Onderka et al., 1992). In our study, *Pseudomonas aeruginosa*, an environmental microorganism (Urano et al., 1995), was isolated from the lung of one animal, suggesting that LeHV-4 may predispose animals to other opportunistic respiratory infections. Respiratory viruses in other species cause similar predispositions to infections with other agents, for example, BoHV-1 and parainfluenza-3 virus in bovine respiratory disease complex (Horwood and Mahony, 2011; Portis et al., 2012). Therefore, the isolation of bacteria from affected lungs should not preclude the possibility of an underlying viral etiology.

Other alphaherpesvirus infections can cause pneumonia, but these infections are usually more sporadic and less severe than observed with LeHV-4 infection. Many of the veterinary varicelloviruses infect epithelium of the upper respiratory tract and can cause descending infections (Narita et al., 1993; Evermann et al., 2011). Pneumonia is a rare complication of HSV-1 infection, usually associated with young or immunocompromised patients. Focal pneumonia can result from descending infection secondary to tracheobronchitis, while diffuse pneumonia results from disseminated infection (Nash, 1972; Ramsey et al., 1982). The widely distributed pneumonia induced by LeHV-4 infection most resembles a descending infection, but differs in the sparing of the bronchial epithelium and does not depend on impaired immunity. Understanding the mechanism underlying this difference may provide clues to the more rare incidence of pneumonia secondary to other alphaherpesviruses.

Intranasal inoculation was designed to mimic aerosolized transmission and was an effective means of LeHV-4 infection. Lack of infection in one of our study rabbits suggests that aerosol transmission may not be 100% efficient, and direct contact with secretions containing virus may be necessary. This has been demonstrated for other alphaherpesviruses such as BoHV-1, for which aerosol transmission is inefficient (Biswas et al., 2013). Transmission of HSV-1 or HSV-2 depends on direct mucocutaneous contact. Direct contact with oculonasal secretions may account for spread to the genital tract and abortions observed in the Alaskan outbreak (Jin et al., 2008a). LeHV-4 may be similar to BoHV-1 and HSV-1, which are able to infect both genital and oral mucosa (Wald, 2006; Steukers et al., 2011). Abortions may be secondary to ascending infection as a sequelae to grooming, but only virgin female rabbits were evaluated in our study.
LeHV-4 Serology

Serum samples collected from the infection trial confirmed that rabbits surviving infections develop neutralizing antibodies, which also provided us with valid positive controls for the development of serologic assays (Chapter 3). From the persistence of serum antibodies to 22 dpi, we concluded that rabbits exposed to the virus would have persistent antibodies and that an estimate of prevalence could be made from a serologic survey. One animal showed neither clinical signs of infection nor produced neutralizing antibodies. Although there are multiple possible explanations for this observation, including that this animal was misdosed, this could also indicate individual resistance to LeHV-4, involving elimination via interferon and natural killer cells, as has been demonstrated for HSV-1 (Chew et al., 2009). The development of serum antibodies in most of the rabbits supports the use of indirect methods to determine prior exposure.

The sera from rabbits infected with live virus was the most appropriate positive control. Unfortunately, polyclonal antibodies generated in rabbits against inactivated virus did not have the same performance in our assays. They were less sensitive in the VN assay and produced increased background in ELISAs. This was surprising as the use of Freund's complete adjuvant typically generates excellent antibody production (Stills, 2005). Infecting rabbits with live virus for the production of positive control antibody is less desirable as the rabbits suffer the effects of the disease, and optimization of the assay for use with polyclonal antibodies generated from inactivated virus is desired.

No commercial diagnostic test is currently widely available for LeHV-4. Basic virology tests, such as virus isolation and virus neutralization, can be developed de novo, as needed, as distinctive cytopathic effects are observed in cells infected with LeHV-4 (Appendix B: Figures B1, B2, B3, B4). However, these tests require propagation of virus and cell culture, which add to biosafety issues in the diagnostic laboratory. Rapid tests that do not require propagation of virus are safer as evidenced by less stringent containment measures recommended by the Center for Disease Control (Miller et al., 2012). Additionally, ELISAs can be automated, reducing human error and biohazard risk. and increasing efficiency (Leland and Ginocchio, 2007).

