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MOLECULAR PATHOLOGY OF FHV-1 INFECTION WITHIN FELINE CORNEA, CONJUNCTIVA, LACRIMAL GLAND, NICTITANS GLAND, TRIGEMINAL GANGLION AND CILIARY GANGLION

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SUSAN JACOBI

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MOLECULAR PATHOLOGY OF FHV-1 INFECTION WITHIN FELINE CORNEA, CONJUNCTIVA, LACRIMAL GLAND, NICTITANS GLAND, TRIGEMINAL GANGLION AND CILIARY GANGLION

By

Susan Jacobi

A THESIS

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ABSTRACT

MOLECULAR PATHOLOGY OF FHV-1 INFECTION WITHIN FELINE CORNEA, CONJUNCTIVA, LACRIMAL GLAND, NICTITANS GLAND, TRIGEMINAL GANGLION AND CILIARY GANGLION

By

Susan Jacobi

Feline herpesvirus 1 (FHV-1) is one of the major causes of feline upper respiratory tract and ocular disease. Despite intensive research in this area, the pathogenesis of FHV-1 infection within ocular and neural tissues remains poorly understood.

In this study we utilized virus isolation, a quantitative real-time polymerase chain reaction, histopathologic examination, and in situ hybridization to quantify the amount FHV-1 present and evaluate the associated morphological changes within the corneas, conjunctiva, lacrimal glands, nictitans glands, trigeminal ganglia, and ciliary ganglia of experimentally inoculated specific pathogen free cats during both the acute and latent phases of infection. We determined FHV-1 is able to infect not only conjunctival epithelium, corneal epithelium, and trigeminal ganglion, but also the ciliary ganglion. The ciliary ganglion may serve as an additional site of latency for FHV-1 and could explain the preponderance of ocular lesions noted during recrudescent infections. However additional studies are required to explore this theory. The theory that lacrimal adenitis contributes to the development of KCS was not supported as there was no evidence of FHV-1 infection or secondary inflammation within the glandular tissues at any time.

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1. CHAPTER - FELINE HERPESVIRUS 1 LITERATURE REVIEW

1.1 Feline herpesvirus 1 – Classification

The family of the *herpesviridae* is comprised of different subfamilies: alphaherpesvirinae, betaherpesvirinae, gammaherpesvirinae, and an unidentified subfamily.¹ Herpesviridae are enveloped viruses that have an icosahedral capsid containing a linear double stranded DNA.¹ Feline herpesvirus 1 (FHV-1) has been classified as a member of the varicellovirus genus of the subfamily alphaherpesvirinae.²⁻⁵ Prototypical members of this subfamily are human herpesvirus-1 (herpes simplex virus, HSV-1, genus simplexvirus), and human varicella-zoster virus (VZV, genus varicellovirus).¹ Alphaherpesvirinae relevant to the veterinary field include equine herpesvirus 1 and 4 (EHV-1 and 4), bovine herpesvirus-1 (BHV-1), canine herpesvirus-1 (CHV-1), pseudorabies virus (PRV, PHV-1), and gallid herpesvirus-2 (GHV-2, Marek's disease virus).^{6,7} Feline herpesvirus-1 is closely related genetically and antigenetically to CHV-1 and phocid (seal) herpesvirus-1 (PhV-1), and cross-protection between feline and PhV-1 has been reported.⁸⁻¹⁶

1.2 Feline herpesvirus 1 – Characteristics

The size of feline herpesvirus particles is 120-180 nm in diameter.¹⁷ Like most alphaherpesviruses, FHV-1 is composed of four major structural elements including an electron-opaque core, an icosahedral capsid, a tegument, and an envelope.^{1,7} A single molecule of linear double stranded DNA is wrapped around the fibrous spoollike core, which is anchored inside the capsid.¹ The capsid is a structural characteristic of all herpesviruses, 100 nm in diameter, and consists of 150 hexameric and 12 pentameric capsomers.^{1,4,7,17} The nucleocapsid is surrounded by an electron dense amorphous material, called the tegument.^{1,4,7} The outer layer of the virion is formed by a fragile lipoprotein envelope, which carries viral glycoproteins.¹

The physical structure of the FHV-1 genome is similar to the genomes of other *alphaherpesvirinae*.¹⁸ Rota et al., first characterized the genome of FHV-1 strain C-27, the strain utilized in the present study, and determined that FHV-1 DNA was approximately 134 kb and was composed of a long (L) and a short (S) segment. The long segment (UI) was 104 kb in size and was composed of unique DNA.¹⁸ The adjacent S segment was approximately 30 kb in size and contained a central portion of unique DNA (Us) which was approximately 8kb in size.¹⁸ The Us region was bounded by inverted repeat sequences which were 11 kb in size.¹⁸ Later Grail et al., determined the size of the genome of FHV-1 strain B-927 to be 126 kb, composed of a long unique region of 99 kb and a short region of 27 kb containing a unique sequence of 8-9 kb flanked by inverted repeats of 7-8.5 kb.¹⁹

1.3 Feline herpesvirus 1 – Replication

The replication cycle of HSV-1 has been studied extensively. Most other *alpha-herpesvirina* follow a similar pattern. The different phases of replication are initiation, early gene expression, DNA replication, late gene expression, assembly, and release.^{1,7,20}

Epithelial cells of the nasal mucosa, conjunctiva, tonsils, and nasal turbinates are the target for primary infection by FHV-1.²¹ During the initiation phase, multiple proteins participate in the attachment of the virus to the host cell.¹ Three viral glycoproteins, gB, gC, and gD, are essential for HSV-1 infection.¹ An FHV-1 gB homolog has been identified and sequenced.²² Via gB and gC the virion binds to heparin sulfate or chondroitin sulfate proteoglycans of the extracellular matrix of the host cell.¹ To facilitate cellular entry, gD binds to a cellular co-receptor.^{1,20} This contact initiates fusion of viral and plasma membranes.¹ Viral nucleocapsids then attach to microtubules and are transported to the nucleus.²³ The viral nucleocapsid docks at the nuclear pore, releasing viral DNA into the nucleus.^{1,7}

Gene expression of the FHV-1 genome is believed to be similar to that of HSV-1 in human cells.^{24,25} VP16, a tegument protein, interacts with host transcription proteins to stimulate transcription of immediate-early genes by host RNA polymerase II.^{1,26} Immediate-early mRNAs are spliced and transported to the cytoplasm where they are translated. The immediate-early proteins (α -proteins) are transported to the nucleus where they activate transcription of early genes and regulate transcription of immediate-early genes.¹ Early gene transcripts are transported to the cytoplasm. The early proteins (β -proteins) function primarily in DNA replication and production of substrates for DNA synthesis. Some β -proteins are transported to the nucleus, while others function in the cytoplasm.¹

DNA replication and recombination produces long, concatemeric DNA, the template for late gene expression. Late mRNAs are transported to the cytoplasm where they are translated.¹ Late proteins (γ -proteins) function primarily as viral structural proteins or participate in virus assembly and particle egress. The precursor glycoproteins are also transported to the Golgi apparatus for further modification and processing. Mature glycoproteins are transported to the plasma membrane of the infected cell. γ -proteins are transported to the nucleus for assembly of the nucleocapsid and DNA packaging. Newly replicated DNA is packaged into nucleocapsids.¹

DNA containing nucleocapsids, together with some tegument proteins, bud from the inner nuclear membrane into the perinuclear lumen, acquiring an envelope.¹ These immature enveloped virions fuse with the outer nuclear membrane, releasing the nucleocapsid into the cytoplasm.¹ The structure is then transported to a late trans-Golgi compartment or endosome that contains mature viral membrane proteins.¹ Here the particles acquire an envelope containing mature viral envelope proteins and the complete tegument layer.¹ The enveloped virus particle then buds into a vesicle that is transported to the plasma membrane for release by exocytosis.¹ Enveloped viruses tend to spread from cell to cell, which accounts for the formation of plaques and syncytia seen during virus isolation in cell culture.²⁷⁻²⁹

1.4 Feline herpesvirus 1 – Latency

Feline herpesvirus-1, similar to other herpesviruses can establish latency, a phase where replication is limited. The virus may become latent for the lifetime of the host. Three general properties characterize latent infections: expression of productive cycle viral genes is absent or inefficient; immune detection of the cell harbouring the latent genome is reduced or eliminated; and the viral genome persists intact so that at some later time a productive acute infection can be initiated.¹ In the latent state, the genome can be maintained as a non-replicating chromosome in a non-dividing cell such as a neuron (e.g., HSV-1, VZV); become an autonomous, self replicating chromosome in a dividing cell (e.g., Epstein-Barr virus), or be integrated into a host chromosome (e.g., adeno-associated virus).¹ During latency, a balance among the regulators of viral and cellular gene expression must be maintained. Therefore only a restricted set of viral gene products is produced.¹

For α -herpesviruses it was generally believed that sensory neurons within ganglia were the primary site of latency.³⁰ However, more recent studies have shown herpes simplex latency within the autonomic pathways of mice.³¹ The trigeminal ganglion is considered a primary site of latency following ocular, oral, or intranasal infection.^{32,33} Once latency is established, viral DNA persists in neurons for the life of

an infected host.³⁰ It is unclear if latency induces neurologic injury.³⁰ However, it has been shown that during reactivation from a latent infection, inflammation and destruction of neurons and surrounding tissue can occur.^{34,35}