We suspected that the virus might be found in a reservoir of domestic rabbits, but found no serologic evidence in the rabbits that were sampled. We tested laboratory, pet and commercial
meat rabbits, and the sera from all these rabbits were negative by VN assay and ELISA. This suggests that LeHV-4 is an uncommon pathogen in Ontario and Quebec rabbits. Although the humoral immune response may wane with time, evidence from HSV-1, HSV-2, and BoHV-1 suggests a long lasting IgG response (Douglas and Couch, 1970; Zweerink and Stanton, 1981; Spilki et al., 2012). Longer infection trials could demonstrate longevity of humoral immune response in rabbits, and may be necessary before implementing an indirect assay as a screening test for LeHV-4. If domestic rabbits are not harbouring the virus, there must be another reservoir that has yet to be discovered.

**Origin of the virus**

The origin of LeHV-4 remains a mystery. All documented cases of infection with LeHV-4 (Jin et al., 2008b; Brash et al., 2010) and a historically similar, but uncharacterized, rabbit herpesvirus (Swan et al., 1991; Onderka et al., 1992) have arisen north of the 49th parallel to date, and an expanded geographic area may be required to find the virus reservoir. Given the profound morbidity and mortality noted in rabbits, it is more likely that the domestic rabbit is not the host-adapted species. Alternatively, the virus may be at a stage before attenuation and host adaptation has occurred. For example, attenuation occurred in myxomavirus after introduction to Australia. Initially the mortality rate was 99.8% mortality rate, but after approximately 50 years, the genome rapidly adapted and mortality is now less than 50% (Kerr et al., 2013). More likely, there may be a reservoir for LeHV-4 within another animal population, which may remain difficult to pinpoint.

One possible host for transmission to domestic rabbits is the wild cottontail rabbit (*Silvilagus floridanus*). It is unknown whether LeHV-4 can infect cottontail rabbits. An uncharacterized herpesvirus was isolated from a moribund cottontail rabbit with bronchopneumonia, splenomegaly, and encephalomalacia (Schmidt et al., 1992). Except for the encephalomalacia, the reported pathology is similar to that observed for LeHV-4 in domestic rabbits. However, unless this particular case was a rare exception, it would appear that LeHV-4 is also not host adapted to cottontail rabbits. Additionally, the range of the cottontail rabbit does not extend to northwestern Canada or Alaska where some of the cases have been reported.
LeHV-4 may be host adapted to another domestic or wild animal. As diagnostic techniques improve, and the ability to detect arrays of viruses becomes less expensive and more readily available, viromes, or the entire set of viruses that infect a species, are being described. Rodents and bats are considered two species that may commonly contribute viruses to other species (Teterina et al., 2009). Simplexviruses have been found in frugiverous bats of South America and Africa (Razafindratsimandresy et al., 2009). One isolate was very closely related to BoHV-2, the nearest neighbour to LeHV-4. Unfortunately, the sequences used to create the phylogeny were not published, and LeHV-4 sequence information was limited at this time. Although it is possible that the virus is emerging from wild animals, there is no overlapping distribution of bats or rodents across the geographic areas in which confirmed or suspected LeHV-4 cases have occurred in domestic rabbits.

Examining the phylogeny of viruses is another way in which virologists explore the origins of emerging viruses (Chan et al., 2013). Genetic information from the LeHV-4 glycoprotein G gene (Appendix A: Figure A3, Table A1) showed a similar relatedness to other herpesviruses based on RR1 and gB analysis (Jin et al., 2008b; Babra et al., 2012). LeHV-4 segregates to the simplexviruses and is most closely related to BoHV-2. Based on the gG phylogeny, LeHV-4 and BoHV-2 appear to be more ancient viruses than the primate viruses. It has been suggested that Chelonid herpesvirus 5, an alphaherpesvirus of turtles, harbours an atypical gene that may be the precursor to the present herpesvirus glycoprotein G gene (Ackermann et al., 2012). It would be interesting to add this gene to the herpetic phylogenetic analysis, but sequence information is not available. Phylogeny is only one method of inquiry and new methods are emerging that may be better at predicting evolution following hybridization of viruses (Chan et al., 2013). If we are able to determine from whence LeHV-4 emerged, additional spontaneous disease outbreaks could be prevented and it might facilitate eradication of the virus.