In neurons latently infected with HSV-1 the only abundantly transcribed gene is the latency-associated transcript (LAT).^{36,37} Therefore, LATs provide molecular markers for latently infected cells.^{30,38} The length of the primary LAT transcripts is 8.3 to 8.5 kb.^{39,40} LAT overlaps the viral genes for ICP0 and ICP34.5 in an anti-sense direction.^{36,37} It has been suggested that LAT is a regulatory RNA that represses expression of ICP0.³⁷ Although the LATs are first detectable at the peak of an acute infection, they are present at much higher levels during latency with high levels in trigeminal ganglia.^{37,41} Several studies suggested that LATs play a role in establishment of latency. It has been demonstrated that HSV-1 LATs enhances establishment of latency in mice.^{42,43} Furthermore, it has been found, that LATs repress productive viral gene expression in trigeminal ganglia of mice during acute infection.^{44,45} Although LATs are not mandatory for the virus to establish latency, viral mutants that are unable to express LATs are reactivation deficient.⁴⁶ Therefore LATs may play a role in reactivation from latency. Different studies using various LAT mutants have shown that LATs increase the induced and spontaneous reactivation phenotypes in the rabbit ocular model^{47,48} and the induced reactivation phenotype in mice.^{42,49-52}

LATs also have anti-apoptotic features and maintain latency by promoting the survival of infected neurons.^{53,54} Gupta et al.⁵⁵ showed that microRNA encoded by the HSV-1 LAT gene confers resistance to apoptosis. The anti-apoptotic activity of LATs is important during the latency-reactivation cycle. Because infected neurons would normally be eliminated by cytotoxic T cells, which kill their target cells in part by

apoptosis, high levels of LAT within latently infected neurons may prevent their elimination.⁵⁶ A functional relationship between the ability of LATs to promote cell survival and their ability to enhance spontaneous reactivation has been demonstrated.⁵⁷

Primary and recrudescent herpetic disease is more prevalent among immunecompromised individuals.⁵⁸ The fact that viral reactivation is associated with immune suppression and the persistence of T cells and cytokines in latently infected human trigeminal ganglia suggest that T cells play a role in control of the virus at the site of latency.⁵⁸⁻⁶⁰ Theil et al.⁵⁹ found T cells positive for CD3 and CD8 markers as well as CD68 positive macrophages in 30 of 42 trigeminal ganglia from 21 healthy humans. The presence of these cells correlated constantly with the occurrence of HSV-1 LATs. Uninfected trigeminal ganglia showed no immune cell infiltration. Quantitative real time PCR revealed an elevated cytokine/chemokine expression (IFN- γ , TNF- α , RANTES, and IP-10) in the latently infected trigeminal ganglia demonstrating that latent herpesviral infection in humans is accompanied by a chronic inflammatory process, but without neuronal destruction. It seems likely that the immune response maintains viral latency and inhibits viral reactivation.

1.5 Feline herpesvirus 1 disease - Epidemiology and pathogenesis

Feline herpesvirus-1 is also known as feline rhinotracheitis virus. Feline viral rhinotracheitis was first recognized in 1957 in 5 to 10-week-old farm kittens that were being raised in a laboratory.^{21,61} Since then it has been recognized as a disease with world-wide distribution within the cat population. Epidemiologic studies indicate that as many as 50-75% of cats have serologic evidence of exposure to the virus.^{62,63} Other studies estimate that over 90% of cats are sero-positive for the virus;⁶⁴ at least 80% of

infected individuals remain latently infected for life; and approximately 45% of latently infected cats shed virus throughout life.⁶⁵

Reactivation of virus may occur spontaneously but is most likely after stress.⁶⁶ Re-excretion of FHV-1 could be stimulated in more than 50% of cats recovered from FHV-1 infection through systemic administration of corticosteroids or a change in housing.⁶⁷ Detectable viral shedding does not immediately follow the stress.⁶⁶ A lag phase of 4-11 days (mean 7.2 days) precedes the shedding of infectious virus.⁶⁵ The period of shedding can range in duration from 1-13 days (mean 6.5 days).^{65,67} Shedding carriers are mainly asymptomatic,^{65,68} although in some cases carriers show recrudescence of mild clinical signs while they are shedding.⁶⁹ During episodes of viral shedding infectious virus is present within oro-nasal and conjunctival secretions and serves as source of infection to other cats.^{65,67}

The enveloped virion is relatively fragile and very susceptible to heat, drying, or disinfectants.^{70,71} At room temperature FHV-1 can only persist in the environment for 18-24 hours in relatively dry conditions.^{70,72,73} Transmission of FHV-1 between cats occurs via direct contact, fomites, or macrodroplets which can be sneezed about 1.3 m.⁷⁴ Management of infected cats within a hospital situation requires appropriate separation of the cats and routine hygiene practices for personnel and facilities.⁷⁵ The virus survives less than one hour in ophthalmic solutions used for routine diagnostics testing, such as fluorescein stain and topical ocular anesthetics. ⁷⁶ However, the virus may persist for up to 5 days in eye wash solutions.⁷⁶

Young cats are most susceptible to primary infection, especially around 8-12 weeks of age when maternal antibodies wane.⁷⁰ Cats with underlying disease (feline leukaemia virus (FeLV), feline immunodeficiency virus (FIV)) are also very susceptible to FHV-1 infection.⁷⁷ Due to limited ability of FHV-1 to replicate at

temperatures of 37 °C or higher, the primary sites of replication are the cooler epithelial surfaces.⁷⁰ The natural routes of infection are via nasal, oral, and conjunctival mucous membranes.^{66,70} In the acute phase of infection virus replication occurs predominantly in the mucous membranes of the nasal septum, turbinates, nasopharynx, and tonsils; other tissues including conjunctivae, mandibular lymph nodes and the upper trachea are also often involved.^{66,78,79} The infection is most commonly restricted to superficial epithelial tissue, which may include the corneal epithelium.^{73,80} Since viral replication is usually restricted to areas of lower body temperature, viremia is rare. It has only been occasionally reported and the virus appears to be associated with peripheral blood leucocytes.^{79,81} Generalized disease is predominantly seen in debilitated animals or neonatal kittens.^{70,82,83}

As the virus replicates its cytopathic effect creates areas of multifocal epithelial necrosis leading to inflammation with neutrophilic infiltration and fibrin exudation.^{27,61,66,70,72,79} Secondary bacterial infection often worsens the condition.⁷⁰ Incubation periods can range from 2-10 days.²⁷ The clinical signs usually resolve within 10-14 days.²⁷ Detection of infectious virus is possible as early as 24 h post infection via oropharyngeal and nasal swabs. The virus generally persists for 1-3 weeks, although detection of viral DNA by PCR might be possible for longer.^{84,85}

In severe cases osteolytic changes within the turbinate can be caused by the virus.⁶⁶ Following intravenous inoculation of young kittens a predilection of the virus for regions of skeletal growth, including the turbinates has been shown.⁸⁶ Experimentally it also has been shown that intravaginal instillation of the virus in pregnant queens can lead to vaginitis and congenitally infected kittens.⁸⁷ Intravenous inoculation of pregnant queens led to transplacental infection and abortion.⁸⁸

1.6 Feline herpesvirus 1 disease – Other species

Feline herpesvirus 1 has been described to possess a very narrow host range.⁸⁹ However, FHV-1 can exist within other non-domestic felids or even non-felidae as well.

In 1971 Thomson et al.⁹⁰ reported the isolation of a herpesvirus from three cheetahs (*Acinonyx jubatus*) living at the Lion Park at Warragamba, New South Wales. Two of the cats developed conjunctivitis and rhinitis.⁹⁰ The third cat showed no clinical symptoms and it was suggested that this animal may have been a carrier.⁹⁰ Virus was isolated from the eyes, nose, and mouth, but not from the rectum. The isolates were identified a herpesvirus by their production of cytopathic effects, including giant cells and Cowdry type A inclusion bodies, and their appearance on electron microscopy.⁹⁰

In 1984 a disease outbreak occurred at the St. Louis Zoological Park, affecting two of three queen-reared Cheetah cub siblings.⁹¹ The cubs had been vaccinated with a killed virus product at three-week intervals from ages 3.5 to 18 weeks.⁹¹ By the age of three months they developed chronic, multifocal, ulcerative cutaneous lesions, which were refractory to treatment.⁹¹ Excisional biopsies suggested a diagnosis of eosinophilic granuloma, but intranuclear inclusions characteristic of herpesvirus were also found.⁹¹ Herpesvirus was also found in these samples by transmission electron microscopy. Virus isolation revealed a virus that induced cytopathology similar to that of alphaherpesviruses. The presence of a herpesvirus was suggested, which was named cheetah herpesvirus (ChHV).⁹¹ In order to compare this virus with FHV-1, further tests (dot blot hybridization, restriction endonuclease digested DNA electrophoretic profiles, antigenic comparison by serum neutralization and western immunoblotting analysis, SDS polyacrylamide gel electrophoresis comparison of viral polypeptides) revealed that the ChHV isolate was closely related to FHV-1.⁹¹ However, at least two detectable differences in the Kpn I digestion patterns were considered to be significant because of the remarkable stability of FHV-1 and lack of restriction fragment length polymorphism.⁹² Whether or not ChHV is truly different from FHV-1 remains to be investigated.