Implications for rabbit health

There are different concerns surrounding LeHV-4 infection depending on the rabbits infected. Economic impact of LeHV-4 infection is of greatest concern in the commercial meat industry. Although rabbits have the potential to be an excellent protein source with their excellent conversion of forage to meat and reproductive potential (McNitt et al., 1996), the
commercial meat rabbit industry in North America is small and lags behind European and Asian counterparts (Lukefahr et al., 2004). *Pasteurella multocida* is a common respiratory pathogen of commercial rabbits. Widespread co-infection with LeHV-4 could have a devastating impact on this sector. Depending on individual on-farm prevalence of LeHV-4 infection, producers might need to consider management and preventive strategies, as disease outbreaks can lead to shed and/or farm quarantine (Jin et al., 2008a). The availability of an indirect ELISA may allow for rapid diagnosis and screening of rabbits for latency.

The possibility of a latent herpesvirus in laboratory rabbits could have devastating impact on experimental results, especially since rabbits are commonly used as models for HSV-1 infection (Webre et al., 2012; Zhou et al., 2012; Flores et al., 2013). Laboratory facilities may consider using the screening test to ensure that rabbits are free of antibody to LeHV-4, as LeHV-4 infection may interfere with animal studies. Because research animals are housed at high densities, facilities are expected to have appropriate plans in place to monitor for infectious disease on a regular basis (Canadian Council on Animal Care, 1993). Pulmonary hemorrhage and congestion similar to that seen with LeHV-4 infection were observed in a study investigating pulmonary effects of methicillin resistant *Staphylococcus aureus* infection (Strandberg et al., 2010). It would seem critical to ensure that the pathology observed experimentally was a result of infection with *S. aureus* and not an undiagnosed viral infection. The development of the rapid antibody screening test in the current body of work will assist research facilities to keep their rabbits free of LeHV-4.

The occurrence of LeHV-4 in pet rabbits raises a potential concern for zoonotic spread to owners. Infection of aberrant hosts with simplexviruses can be fatal. For example, frequent reports of fatal herpetic encephalitis due to HSV-1 transmitted from owners have been reported in rabbits (Muller et al., 2009; Sekulin et al., 2010). Although rabbit to human transmissions of herpesviruses have not been reported, humans can acquire fatal herpetic encephalitis from macaques (Elmore and Eberle, 2008) and the possibility of transmission of LeHV-4 to humans exists. In vitro, LeHV-4 infects Vero cells but not HeLa cells (Appendix B: Figure B4), and it may not pose zoonotic risk, but this cannot be definitely concluded without further study. No illness has been reported in pet owners associated with the LeHV-4 infection in their pet rabbits, but owners may not have thought to correlate potential lack of wellness within themselves to
illness in their pet rabbits. Therefore protecting the pet population may be very important in preventing human infections with LeHV-4.

**Future Directions**

The present study describes pathology of LeHV-4 infection in domestic rabbits, serosurveillance of Ontario rabbit populations, and development of a diagnostic serological assay for accurate LeHV-4 antibody detection, but many questions still remain about pathogenesis of disease, development of latency, and the evolutionary origin of the virus. Our data has indicated that there is a negligible presence of LeHV-4 in Ontario based on the seroprevalence assay and that priority should be placed on ongoing surveillance and rapid diagnosis, such that any change in prevalence may be detected, prompting further investigation.

**Further diagnostic test development**

We have developed a subunit indirect ELISA that uses glycoprotein G expressed in a baculovirus vector as target antigen. The ELISA performs well in our hands, with 100% sensitivity and 99% specificity compared with the VN assay. External validation, including between lab reproducibility (OIE, 2012), remains to be investigated. The specificity of the assay should also be tested using rabbit serum containing HSV-1 antibodies, as HSV-1 is also capable of infecting rabbits (Weissenbock et al., 1997; Muller et al., 2009; Sekulin et al., 2010).

There are several refinements of the indirect ELISA that could be explored. Further purification of glycoprotein G from insect cell lysates may result in less background noise when testing polyclonal antibody to inactivated virus as positive control. The antibodies generated in our study required animals to suffer the effects of LeHV-4 disease. It would improve the welfare of rabbits if effective antibodies could be generated from inactive virus. The use of other glycoproteins may provide additional information. In the diagnosis of macacine herpesvirus 1 infections, it was found that using multiple target antigens improved diagnostic specificity and sensitivity (Katz et al., 2012). Earlier detection of antibodies, which could allow for diagnosis and treatment of active infection, may be found with an IgM-based ELISA.

A conventional, gel-based PCR test has been used in our laboratory to track the presence of virus in tissues, secretions, and cell culture (Appendix C: Figure C1). Using this assay, we have
demonstrated the presence of LeHV-4 in an archival formalin-fixed, paraffin-embedded sample of rabbit lung from 1991 (Figure C2, C3). Confirmation was obtained by sequencing the amplicon. The detection of LeHV-4 in this sample suggests that the virus has been around longer than originally suspected. We would like to investigate other archived samples to get a better picture of LeHV-4 epidemiology.