In 1977 Boever et al.⁹³ reported viral rhinotracheitis in 4 of 5 clouded leopards (*Felis nebulosa*) housed at the St. Louis Zoo. The leopards had clinical signs which consisted of serous nasal and ocular discharge and sneezing. Two of the cats also had ulcerative glossitis. One pregnant animal developed vaginitis and aborted. Nasal and rectal swabs were obtained for virus isolation from three severely affected animals that died due to progression of their illness. A cytopathogenic agent, which was identified as feline rhinotracheitis virus, was isolated from the nasal swab of one animal.⁹³

Truyen et al. in 1990⁹⁴ reported an acute outbreak of encephalomyelitis in 10 lions from a safari park in Germany. Three lions were euthanized and different diagnostic tests (histopathology, virus isolation, serum neutralization) were performed. Apart from mild swelling and reddening of the tonsils, alterations were mainly found in the brain. The pathological diagnosis was non-suppurative polioencephalomyelitis with demyelinization of the spinal cord. In the nuclei of some glial cells small intranuclear, acidophilic, round and ovoid structures suggestive of intranuclear inclusion bodies were found. Virus isolated from the tonsils of one animal demonstrated cytopathic effects. The cytopathic agent was serologically characterized as FHV-1. However, FHV-1 was not isolated from any brain tissues. Ultimately it was concluded that FHV-1 was unlikely to be the cause of the encephalitis outbreak.⁹⁴ Surveys of antibody titers in free-ranging lions in Africa revealed a seroprevalence for FHV-1 of 67% and 91% in Etosha National Park and Kruger National Park, respectively.^{95,96} Antibodies to FHV-1 were found in 309/310 samples in lions living within Serengeti National Park and Ngorongoro Crater.⁹⁷

In 1991, Eberle et al.⁹⁸ evaluated sera from 19 free-ranging bobcats (*Lynx rufus*) to determine the seroprevalence of FHV-1 via an enzyme linked immunosorbent assay (ELISA). All samples were negative suggesting that FHV-1 was uncommon or not present at all in this species. Four free-ranging bobcats were captured, and ELISA for anti- FHV-1 antibodies and attempts at virus isolation from oropharyngeal swabs were negative. The cats were inoculated by instilling 200 μ l of the viral suspension in each nostril. None of the animals developed clinical signs of disease. However, virus was isolated from the oropharynx and nostrils on days 3-4, 7-8, and 14-15 post-inoculation. Although the bobcats did produce anti-FHV-1 IgG, titers were generally lower than those seen in domestic cats.⁹⁸ The experiment demonstrated that bobcats are susceptible to FHV-1 infection, but clinical signs of disease do not accompany the infections. Paul-Murphy et al.⁹⁹ demonstrated a seroprevalence of 19% for FHV-1 in 58 mountain lions (*Felis concolor*) in California.

In 1999, Pratelli et al.¹⁰⁰ reported an outbreak of respiratory tract disease characterized by sneezing, nasal and ocular mucopurulent discharge, anorexia and prostration in three tigers and one leopard from the Fasano Zoo in southern Italy. Nasal swabs were obtained from all four animals for virus isolation. Characteristic cytopathic effects and intranuclear inclusions were observed. Virus neutralization tests using a goat antiserum, monospecific to FHV-1, identified the isolates as FHV-1 strains. Restriction patterns were compared with those of a vaccinal and a field strain of FHV-1. Restriction patterns obtained with *Pst*I and *Bam*HI revealed that the genomes of the four isolates, the field strain, and the vaccination strain were identical. It could not be concluded if the disease was caused by a FHV-1 strain reactivated in a wild felid or by a strain transmitted by domestic cats.¹⁰⁰

Nakamura et al.¹⁰¹ investigated the seroprevalence of FHV-1 in domestic cats (*Felis catus*) and leopard cats (*Felis bengalensis*) in Vietnam. All cats were considered to be unvaccinated. In the Hanoi area only 1.4% (1/69) of domestic cats were positive for FHV-1 antibodies, compared to 44% (22/50) in the Ho Chi Minh City area. Of the tested leopard cats, 11.1% (1/9) were positive in the Hanoi area, whereas 50% (2/4) were positive in the Ho Chi Minh City area. The authors suggested that the higher cat population density in Ho Chi Minh City area explained in part the higher prevalence of FHV-1.

Evermann et al.¹⁰² isolated a herpesvirus from dogs with diarrhea. Virus neutralization tests revealed a relation of the isolated viruses to FHV-1. Rota et al.¹⁰³ compared five herpesvirus isolates from these dogs to the DNA and polypeptide patterns of FHV-1. The genomes of these five canine isolates showed restriction enzyme digestion patterns that were nearly identical to those of FHV-1, but completely different from CHV. The disease potential of these canine FHV-1-like isolates remained unclear in this study.

Kramer et al.¹⁰⁴ isolated FHV-1 from the thymus and spleen of a 17 week old cross breed dog with a history of distemper-like illness, diabetes mellitus, and pancreatic atrophy. Following culture of the virus in Crandell feline kidney cells, three healthy puppies were inoculated intravenously and intranasally with the viral suspension. No clinical signs were produced. In a study investigating spotted hyenas, only one of 240 animals had serum antibodies against FHV-1.¹⁰⁵ This suggests that hyenas unlikely play a role as a reservoir for FHV-1 in the Serengeti ecosystem.

1.7 Feline herpesvirus 1 disease - Systemic manifestations

Feline herpesvirus-1 infection causes upper respiratory tract symptoms, ocular manifestations and reproductive failure.¹⁰⁶ The primary, acute disease affects kittens more commonly and more severely. Following an incubation period of 24-48 hours, a sudden onset of sneezing, followed by profuse serous nasal and ocular discharge, hypersalivation, fever, anorexia and lethargy may be observed.^{7,21,27,70,79,106} Due to secondary bacterial invasion, the discharge may progress from serous to mucopurulent.¹⁰⁶ In severe cases, the virus may invade the lungs leading to viral pneumonia and possibly secondary bacterial pneumonia.^{70,82,83,107} *Bordetella bronchiseptica* has been identified as a frequent secondary invader.¹⁰⁸ Primary FHV-1 infection is self limiting in most cases, and symptoms regress within 10-14 days.⁷⁰ Chronic sinusitis may follow severe primary infection and may result in necrosis and distortion of the turbinates.^{65,66}

In rare cases of FHV-1 infection, oral ulceration has been observed. However, oral ulceration is more commonly associated with feline calicivirus (FCV) infection.^{69,106} As a rare sequela to FHV-1 infection, neurological signs have been described.¹⁰⁹ Abortion has been associated with FHV-1 infection. However, experimental studies have suggested that abortion is most likely due to the severe systemic illness, rather than a direct effect of the virus itself.⁸⁸ Hickman et al.¹¹⁰ investigated a natural outbreak of FHV-1 infection in a specific pathogen free cat colony. No cases of abortion were seen, even in severely affected pregnant queens.¹¹⁰

Cutaneous ulceration has been described with FHV-1 infection in domestic cats and also in Cheetahs.¹¹¹⁻¹¹⁶ Histopathologic examination of the lesions reveals necrotizing and ulcerative changes.^{115,116} Typically a marked eosinophilic inflammatory response is noted within these lesions. However, the infiltrate may also be neutrophilic in some cases.¹¹¹⁻¹¹⁶ Holland et al.¹¹⁷ showed that FHV-1 DNA can be detected in the skin of cats with herpetic dermatitis and suggested that the virus may play a causative role in the disease. Furthermore, a PCR assay for FHV-1 was shown to be useful in the diagnostic process with high sensitivity and specificity.¹¹⁷

1.8 Feline herpesvirus 1 disease - Ocular manifestations

1.8.1 Conjunctivitis

Feline herpesvirus-1 infection is the most common cause of conjunctivitis in cats and is often accompanied by respiratory disease.¹¹⁸ Bilateral conjunctivitis characterized by blepharospasm, chemosis, serous ocular discharge, and hyperemia is the most common ocular manifestation during acute FHV-1 infection.^{4,21,70,71,119-122} Diffuse necrosis of the conjunctival epithelium and intranuclear inclusions within conjunctival epithelial cells are present by day four post-infection.⁴ Around day 8 post-infection, cytopathic effects of the virus cause the conjunctival epithelium to slough.⁴ The serous ocular discharge can progress over several days to mucopurulent discharge as polymorphonuclear cells as well as low numbers of macrophages infiltrate the conjunctival stroma.^{4,120} Bacterial infections may worsen the mucopurulent discharge, and pseudodiphtheritic membrane formation may occur.¹¹⁸ Most cats recover within 10-20 days with no other ocular sequelae.^{4,120} However, more severe infection or immunosuppression may result in chronic or recurrent

conjunctivitis.¹²³ FHV-1 infection in adult cats typically causes conjunctival hyperemia, intermittent blepharospasm, and mild serous ocular discharge.¹²⁴

Conjunctival cytologic findings depend on the duration of the disease process and the presence or absence of other infectious agents.¹¹⁸ The predominant cell type is the lymphocyte, although macrophages are also a common finding.^{118,121} With chronic or secondary infection, more neutrophils and giant epithelial cells may be seen.^{118,121} Epithelial intranuclear inclusion bodies may occur, but are rarely observed in clinical cases.¹²¹

1.8.2 Keratitis

Epithelial keratitis commonly occurs during primary infection in young cats, which resolves spontaneously in most cases.¹²⁵ Feline herpesvirus-1 induced corneal ulceration in adult cats is likely due to reactivation of the virus.^{119,120} This condition is accompanied by varying degree of conjunctivitis.⁷⁰ usually Systemic immunosuppression, environmental stress, and systemic administration of corticosteroids have been associated with reactivation of latent virus.^{120,125} In the adult cat, herpetic keratitis can be unilateral or bilateral and is usually not associated with upper respiratory disease.^{118,125} Corneal edema may precede the formation of corneal ulcers.¹¹⁸ During the acute stage of ocular infection, the corneal lesions tend to be microdendritic.¹¹⁹ Early corneal ulcers may also be dendritic. However, they often progress quickly to larger geographic ulcers.^{4,70} Dendritic shaped corneal ulcers are the classical pattern of corneal ulcerations induced by FHV-1.^{4,70,119,126,127} They result from the direct cytopathic effect of the virus.^{125,127} Sometimes, ulcers may also occur as numerous punctate lesions, that may coalesce to form a larger superficial ulcer.¹¹⁸ Occasionally herpetic ulcerations progress, likely due to secondary bacterial infection, to involve deeper stromal layers or cause corneal perforation.⁷⁰ Chronic ulcers may incite a corneal vascular response, although the intensity varies greatly.⁷⁰ FHV-1 induced corneal ulcerations may resolve spontaneously, whereas others may develop into indolent ulcers.⁷⁰