Quantitative PCR assays are in development for two separate purposes. In the research setting, we would like to quantify the amount of virus in various tissues throughout the course of infection and are using a SYBR green based method. For the diagnostic laboratory specificity is essential. We wish to develop a qPCR test that utilizes hydrolysis target specific probes, which is consistent with the standard of care for diagnostic laboratories (Belak, 2007). These can also be used to quantify virus titers to detect viremia in blood and virus load in tissues.

The glycoprotein G antigen produced for the indirect ELISA could be used to produce a more robust antibody for use in immunohistochemistry (IHC) and immunocytochemistry assays. IHC is considered more sensitive for detection of feline herpesvirus infections that histologic detection of inclusion bodies (Persico et al., 2011). Development of an IHC protocol would also provide supporting evidence when investigating tissue distribution of the virus in experimental or natural infection samples, differentiating it from viremia.

**Elucidating the pathogenesis of LeHV-4 infection**

There are many particulars of LeHV-4 infection that remain to be investigated. Different routes of infection may reveal more information about some of the pathology seen in the Alaskan outbreak, including intraocular, intradermal, or intravaginal routes. It would also be of interest to examine the effect of LeHV-4 infection in different cohorts of animals, such as juveniles, males or pregnant does. Specific studies on how the virus can be transmitted between animals may help understand and prevent the high morbidity rates observed in the Alaskan outbreak.

Recurrence of disease in the year following the Alaskan outbreak implies that latency and recrudescence occurs following LeHV-4 infection, as for other herpesviruses (Jin et al., 2008a). Tracking LeHV-4 in tissue samples of recovered animals may reveal sites of latency in particular tissues, but longer time courses may also be necessary to induce latency. Before extended animal studies are conducted, latency should be induced *in vitro* as has been previously described for HSV-1 (Su et al., 1999).
Infection of the spleen indicates that there was systemic distribution of the virus throughout the body, but a specific mechanism for virus distribution was not evaluated in this study. We tested serum for the presence of virus, but testing of whole blood or plasma may be necessary if LeHV-4 is carried in a blood cell component. HSV-1 viremia in immunosuppressed humans occurs in whole blood or plasma (Stanberry et al., 1994; Harel et al., 2004), and mouse models indicate that the virus is primarily associated with platelets (Forghani and Schmidt, 1983). Lymphocyte-associated viremia with subsequent infection of endothelium is common among varicelloviruses, including VZV (Ku et al., 2004) and EHV-1 (Pusterla et al., 2009). Hemorrhagic myocarditis and abortion may be the result of viremia and infection of endothelium.

Summary and Conclusions

1. LeHV-4 causes necrohemorrhagic pneumonia and splenic necrosis in rabbits, with peak pathology and presence of intranuclear inclusion bodies between 5 to 7 dpi.

2. Actual clinical disease in rabbits extends over a longer period with clinical signs occurring between 3-7 dpi, including serous oculonasal discharge, dyspnea, cyanosis, pyrexia and inappetence.

3. LeHV-4 is shed in nasal secretions from rabbits between 2 to 10 dpi following intranasal inoculation and can be detected by virus isolation and PCR.

4. Neutralizing serum antibodies are produced by 11 dpi, and can be detected by virus neutralization assay, whole virus indirect ELISA, and glycoprotein G subunit indirect ELISA.

5. Serum antibodies have not been detected in otherwise unaffected laboratory, pet or commercial rabbits. This finding suggests that LeHV-4 is an uncommon pathogen in Ontario and Quebec.
6. Immunoreactive glycoprotein G from LeHV-4 can be produced from Sf-21 cells infected with recombinant baculovirus.
References


McNitt JI, Patton NM, Lukefahr SD, Cheeke PR (1996). Rabbit Production (Danville, IL: Interstate).


Taddeo B, Roizman B (2006) The virion host shutoff protein (UL41) of herpes simplex virus 1 is an endoribonuclease with a substrate specificity similar to that of RNase A. *J. Virol.* 80: 9341-9345.