1.8.3 Stromal keratitis

Herpetic stromal keratitis caused by HSV-1 is the leading cause of infectious corneal blindness in the industrialized world. Changes associated with stromal keratitis are thought to be due to the inflammatory response to chronic or repeated recrudescent infection. Several observations support the conclusion that the pathogenesis of herpetic stromal keratitis is immune mediated, including the observation that this manifestation of ocular herpetic disease is uncommon in humans who are immunodeficient.¹²⁸ Furthermore, large numbers of inflammatory cells infiltrate the cornea, including polymorphonuclear neutrophils, macrophages, Langerhans cells, natural killer cells, plasma cells and T cells.¹²⁹⁻¹³² The importance of the immune response in stromal keratitis is also supported by observations that in T cell-deficient mice, ocular infection with HSV-1 was not associated with the development of marked stromal disease.¹³³

The exact immunological mechanism of the development of stromal keratitis is not known. Originally the chronic changes believed to result from immune complex deposition because immunoglobulins and herpesvirus particles were found in the stroma.¹³⁴ However, there is evidence, that a chronic low-level viral infection persists within stromal keratocytes providing a continuous stimulus to antigen-specific T lymphocytes that kill the keratocytes by a cytotoxic mechanism.¹³⁵ Furthermore, CD4 T cells reactive against HSV-1 antigens have been isolated from chromic stromal keratitis lesions.^{136,137} The pathogenesis of corneal scarring and vascularization in stromal keratitis is still uncertain^{138,139} HSV-1 infection can induce production of different angiogenic factors such as vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMP) 2 and 9, platelet-derived growth factor (PDGF), β fibrosing growth factor (bFGF), macrophage inflammatory protein 2 (MIP-2) and monocyte chemotactic protein 1 (MCP-1).¹⁴⁰⁻¹⁴² These factors are mainly produced by polymorphonuclear neutrophils. However, VEGF is also expressed in corneal epithelial and stromal cells. It appears to be produced by cells close to those infected by the virus.¹⁴⁰ It is possible that interleukin 1 (IL-1) and interleukin 6 (IL-6), produced initially by infected cells, induced the production of VEGF.

Stromal keratitis is a rare manifestation of corneal FHV-1 infection.^{4,80} However, due to its potential to cause vision threatening corneal scarring, it is considered the most significant manifestation of feline ocular herpetic disease.⁴ It is usually associated with chronic epithelial ulceration and characterized by stromal edema, deep vascularization and the presence of inflammatory cells.⁴ In experimentally induced ocular feline herpesvirus-1 infection, the virus was observed to preferentially infect conjunctival epithelium. However, FHV-1 was able to replicate in corneal epithelium, and only minimal viral antigen was detected in the corneal stroma.⁸⁰ When cats were treated with subconjunctival administration of betamethasone followed by topical application of FHV-1 to the scarified cornea, a stromal keratitis developed, characterized by geographic epithelial ulceration, interstitial edema, deep vascularization, and inflammatory cell infiltrates.^{4,80}

To elaborate the mechanism through which stromal disease occurs, Nasisse et al.¹⁴³ studied the histologic, immunologic, and virologic features of FHV-1 induced keratitis in 18 experimentally infected cats. Stromal disease was induced by local immunosuppression via application of three consecutive daily subconjunctival injections of 4 mg betamethasone prior to inoculation with FHV-1.¹⁴³ Prior to the

development of stromal keratitis, prolonged absence of the corneal epithelium, decreased numbers of circulating lymphocytes, decreased mitogen responses, and acquisition of viral antigen by the corneal stroma were noted.¹⁴³ The first cells to arrive in infected corneas were neutrophils. The subsequent arrival of B and T lymphocytes was supportive of a specific antiviral immune response.¹⁴³ Fibrosis and scarring developed secondary to the stromal inflammation. The authors concluded, that it is probable that the corticosteroid-mediated suppression of the normal immune function allowed uncontrolled local virus replication, which combined with delayed corneal epithelial healing led to stromal accumulation of virus.¹⁴³ The development of stromal keratitis was likely a result of the immune response to viral antigen.¹⁴³

1.8.4 Keratoconjunctivitis sicca

Decreased tear production in cats has long been suspected to be a potential sequelae to FHV-1 infection.¹¹⁹ Clinical signs can include conjunctival hyperemia, a dry appearance of the cornea, corneal epithelial hyperplasia and corneal ulceration.¹²⁰ The cause remains speculative, but it has been assumed that excretory duct occlusion or lacrimal adenitis is responsible.^{4,70,80} In most cats, keratoconjunctivitis sicca is transient, with tear production increasing with resolution of active FHV-1 related disease.⁷⁰ However, a decrease in tear production associated with FHV-1 infection can also be permanent.^{4,70} Experimentally, a temporary keratoconjunctivitis sicca developed in 5 of 10 cats treated pre-inoculation with three consecutive daily subconjunctival injections of betamethasone.⁸⁰ Aqueous tear production returned to normal in all cats by day 60 post-infection.

Using specific fluorescent antibody test and virus isolation, Bistner, et al. failed to demonstrate the presence of FHV-1 within the lacrimal gland.¹²¹ However, using a PCR assay, Reubel et al, detected FHV-1 DNA in two of five lacrimal glands of latently infected cats, as defined by negative virus isolation from swabs of oral and ocular secretions.¹⁴⁴ Fluorescein labeled antibody has been used to examine human lacrimal glands during both acute and asymptomatic stages of HSV-1 corneal infection. Viral antigen was detected in 2/3 patients with acute disease and in 4/7 asymptomatic patients with a history of previous herpetic infection.¹⁴⁵ A case of dacryoadenitis was described in an immunodeficient patient.¹⁴⁶ A biopsy sample from the right lacrimal gland was positive on immunohistochemisty for HSV-1. However, virus isolation was negative for HSV-1, VZV, cytomegalovirus, and enterovirus, but positive for adenovirus. The patient was treated with systemic antiviral therapy (acyclovir). The patient recovered with the exception of mild bilateral dry eye symptoms. Duration of the dry eye could not be evaluated because the patient was lost to follow-up.

Lim and Cullen¹⁴⁷ evaluated Schirmer tear test values and tear film break up times in cats with conjunctivitis. Lower mean tear film break up times, but not lower Schirmer tear test values were found in cats that had FHV-1 DNA detected by PCR in their blood compared to cats that did not. The authors concluded that tear film instability may play a role in the pathogenesis of feline conjunctivitis, although it is not clear if it is a predisposing factor or a result of it.

In a recent study the effect of primary FHV-1 infection on tear film break up time and Schirmer tear test values, as well as the relationship between these parameters and conjunctival goblet cell density, histological inflammation, and clinical disease severity was evaluated.¹⁴⁸ Tear film break up time and goblet cell density decreased dramatically during the first week following viral inoculation, continued to decline until 15 and 21 days post-inoculation and remained abnormal for the duration of the study (29 days).¹⁴⁸ The total disease score and clinical and

histological evidence of conjunctivitis increased in all 6 cats during the first week, but began to normalize thereafter. Schirmer tear test values remained elevated for 29 days following inoculation, likely due to the continued tear-film instability.¹⁴⁸

1.8.5 Ophthalmia neonatorum and symblepharon

Ophthalmia neonatorum as a presentation of early FHV-1 infections was initially described by Bistner et al.¹²¹ Two to four week old kittens with ocular lesions had generalized disease as well characterized by rhinitis, tracheitis, bronchopneumonia, and hepatic necrosis.¹²¹ Infection with FHV-1 before the eyelids are open can result in mucopurulent conjunctivitis which causes distension of the eyelids.¹²⁰

Symblepharon formation, the adhesion of conjunctiva to itself or to the cornea, may be observed as a sequela to FHV-1 infection.^{4,120} The profound epithelial necrosis caused by FHV-1 precedes this condition, leading to adhesions and scar formation.^{4,119,120}

1.8.6 Corneal sequestrum

Feline corneal sequestrum (FCS) is a disease unique to the cat^{149,150} characterized by degeneration of collagen and accumulation of brown pigment.^{118,151,152} The pigment appears to be water soluble and not melanin or hemosiderin.^{119,151,153} However, Featherstone et al.¹⁵⁴ found particles within corneal sequestra which were consistent with the appearance of melanin particles. The clinical appearance is characteristic. Lesions are variable in pigmentation, round to oval shaped and usually located centrally or paracentrally.^{149,155} Corneal vascularization, perilesional stromal edema, and inflammation are common in chronic disease.^{118,119,156} Clinical signs include blepharospasm, epiphora, enophthalmos, elevation of the

nictitans, chemosis and hyperemia of the conjunctiva as well as photophobia and decreased activity.^{149,150,157,158} The condition can occur unilaterally or bilaterally.^{149,150,158} Predispositions for Persian,^{118,119,150,158,159} Himalayan,^{118,158} Colorpoint,¹⁶⁰ American Shorthair ¹⁵⁸ and Siamese cats^{118,150,158} are reported. Histopathologically the sequestrum is characterized by stromal degeneration, cell loss and dessication, often surrounded by a zone of inflammatory cells.^{118,151-153,156,161}

The etiology of FCS is unknown. Feline herpesvirus-1 DNA has been detected in 18-55.1% of keratectomy specimens from cats with corneal sequestrum using polymerase chain reaction (PCR).^{162,163} Using immunofluorescent antibody tests (IFA), FHV-1 was detected in 22.9% of corneal sequestra examined.¹⁵⁸

1.8.7 Eosinophilic keratitis

Infection with FHV-1 has also been implicated in the development of eosinophilic keratitis.¹⁶⁴ Clinically, the patient presents with a pink to white, irregular, vascularized lesion in the peripheral limbal area that may invade the superficial cornea, conjunctiva, and nictitating membrane and be present unilaterally or bilaterally.^{118,119,165} Cytologic examination usually demonstrates the presence of eosinophils, mast cells, lymphocytes, or plasma cells.^{124,166} Histopathological findings are chronic granulomatous inflammation characterized by infiltration of plasma cells, lymphocytes, numerous eosinophilic granulocytes and occasionally mast cells.^{119,166}

Using IFA, corneal scraping specimens from cats affected with eosinophilic keratitis were positive for FHV-1 in 9 of 27 cases (33.3%).¹⁶⁴ Nasisse at al.¹⁶³ detected FHV-1 DNA using PCR assay in 76.3% of corneal scrapings from cats with eosinophilic keratitis, but only in 5.9% of those from clinically normal cats. The higher detection rate in the second study is explained by the higher sensitivity of PCR

compared to IFA. The role of FHV-1 in the development of eosinophilic keratitis has to be further elucidated.¹²⁰

1.8.8 Anterior uveitis

Herpes infections that involve the anterior uvea have been described in humans and are generally not associated with simultaneous or past keratitis.¹⁶⁷ Utilizing PCR and hybridization techniques, Bustos et al. detected HSV-1 DNA in 20/30 (66.6%) human ciliary ganglia from patients with no known active HSV-1 infection at the time of death.¹⁶⁸ This suggests that the ciliary ganglion may be an additional site of HSV-1 latency in humans.¹⁶⁸ Latent HSV-1 was also found in ciliary ganglia from experimentally infected mice.³¹ This suggests that anterior uveitis may result from virus reactivation within the ciliary ganglia rather than within the trigeminal ganglion. However, Labetoulle et al.¹⁶⁹ concluded from another study, that infection of the iris and the ciliary body results from transfer of virus through the sympathetic system via the superior cervical ganglion. If additional sites of latency within the autonomic system exist in cats, a possible role of FHV-1 in feline anterior uveitis could be explained.

Maggs et al.¹⁷⁰ utilized a PCR assay to detect FHV-1 DNA in the aqueous humor of 11 of 73 cats (14%) with idiopathic anterior uveitis or uveitis due to *Toxoplasma gondii*. Intraocular production of FHV-1 antibodies was noted in 22/44 cats with idiopathic uveitis and 11/29 cats with toxoplasma uveitis, whereas intraocular production of FHV-1 antibodies was never detected in cats without uveitis.¹⁷⁰ In a study by Powell et al.,¹⁷¹ four specific pathogen free kittens were inoculated with FHV-1 by use of an atomizer administering ½ of the volume into the oropharynx and ¼ of the volume into each nostril. Two of the cats developed mild anterior uveitis 11 and 25 days post-infection respectively that resolved within one week of appearance. No posterior segment lesions were detected.¹⁷¹ These findings suggests that FHV-1 may play a role in feline anterior uveitis.

1.9 Feline herpesvirus 1 disease – Diagnosis

The characteristic clinical signs and the self-limiting nature of most acute phase FHV-1 infections often make obtaining a definitive diagnosis less critical.^{75,120} Application of Rose Bengal stain, a vital dye that stains dead and degenerating cells, to the ocular surface can be helpful to identify early dendritic lesions prior to the disruption of the corneal epithelium.^{120,126,172} Application of sodium fluorescein to the ocular surface will identify corneal epithelial disruption.¹²⁰ Conjunctival cytology may be performed in acute, primary FHV-1 infections particularly for the presence of intranuclear inclusion bodies.¹¹⁸ May-Gruenwald-Giemsa stain has been recommended for identifying intranuclear inclusions.¹²¹ Conjunctival cytology is rarely diagnostic because the predominant cell type observed is the neutrophil and intranuclear inclusions are infrequent.¹²⁶

The diversity of clinical signs in chronic FHV-1 disease make a definitive diagnosis with viral identification more desirable.^{75,120} Tests that are currently available either rely on detection of the organism or demonstration of an immunologic response to the organism. The methodologies available include virus isolation (VI), detection of virus neutralizing serum antibodies (SN), enzyme-linked immunosorbent assay (ELISA), immunofluorescent antibody testing (IFA), or polymerase chain reaction assays (PCR).⁷⁵

1.9.1 Virus isolation

In 1958, Crandell and Maurer²¹ isolated a feline virus associated with intranuclear inclusion bodies, which was designated as the C-27 virus. In 1959, the

cytopathogenic features of feline rhinotracheitis virus in tissue cultures of feline renal cells were described as: development of intranuclear inclusion bodies, formation of giant cells, and a slight reduction in the metabolic rate of infected cells.²⁸ Virus isolation (VI) has been the gold standard for the diagnosis of alphaherpesviruses.⁷⁵ Due to FHV-1's rapid replication and production of characteristic cytopathic effects in cell culture, VI is relatively simple to perform provided viable virus was collected and transported to the laboratory.⁷⁵ Dacron or cotton swabs are recommended to collect the samples as alginate swabs can be inhibitory to some herpesviruses.¹⁷³ After collection of the sample, it should be refrigerated and shipped promptly.⁷⁵

1.9.2 Serology

Regardless of methodology, serologic titers are not considered useful in the diagnosis of FHV-1 infection because of the high rate of vaccination against FHV-1 with resultant systemic antibody titers.^{70,75,123} Therefore titers are unlikely beneficial, because serology does not differentiate between immune response to vaccination or natural infection.⁷⁵ In one study, no correlation between FHV-1 seropositivity or titer magnitude and the likelihood of detecting virus or presence of clinical signs could be demonstrated.⁶⁴

The serum neutralization (SN) test is commonly used to detect FHV-1 infection in cats.^{61,174-176} However, FHV-1 is difficult to detect with this method, because of the poor immunogenicity of the virus.¹⁷⁷ The presence of antibody in serum suggests prior infection but does not necessarily correlate with clinical disease.¹⁷⁸ On the other hand FHV-1 has been documented in ocular tissues of many cats negative for antibody by serum neutralization test.¹⁷⁹

Enzyme-linked immunosorbent assays (ELISA) for detection of feline herpesvirus-1 antibody have been developed.^{177,178} These tests are more sensitive than serum neutralization.¹⁷⁸ However in one study, seroprevalence, as assessed by ELISA did not vary significantly between normal cats and cats suspected to be undergoing acute or chronic FHV-1 infections, nor did it correlate with the likelihood of viral detection utilizing IFA or VI.⁶⁴

1.9.3 Fluorescent antibody test

Fluorescent antibody tests can be performed as a direct test (polyclonal fluorescein conjugated antibody) or an indirect test (polyclonal unlabelled antibody, which then reacts with a conjugated antibody). The antibody then detects FHV-1 epitopes on the cell surface.⁷⁵ However, the indirect test is more sensitive than the direct test.¹⁸⁰ Samples are usually obtained by conjunctival or corneal scraping or by biopsy.^{75,120} It is necessary to collect the sample prior to topical application of fluorescein dye, as this will interfere with the interpretation of the test result.¹⁸¹ Carlson and Scott found the test specific and sensitive for diagnosing cats showing clinical sings of FHV-1 infection.¹⁸² In contrast, other studies found different limitations of the fluorescent antibody test, such as lack of significant differences in detection rates between acutely or chronically affected and normal animals.⁶⁴ and the subjective nature of the laboratory interpretation.^{64,126} Particularly in chronic infections, it is possible that viral epitopes may be bound to host antibody, which results in a false negative test.¹²⁶ Burgesser et al.¹⁸³ determined the sensitivity and specificity for indirect immunofluorescence to be 29% and 96%, respectively, compared to PCR. These limitations and the availability of other diagnostic methods, make fluorescent antibody tests appear to be of limited clinical application.⁷⁵

1.9.4 PCR assays

The polymerase chain reaction was introduced in 1987 by Mullis et al.¹⁸⁴ and dramatically changed the field of molecular biology as well as disease diagnosis in man and animals. To obtain a sample for FHV-1 detection, host cells need to be sampled, since the virus is an obligate intracellular agent.⁷⁵ Biopsy samples are recommended.⁷⁵ However, in other studies, FHV-1 DNA was obtained by the use of Schirmer tear test strips or microbrush swabs.^{84,185} Laboratories often suggest the samples are placed in a small volume of a physiological fluid such as phosphate buffered saline, frozen, and shipped overnight on ice for analysis.⁷⁵ However, Maggs et al.¹⁸⁶ assessed the effect of shipping temperature on results of one PCR assay for detection of FHV-1 DNA. PCR results did not differ for identical samples sent overnight on ice or mailed over several days at ambient daytime maximum temperatures between 17 an 47 °C.¹⁸⁶

Multiple PCR assays, some of which are commercially available, have been described for use in the detection of FHV-1 DNA.^{85,144,183,187-189} PCR analysis for FHV-1 is commonly based on DNA-amplification of the thymidine kinase gene.^{126,144} PCR and nested PCR are highly sensitive for the detection of active or latent FHV-1 infections.^{126,144,162,183} These PCR techniques have shown to be more sensitive than the use of immunofluorescent antibody testing or virus isolation, but detection limits vary greatly.^{85,144,183,187-189}

Nested PCR utilizes a second primer pair internal to the initial primer. Stiles et al.¹⁸⁸ demonstrated a nested PCR assay to be more sensitive for identification of FHV-1 in cats with conjunctivitis than virus isolation or fluorescent antibody tests. In another study comparing virus isolation, single PCR, and nested PCR for the detection of FHV-1 in ocular and nasal secretions, nested PCR was shown to be 4.8 times more sensitive than single PCR.¹⁸⁹ When 22 samples that were negative for virus isolation were assayed, 19/22 (86.4%) were positive by nested PCR and 2/22 (9%) were positive by single PCR.¹⁸⁹