Appendix A. Sequence of glycoprotein G from the LeHV-4 ON1 isolate

```
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76 CCGCGGCGCGCCAGC ACCCCGGGGAAGGCC CCCCCCTCGCTACCCT TCCCCGTTTTTTTCT AGCCGGCGGTCCGCTCC
26 P R P P S T P A K A P L C Y P F P V F S S R G P S
151 GGCGGATCCGCGTAC GCTTCCGCGCAGAAG CCACGTCCGGGTGTG CACACCCCGCGACA TACGGCAGGCGGCCCA
51 G P I R V A S A H K P G R V V H H P Y T Y A P A P
226 AGCTGCGAGATCAGT ACTGCTGAGGGCGCGCC CTTCGTCATTATGGG AACAGACAGCCGGTAC AATGAGCAGGCGTGG
76 T C Q I T L S P P L R H Y G K R R Y N A T L V
301 TACTACCCATCCTGTA GGAAGCAGGCGCCGG GCCCTCATAATGAGG CATTACTCCGATGCG GCCCGCGGACACCGG
100 Y T I V G T A R R P L I M R H Y S G C A P G H P
376 CCCCCTGCGAAACAC ACCGCACTCATATTCC TTTAAGCTATTTAT GGGCGGCGCGCCAGC GCAATGCGATCGCGCC
126 P S P N T C N L Y S F T Y F N G A A P S A Y A I A
451 AACCCTGCTCTGATGC GGCGGCGCGGGGAC CGGTTCCGACTGCAAA TTTGGGTAGATTGTG GCGATGCGAGGACCG
151 N A S L V G P A A P F P R Q F G Y D L R I G R T
526 CTGCAGATCAGGAGAC ATAGCGGTGTCGCCGG GCGCGCGCGCCGGCC CCCTGAGCAACGTG TGGCGCGATCGGCTC
176 L H T G E I R V A A G G A A P C T T V C A T S L
601 AAAGCTGCGAGCGCCCTCCGAT GGGCTGGGAAATGGC AGGTATAGGAGGGC GAGCGGCGGTCCGCA
251 N W D A V P R C S L G L A N A R Y R K A A T G W H
676 AACCAGCGGCGGAT GTGACCGACTTTCGT TGCGTACTAAGGC TGGCAAGCGTGCTG GCGCGCTCGCGGAAG
226 N R T A D V T D F V C V A S A S Q S V L A A S E G
751 GCGGTGCGTGAGAGAC GACATTCCGTCGGAG CGCGGCGCTTGACCT CCACCGCCCGCGC CCGCGCGGCTTCCG
251 A V V R D A F R V T R V P H S P P T P R P G R V R
826 CCTACTGAGTAATGCG CCGGAAATCTCGCCGG CTGATAGAAGCTCGAT GCTGCCCCCTCCGGA AACAGCTCTCGCGGA
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901 CTTTTGTCCCTGGAA ATGTCCGTACATCGC GATCGGAATAGTGC TGGCGGGTGCTCCT CTGGCACGGCGGGG
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351 R H T T G P A P T S T T G A A R T P K S G S G
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376 E A E G T L R H L T L Y G R H T T T R P A P T
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1276 CTGGACTTCCGCGCG GAGGGCGGAGGGCGG GTCAGGACAGATGC GCGCGCGCCGCGCA GACCGCGGTCGGAAC
426 L D S A A E A G P G S T T E S G A A P T D P G A N
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451 A T A A M P E V S P R E A S P A G A A D N A T A
1426 ACCCGGGGATGCGCG GGGCGAGGCTGCGG GAATCGCGCGAGTG GAGTCCAGACAGGG GAGCGCTGACGACT
476 T A A G G G P G M A E S P A M E S T T G A A L D T
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501 S A A T V F P P A A P V T K N D S V A N S D T T A A
1576 GCGGAGCCCGTTTGC GGTGCCGAGAACCC TCAACACGTATTCT CGCTTCTCGGAAGCC GAGCGCGACACCGCG
596
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Figure A1. US4 nucleotide and predicted amino acid sequence from Ontario isolate of LeHV-4 (generated by http://www.fr33.net/translator.php)
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*Figure A2. Amino acid alignment of glycoprotein G homologues from LeHV-4, BoHV-2 and HSV-2. The predicted protein sequence of glycoprotein G from the ON1 LeHV-4 isolate (LeHV-4ON1gG), the Alaska LeHV-4 isolate (LeHV-4AKgG), BoHV-2 (BoHV-2gG) and HSV-2 (HSV-2gG) were aligned using ClustalW (Larkin et al., 2007). The predicted transmembrane region is underlined.*
Figure A3. Evolutionary relationships of select alphaherpesviruses based on glycoprotein G amino acid sequence. The evolutionary history of select alphaherpesviruses as indicated in Table A1 was inferred based on glycoprotein G sequence using the Minimum Evolution method (Rzhetsky and Nei, 1992), and a bootstrap consensus tree was inferred from 1000 replicates (Felsenstein, 1989). Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). The nearest neighbour to LHV-4 remains BoHV-2.
Table A1. List of alphaherpesviruses used for generation of phylogenetic tree (Figure A3) with GenBank accession numbers for glycoprotein G