Real time PCR not only allows detection of, but also the quantification of a given DNA sequence.¹⁹⁰ Accumulation of PCR product is measured through a labelled fluorogenic probe.¹⁹⁰ Advantages of real time PCR over conventional PCR include simultaneous quantification of input target DNA within the PCR process, no necessity for post-amplification steps, and therefore reduced chance for cross contamination.¹⁹⁰ The procedure is fast and highly efficient.¹⁹⁰ A real time PCR assay was developed for the quantification of FHV-1 DNA in ocular fluid samples of clinically diseased cats.⁸⁴ A good correlation between viral titer and quantitative PCR was observed during early infection.⁸⁴ In the second stage of disease, the viral titers decreased, while the PCR signal remained high, and in the final stage of the disease, virus isolation was negative and the PCR signal decreased consistently.⁸⁴ The authors concluded that comparing the results of virus isolation and quantitative PCR in consecutive samples would allow one to track the course of the infection.⁸⁴ Low et al.¹⁹¹ assessed the prevalence of FHV-1 DNA in cats with and without conjunctivitis using conventional PCR and quantitative PCR. The concordance between the two assays for detection of FHV-1 DNA was 92.5%.¹⁹¹ No significant difference for FHV-1 prevalence was found between cats with conjunctivitis and cats without conjunctivitis.¹⁹¹

Another study compared six published PCR assays for detection of FHV-1 DNA in 15 clinical samples and five vaccines licensed for use in preventing FHV-1 associated disease.¹⁹² Detection rates for the clinical samples varied between 29% and 86%.¹⁹² All six PCR assays detected FHV-1 DNA in all five vaccines tested.¹⁹²
Therefore, a positive PCR results cannot differentiate a vaccinal strain from natural strains and does not differentiate sub-clinically infected cats from those with clinical disease as a result of FHV-1 infection.^{64,192}

1.9.5 In situ Hybridization

In order to obtain information regarding latent FHV-1 infection, Ohmura et al.¹⁹³ utilized *in situ* hybridization to detect FHV-1 LATs in the trigeminal ganglia of experimentally infected cats. Such yet al.¹⁹⁴ performed in situ hybridization with a digoxigenin-labeled double-stranded DNA probe for FHV-1 on different tissues from a severely affected cat. Hybridization signal was found only in nuclei of intact epithelial cells in the basal layers of the epidermis, hair follicles of the eyelids, nose, superior lip and paw, as well as in conjunctival epithelium of palpebrae and nictitating membrane and in a few superficial cells of both corneas.¹⁹⁴ Kim and Yi¹⁹⁵ optimized an *in situ* hybridization protocol for the detection of FHV-1 DNA by using microwave irradiation as pre-treatment. Although this technique may be of limited value in diagnosis of clinical cases, it offers great potential in studying FHV-1 infection of different tissues as in situ hybridization allows for specific localization of the virus and evaluation of tissue morphology simultaneously. Sensitivity of in situ hybridization largely depends on many variables in the utilized protocol including fixation of the tissue, exposure of target nucleic acid, selection of probe, type of probe labelling, denaturation of doublestranded DNA, hybridization conditions, and detection conditions.¹⁹⁶

1.9.6 FHV-1 detection in different tissues

Despite the high incidence of FHV-1 infection within the feline population and many investigations into its pathology, much remains unknown. For example, FHV-1 latency is stated to occur only in the trigeminal ganglia.² During the latent period, clinical signs are absent and the virus cannot be isolated using standard cell culture techniques.^{38,197} However, several studies have detected FHV-1 DNA by PCR in 13%¹⁸⁸ to 31%¹⁸³ of conjunctival and 5.9%,¹⁶³ 46%,¹⁶² and 49%¹⁹⁸ of corneal samples from clinically normal cats. Despite the PCR positive results of conjunctival samples, FHV-1 was detected either infrequently¹⁸³ or not at all¹⁸⁸ by virus isolation (VI) or fluorescent antibody (FA) staining. In these samples, FHV-1 was not quantified, nor was the active or latent viral status or specific viral location within each tissue identified.

It is known that FHV-1 demonstrates a tropism for conjunctival epithelium and also replicates within corneal epithelium.^{79,80} In one study, immunohistochemical staining of corneal and conjunctival epithelial cells revealed the diffuse presence of viral antigen during the acute stage of infection.⁸⁰ In cats with experimentally induced stromal keratitis, viral antigen persisted within the corneal stroma between collagen lamellae.¹⁴³ However these cats were immunosuppressed by three consecutive daily subconjunctival injections of betamethasone prior to inoculation with the virus. In clinically normal cats with PCR detectable virus, whether the virus persists in epithelial cells, becomes localized in the underlying stroma, or has any effect on the surrounding tissues is not known. Viral location is critical, as acquisition of viral antigen by the corneal stroma is the factor currently thought to precede development of stromal keratitis.¹⁴³ The ability of the virus to infect other ocular tissues has not been widely explored.

Studies to identify sites of FHV-1 latency have concentrated particularly on the trigeminal ganglion as this is an important site of viral latency for many other herpesviruses.^{197,199} Gaskell et al.¹⁹⁹ isolated FHV-1 from the trigeminal ganglia of 3/17 cats latently infected with field virus using a tissue fragment culture technique. FHV-1 could not be detected in four cats previously infected with a vaccine strain of FHV-1 or in four cats which were administered the vaccine and 48 hours later were inoculated with field virus.¹⁹⁹ The authors suggest that the vaccine may prevent latent infection.

Nasisse et al.²⁰⁰ isolated FHV-1 in trigeminal ganglia from 5/26 latently infected cats using a similar tissue culture method. FHV-1 was also isolated from the trigeminal ganglia from 4/4 cats with active infection.²⁰⁰

Reubel et al.¹⁴⁴ developed and utilized a PCR assay to detect FHV-1 in various tissues in latently infected cats. Latency was defined by concurrent negative virus isolation for those tissues. FHV-1 was detected by PCR in optic nerves (6/6), trigeminal ganglia (8/9), olfactory bulbs (7/9), nasal turbinates (7/9), corneas (6/6), lacrimal glands (2/5), salivary glands (1/9), oral fauces (3/7), tonsils (1/9), cerebrum (1/9), conjunctiva (1/9), and mesenteric lymph node (1/9). No virus was detected by PCR in cerebral cortex, thymus, trachea, lung, liver, spleen, kidney, or peripheral blood monocular cells. However, 5/9 cats received intravenous methylprednisolone acetate 10 days prior to necropsy. In all five cats virus could be isolated from oral swabs obtained at the time of necropsy. In these cases, it is likely that a recrudescent infection was present at the time of euthanasia. In 4/4 cats viral DNA was detected utilizing PCR from oral swabs. Vaginal swabs were negative in all 5 cats.

In a similar study conducted by Weigler et al.⁸⁵ various tissues from 10 cats latently infected with FHV-1 were evaluated for the presence of FHV-1 by PCR. Latency was confirmed by concurrent negative virus isolation. All 20 trigeminal ganglia, 15/20 optic nerves, and 6/20 corneas were positive for FHV-1 DNA on PCR. FHV-1 was detected in 4/10 optic chiasma and 2/9 olfactory bulbs. Virus was not detected within the uvea.

Detection of latency associated transcripts (LATs) is probably the most accurate way to truly define the latent stage of infection.^{30,197} With the detection of LATs it is also possible to distinguish true latency from low-level persistent infection.²⁰¹ Using *in situ* hybridization and northern blot analysis, Ohmura et al.¹⁹³ detected transcripts of opposite strand to the immediate-early mRNA in the trigeminal ganglia of 3/5 experimentally infected cats.

In another study,¹⁹⁸ FHV-1 DNA was detected by PCR in 45/92 (48.9%) corneas and in 38/92 (41.3%) trigeminal ganglia of cats without clinical signs of FHV-1 infection. The investigators also developed a reverse transcriptase PCR (RT-PCR) to detect FHV-1 LATs. None of the 39 tested corneas and four of the 16 tested trigeminal ganglia had positive results for FHV-1 LATs.¹⁹⁸

These studies to identify FHV-1 sites of latency suggest that FHV-1 follows a pattern similar to HSV-1 during latency. However, studies to evaluate whether FHV-1 infects and establishes latency in autonomic ganglia have not yet been undertaken.

1.10 Feline herpesvirus 1 disease - Treatment and prevention

Upper respiratory tract infection during the acute stage of the disease is in most cases self-limiting. Systemic broad-spectrum antibiotics are indicated to prevent or treat secondary bacterial infections.^{66,70,71} Supportive care is also very important.¹²⁰ It is necessary to keep oro-nasal pathways clear. Frequent removal of nasal and ocular discharge is recommended.^{71,106,202} Intravenous or subcutaneous fluid administration can become necessary to achieve rehydration.^{66,71,202} Nutritional support is indicated in cases of severe anorexia.^{66,202}

Corneal ulceration should be treated with topical broad spectrum antibiotics to prevent secondary bacterial infections, and topical antiviral therapy should be considered.^{66,70,118,203} In kittens with primary FHV-1 infection with corneal ulceration or early symblepharon formation, topical antiviral therapy can be beneficial.^{66,70,203} In these cases frequent mechanical break-down of the symblepharon is also recommended.^{70,203} In older cats with recurring bouts of FHV-1 conjunctivitis, therapy depends on the severity, duration, and frequency of the outbreaks.^{70,203} Mild transient conjunctivitis does not require specific treatment, as it is usually self-limiting.^{70,120} If the conjunctivitis does not resolve or a corneal ulcer is present, topical antiviral therapy, as discussed below, is indicated.^{70,118,125} Mechanical debridement of the corneal ulcer can be helpful^{70,203} to enhance the healing by removal of any loose epithelium, as well as removal of viral particles. Specific antiviral and adjunctive therapy, such as oral supplementation with the amino acid L-lysine, or application of interferons are described below in detail.

Topical application of corticosteroids is the recommended initial treatment in cases of eosinophilic keratitis;^{118,125} however, in cats where an association with FHV-1 is suspected, other treatment options should be considered instead.^{164,204} Oral administration of megestrol acetate, 5 mg/day during the first week and 2.5-5 mg/week for maintenance dosage as treatment of eosinophilic keratitis has been recommended.^{119,204} Due to the potential of serious systemic side effects, such as temperament changes, polyphagia, diabetes mellitus, iatrogenic Addison's syndrome, endometritis, cystic endometrial hyperplasia, mammary hypertrophy and neoplasia, megestrol acetate is not recommended as the initial choice of therapy.^{119,205} Topical application of cyclosporine can be used alternatively.^{204,206} In a recent study by Spiess et al.,²⁰⁶ 33 cats were treated with topical cyclosporine 1.5% two or three times daily. 83% of these cats showed marked improvement and 6% showed mild improvement of the disease. While, three animals showed no response, the likely cause was thought to be poor owner compliance. Two animals developed blepharitis, which resolved after treatment.

1.11 Antiviral therapy

A multitude of antiviral drugs are licensed for treatment of viral diseases in humans. As a result of the many similarities between HSV-1 and FHV-1, several of the drugs are employed in the treatment of herpetic disease in cats. However, differences in the biologic behaviour of these viruses support the need for continued research into and development of antiviral drugs specifically for use against FHV-1 in cats.

Frequent administration of the antiviral compounds is crucial in order for therapy to be effective.²⁰⁷ This is often a problem for cat owners, as many of the available topical antivirals are topically irritating.¹²⁰ The presently available antiviral drugs are also virustatic. Therefore the drugs are effective only during the replicative phase of infection and latent FHV-1 remains unaffected.^{1,207,208} Precise targeting of viral specific replicative processes is difficult as the virus utilizes host cell machinery for replication.²⁰⁷ For many antiviral agents, the mechanism of action does not adequately differentiate infected from non-infected cells, which can lead to host toxicity.²⁰⁷

1.11.1 Nucleoside analogues

The structure of these medications is similar to the nucleosides required for viral and host DNA and RNA synthesis. However, due to slight modifications the nucleoside analogues are able to disrupt the viral replication cycle.²⁰⁷ Following an

intracellular phosphorylation, the nucleoside analogues are incorporated into newly synthesized mammalian and viral DNA strands.¹ DNA synthesis is then disrupted by mechanisms that are similar for all nucleoside analogues and are described in further detail below.

1.11.2 Acyclovir

Acyclovir (9-(2-hydroxyethoxymethyl)guanine (acycloguanosine)) is similar to guanosine, but it contains an acyclic sugar group.¹ Acyclovir is a specific, nontoxic drug, that is highly efficacious against herpes simplex virus and, to some extent, varicella zoster virus.¹ Activation of this pro-drug requires three kinases to be present in the cell to convert acyclovir to a triphosphate derivate, the actual antiviral drug.¹ The first step, phosphorylation of acyclovir into the monophosphate, is catalyzed by a herpesvirus-specific thymidine kinase.²⁰⁹⁻²¹¹ Cellular kinases perform the two remaining phosphorylation steps to produce acyclovir triphosphate.^{1,207} Acyclovir triphosphate has a greater affinity for the viral DNA polymerase than the naturally occurring deoxyguanine triphosphate, and is therefore preferentially incorporated into growing DNA via a competitive mechanism.^{1,207}

The primary activities of acyclovir are related to its ability to decrease viral replication by chain termination of replication DNA by inactivating viral DNA polymerase.²⁰⁷ As acyclovir lacks the 3'- hydroxyl group present in the cyclic sugar ring of naturally occurring nucleotides, the growing DNA chain terminates, and further DNA replication is blocked.^{1,207} When acyclovir triphosphate is bound to the DNA template in the presence of the next nucleotide to be attached, the viral DNA polymerase is inactivated by the formation of an irreversible complex.^{212,213} Further DNA replication is shut down by these mechanisms; therefore, no further production of infectious virions is possible.²⁰⁷

Acyclovir is highly specific for cells infected with herpes simplex virus or varicella zoster virus.¹ The specificity depends on the virus-encoded kinase.¹ Differences or mutations in the viral thymidine kinase prevent the initial step of monophosphorylation leading to antiviral resistance.²⁰⁷ Since the development of acyclovir in 1974, many variations of chain-terminating nucleoside analogues have been developed in order to increase efficacy and bioavailability.¹ Information regarding efficacy of acyclovir against FHV-1 is described below.

1.11.3 Valacyclovir

Valacyclovir (L-valine, 2-[(2-amino-1,6-dihydro-6-oxo-9H-purin-9yl)methoxy]ethyl ester, monohydrochloride) is the hydrochloride salt of the L-valyl ester derivate of acyclovir.^{1,207,214} Following oral administration, valacyclovir is nearly completely converted by a liver hydrolase to acyclovir and L-valine during intestinal or hepatic metabolism.²¹⁵⁻²¹⁷ It is therefore considered to be a pro-pro-drug.¹ The mean absolute oral bioavailability of acyclovir after administration of valacyclovir is four-fold higher (54.4 \pm 9.1%)^{214,218} than the limited oral bioavailability of acyclovir (15 to 21%).²¹⁹ The bioavailability of valacyclovir in cats is 2.3-fold greater than that of acyclovir;²²⁰ however, severe systemic side effects such as bone marrow suppression, hepatic necrosis, and renal tubule epithelial necrosis have been documented.²²¹ Due to its extremely toxic properties, valacyclovir is considered unsuitable for use in cats.²²¹

1.11.4 Ganciclovir

Ganciclovir (9-(1,3-dihydroxy-2-propoxymethyl)guanine) is an acyclic guanosine analogue, which is structurally closely related to acyclovir.^{207,222} Following phosphorylation to its triphosphorylated form, ganciclovir inactivates viral DNA

polymerase by competing with deoxyguanosine triphosphate for incorporation into the replicating DNA.²⁰⁷ Its incorporation into viral DNA-strands also results in termination of elongation.²²³ The toxicity of ganciclovir is greater than that of acyclovir *in vivo* and *in vitro*. It can cause severe neutropenia in humans.²⁰⁷ Similar to acyclovir, the oral bioavailaility is limited. Therefore a valine ester of ganciclovir (valganciclovir) has been synthesized.²²² Like valacyclovir, valganciclovir will be converted to release the parent compound, ganciclovir.²²²

1.11.5 Penciclovir

Penciclovir (9-(4-hydroxy-3-hydroxymethyl-but-1-yl)guanine) was first synthesized in 1972.²²⁴ The activity of penciclovir against several herpesviruses was first evaluated in 1987.²²⁵ It is an acyclic guanosine nucleoside analogue with a structure similar to acyclovir.^{207,222,225} Like acyclovir and ganciclovir, penciclovir must be initially phosphorylated by a viral thymidine kinase, followed by two more phosphorylation steps to reach its active form, penciclovir-triphosphate.^{207,222} This active form has been shown to inhibit herpesvirus DNA polymerase. However the possibility exists for internal incorporation of penciclovir residues into viral DNA and further chain extension.²²⁶ The first phosphorylation step of penciclovir to penciclovir-monophosphate is more efficient than the phosphorylation of acyclovir. However, the penciclovir-triphosphate formed in infected cells is a less effective inhibitor of herpes simplex virus DNA polymerase than acyclovir-triphosphate.²²⁷ Earnshaw et al.²²⁷ showed that for HSV-1 DNA polymerase, the K_i for penciclovir triphosphate is about 100-fold greater than that for acyclovir triphosphate. Therefore, a greater drug concentration is needed to inhibit viral DNA polymerase and hence inhibit the virus. Although penciclovir showed a similar spectrum of antiviral activity and selectivity when compared to acyclovir, the oral bioavailability was even lower than that of acyclovir.²²² Attempts at improving bioavailability led to the development of famciclovir.²²²

1.11.6 Famciclovir

Famciclovir (Diacetyl-6-deoxy-9-(4-hydroxy-3-hydroxymethyl-but-1yl)guanine) is the diacetyl derivate of penciclovir.²²⁸ Following oral administration of famciclovir, it is efficiently converted to penciclovir.²²⁹ The absolute bioavailability of penciclovir from famciclovir following a single oral dose is 77%. The final products are penciclovir and the harmless by-product, acetic acid.²²⁹ Administered orally, famciclovir is highly effective against HSV-1 in a variety of different animal models.²³⁰ In a quantitative study on the prevention of HSV-1 latency in mice, significantly less latent virus was detected in the ganglia of mice treated with famciclovir as compared to those treated with valacyclovir.²³¹ Oral administration of famciclovir appears to be well tolerated in cats.²³²

1.11.7 Idoxuridine

Idoxuridine (IDU, 5-iodo-2'-deoxyuridine) was the first available antiviral agent, initially synthesized in 1959.²³³ It is still used in veterinary medicine. In human medicine it has largely been replaced by newer agents, such as acyclovir.²⁰⁷ Idoxuridine is a thymidine analogue, which does not require a virally encoded thymidine kinase for phosphorylation.^{1,211} Since it can be phosphorylated by both cellular and viral kinases, it has less specificity than acyclovir.^{1,211} Idoxuridine has a direct inhibitory effect on virus-specific DNA polymerase causing suppression of viral DNA synthesis.²¹¹ Idoxuridine also causes disruptive replicative events as incorporation of the thymidine analogue into newly synthesized viral DNA produces abnormal viral proteins.²¹¹ When administered systemically, it combines with cellular

DNA and can cause severe systemic side effects, including neoplastic changes, genetic mutations, or infertility.²¹¹ Ophthalmic preparations are often compounded as 0.5% ointment or 0.1% solution,²³⁴ which are well tolerated and reasonably effective in the treatment of FHV-1 disease in cats.²⁰⁷

1.11.8 Trifluridine

Heidelberger et al. first synthesized 5-trifluoromethyl-2-deocyuridine (TFT) in 1964.²³⁵ Like idoxuridine, trifluridine is structurally similar to deoxythymidine, a pyrimidine nucleoside used in DNA synthesis.²⁰⁷ It acts by incorporation into viral DNA, followed by inhibition of transcription of late virus-specific DNA.²¹¹ As with idoxuridine, trifluridine is also independent of viral thymidine kinase for phosphorylation because it can be phosphorylated by cellular or viral kinases to its active form.²³³ Trifluridine is commercially available as a 1% ophthalmic solution.²³⁴