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* glycoprotein G predicted to be truncated to a non-functional protein, amino acid sequence inferred from nucleotide sequence
Appendix B. Characterization of LeHV-4 ON1 isolate

Figure B1. Cytopathic effect of LeHV-4 in CRFK cell culture. CRFK cells (A) were infected with LeHV-4 at an MOI of 0.01 and phase contrast micrographs taken at various time points. At 12 hpi (B), a few small syncytia are present. Much larger syncytia are present at 18 hpi (C) with complete detachment of syncytia by 60 hpi (D).
Figure B2. Multi-step growth curve of LeHV-4 in CRFK cells. CRFK cells monolayers in 35 mm dishes were inoculated with LeHV-4 at an MOI of 0.01 and incubated at room temperature for 60 minutes. All monolayers were washed three times with PBS and 1 mL of maintenance media (IMDM/EMEM/L-15 with 5% FBS) was added. At each time point, the supernatant was collected and the amount of extracellular virus was determined by 96-well endpoint titration (closed circles). To determine the amount of intracellular virus, monolayers were washed twice and stored at -80 °C in 1 mL of PBS. The monolayers underwent two freeze-thaw cycles and the amount of intracellular virus was also determined by 96-well endpoint titration (open circles).
Figure B3. One-step growth curve of LEHV-4 in CRFK cells. CRFK cells monolayers in 35 mm dishes were inoculated with LHV-4 at an MOI of 1.3 and incubated at room temperature for 60 minutes. All monolayers were washed twice with PBS and 1 mL of maintenance media (IMDM/EMEM/L-15 with 5% FBS) was added. At each time point, the amount of extracellular virus (closed circles) and intracellular virus (open circles) was determined as described above.
Figure B4. Cytopathic effect of LHV-4 in Vero cells. A monolayer of Vero cells were either inoculated with LHV-4 at an MOI of 0.2 and or sham-inoculated (A). Significant cytopathic effect including development of syncytia and detachment from substrate was observed at 18 hours post-infection (B). No cytopathic effect was observed in HeLa cells (data not shown).
Appendix C. PCR for diagnosis of LeHV-4

Figure C1. Agarose gel electrophoresis of PCR products of DNA isolated from nasal secretions, frozen and formalin-fixed, paraffin embedded (FFPE) tissues. Conventional PCR was performed in 25 μL solution volume containing up to 100 ng of template DNA, 1.0 U of Platinum Taq polymerase, PCR Buffer, 1.5 mM MgCl2, 0.2 mM dNTP, and 0.4 μM forward (5’-CCACCAACGTCTCCGCCGTGTT-3’) and reverse (5’-AGCTTTGGCGCGGTGCAGAAGC-3’) primers.
Figure C2. Histopathology from suspected herpesvirus case. Paraffin blocks containing the lung and spleen of a two-year old rabbit from Prince Albert, Saskatchewan that was diagnosed in 1991 with an unconfirmed herpesvirus infection were obtained from Dr. Hélène Philibert (Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, SK). The initial diagnosis of herpesvirus infection was made based on the presence of intranuclear inclusion bodies present within endothelial and epithelial cells of the lung (A, 10X; B, 100X) and spleen (C, 10X; D, 60X).
Figure C3. Agarose gel electrophoresis of PCR products from archival formalin-fixed paraffin-embedded tissues from 1991 Saskatchewan case. DNA was extracted from 3 scrolls of paraffin-embedded tissue. Conventional PCR was run as indicated above (Figure B1). Lanes: MW: Molecular weight markers; A: Paraffin-embedded tissues from Saskatchewan case; B: no template; C: Paraffin-embedded lung from index case (Brash et al, 2010); D: concentrated LeHV-4; E: Paraffin-embedded lung from a healthy rabbit. Sequencing of the anticipated 140 bp PCR product demonstrated 100% identity with the ON1 isolate of LeHV-4.