1.11.9 Vidarabine

Vidarabine (9-Beta-d-rabinofuranosyladenine (Ara-A, adenine arabinoside)) was first synthesized in 1960 as a potential anti-neoplastic agent.²¹¹ In 1964, its antiviral properties were reported.²³⁶ Vidarabine is a nucleoside analogue of adenosine, which does not require viral thymidine kinase for phosphorylation and activation.²³⁷ It selectively inhibits the replication of DNA viruses and some RNA viruses.^{207,211} Following the three successive phosphorylation steps performed by host cellular enzymes, it is a competitive inhibitor of cellular and viral DNA polymerases, but has more potency against viral enzymes.^{211,237} Systemic use is limited by its toxic neurologic and gastrointestinal side effects.²¹¹ Vidarabine is available as a 3%

ophthalmic ointment with a recommended application frequency of every three hours.²³⁴ Clinically, it is reasonably tolerated by cats.²⁰⁷

1.11.10 Cidofovir

Cidofovir ((S)-1-(3-hydroxy-2-phosphonylmethoxypropyl)cytosine) was first described in 1987 as a broad-spectrum anti-DNA viral agent active against herpesviruses (HSV-1, HSV-2, VZV, CMV), adeno- and poxviruses.²³⁸ Cidofovir does not require viral thymidine kinase for phosphorylation as cellular host enzymes perform the two phosphorylation steps required to reach its active form.²⁰⁷ Nephrotoxicity of cidovovir when given orally necessitates limitation of the dosage in humans.²²² 1-[((S)-2-Hydroxy-2-Oxo-1,4,2-Dioxaphosphorinan-5-yl)Methyl]cytosine is the chemically stable pro-drug of cidofovir, which has reduced nephrotoxicity as compared to cidofovir in rats²³⁹ and in humans.²⁴⁰ Within the cells, the pro-drug is converted to cidofovir by an intracellular cyclic CMP phosphodiesterase.²⁴¹ To increase the limited oral bioavailability, alkoxyalkyl esters have been prepared.²⁴²

Cidofovir was able to reduce clinical disease and viral shedding in cattle with bovine herpesvirus type 1 infection.²⁴³ Topical administration of cidofovir was highly effective in rabbit models in the treatment of experimental herpetic keratitis.²⁴⁴⁻²⁴⁷

1.11.11 Bromovinyldeoxyuridine

Bromovinyldeoxyuridine, BVDU [(E)-5-(2-bromovinyl-2'-deoxyuridine)] is a pyrimidine analogue of the nucleoside thymidine.²⁰⁷ For phosphorylation it requires viral-encoded thymidine kinase like acyclovir.²⁰⁷ FHV-1 thymidine kinase does not effectively phosphorylate this agent, making BVDU relatively ineffective against FHV-1.²⁰⁷ Because of the low potency, BVDU is not used in veterinary medicine.²⁰⁷

1.11.12 Ribavirin

1-Beta-d-ribofuranosyl-1,2,4-triazole-3-carboxamide, (ribavirin or virazole) is a triazole purine analogue, which inhibits a broad spectrum of RNA and DNA viruses.²¹¹ Following phosphorylation to its 5'-monophosphate form, it directly inhibits the synthesis of guanine nucleosides. Ribavirin triphosphate also blocks ATP and GTP binding to RNA polymerase.²⁴⁸ Systemic administration may lead to a number of side effects, such as extravascular hemolysis, bone marrow suppression, gastrointestinal toxicity, and central nervous signs.^{208,248} These side effect make it unsuitable for clinical use.

1.12 Efficacy of nucleoside analogues against FHV-1

Nasisse et al.²⁴⁹ evaluated the *in vitro* susceptibility of feline herpesvirus-1 to various antiviral drugs and found the relative potency of these compounds was trifluridine >> idoxuridine > vidarabine > bromovinyldeoxyuridine >> acyclovir. Maggs and Clarke²⁵⁰ conducted a study in order to establish the *in vitro* efficacy against FHV-1 of ganciclovir, cidofovir, penciclovir and foscarnet. Furthermore the results were compared to the *in vitro* efficacy of acyclovir and idoxuridine. The relative potency of these compounds was idoxuridine = ganciclovir >> cidofovir > penciclovir >> acyclovir >> foscarnet. The authors concluded that clinical trials with ganciclovir, cidofovir are warranted.

Van der Meulen et al.²⁵¹ compared the *in vitro* efficacy of different antiviral drugs against FHV-1. Ganciclovir, 9-(2-phosphonylmethoxyethyl)-2,6-diaminopurine (PMEDAP) and cidofovir were most potent; adefovir and foscarnet were intermediately potent; and acyclovir was least potent. However, all antiviral drugs were able to significantly reduce plaque size in Crandell-Rees feline kidney (CRFK)

cells when compared with the untreated control. None of the compounds were toxic for the CRFK cells at antiviral concentrations.

A study to assess the effect of cidofovir on viability of feline corneal epithelial cells, replication of FHV-1, and virus-induced cytopathic changes revealed that cidofovir *in vitro* was highly efficacious against FHV-1 infection of a primary culture of feline corneal epithelial cells, but had cytostatic effects on the cultured cells.²⁵² A recent study by Fontenelle et al.²⁵³ found that a twice daily application of 0.5% cidofovir solution in both eyes significantly decreased the amount of viral shedding, as determined by quantitative real time PCR, and the severity of clinical disease in cats with experimentally induced ocular FHV-1 infection.

Owens et al.²²⁰ studied the pharmacokinetics of acyclovir and valacyclovir in cats. Nephrotoxicity and bone marrow suppression were observed with repeated dosing of acyclovir and valacyclovir in cats with experimentally induced FHV-1 infection. Futhermore, neither compound appeared efficacious against FHV-1 infection. In another study conducted to determine whether orally administered valacyclovir could be used safely and effectively to treat cats with primary FHV-1 infection, severe systemic side effects such as coagulative necrosis of the renal tubular epithelium, centrilobular atrophy and hepatic necrosis, and severe bone marrow depression were noted.²²¹ The experiment had to be terminated after 12 days for humane reasons. Therefore treatment of cats with systemic acyclovir or valacyclovir is not recommended. However, Williams et al.²⁵⁴ evaluated the efficacy of topical ophthalmic acyclovir applied five times daily as a treatment for FHV-1 keratitis in cats and concluded that topical acyclovir can have a beneficial effect against FHV-1 conjunctivitis and keratitis.

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A study evaluating the pharmacokinetics and safety of penciclovir following oral administration of famciclovir in cats revealed that famciclovir dosages of 15 mg/kg orally administered every eight hours are unlikely to result in plasma penciclovir concentrations with activity against FHV-1.²³² No adverse side effects with the oral administration of famciclovir were observed. However, anecdotal reports suggestive favourable responses after administration of 6-10 mg/kg orally of famciclovir every 12 to 24 hours to clinically affected animals.²³²

1.13 Non-nucleoside antivirals

1.13.1 Foscarnet

Phosphonoformic acid (PFA) is a pyrophosphate analogue inhibiting in a noncompetitive way the pyrophosphate binding site of DNA polymerase.²⁵⁵ Foscarnet does not require phosphorylation. Its efficacy is therefore not dependent on viral thymidine kinase.¹ It has been shown to have activity against herpesviruses, human immunodeficiency virus, and other RNA and DNA viruses.²⁵⁵ Foscarnet is administered intravenously, and due to its severe renal toxicity it is recommended only for life threatening infections for which other antiviral drugs are no longer effective.¹ Maggs and Clarke²⁵⁰ investigated the antiviral activity of foscarnet against FHV-1 *in vitro* and reported it to be relatively ineffective.

1.13.2 L-Lysine

Tankersley²⁵⁶ in 1964 studied the amino acid requirements of herpes simplex virus in human cells. Results showed that cells infected with herpes simplex virus deprived of arginine support neither cytopathic effects nor virus replication. However, when arginine was replaced, a prompt and extensive infection followed. Lysine was

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not required but rather exhibited a partially inhibitory effect upon virus multiplication.²⁵⁶

A later study confirmed that the yield of several DNA viruses in cell culture is largely dependent on the presence of arginine in the medium.²⁵⁷ Arginine deprivation in the media of HSV cell cultures causes a selective decrease in the synthesis of viral macromolecules. It seems that deprivation of arginine may be more detrimental to virus multiplication than restriction of other amino acids because several specific viral proteins are large in size and rich in arginine.²⁵⁷

A study evaluating the relationship of arginine-lysine antagonism to herpes simplex growth in tissue culture concluded that arginine deficiency suppressed HSV replication while lysine antagonized the viral growth-promoting action of arginine.²⁵⁸ Walsh et al.²⁵⁹ investigated lysine's therapeutic response when administered to patients with herpes simplex and found lysine to be an effective form of treatment for herpetic infection. L-lysine was shown to reduce the occurrence of, severity of and healing time for recurrent HSV infection.^{260,261}

Multiple studies have investigated the mechanism of the arginine-lysine antagonism. Kadirvel and Kratzer²⁶² concluded that the lysine-induced arginine deficiency is a metabolic effect rather than an inhibition of gastrointestinal tract absorption of arginine. Another study supports arginine and lysine competition for a common transport system in the blood.²⁶³ Therefore an increase of L-lysine plasma concentration saturates the transport mechanism and thereby reduces the availability of arginine.

Arginine is an essential amino acid for cats and cannot be restricted in feline diets due to their extreme sensitivity to an arginine deficiency. Morris and Rogers²⁶⁴ fed cats that were fasted overnight a single meal of a complete amino acid diet

without arginine. Signs of severe hyperammonemia developed and one cat died 4.5 hours after ingestion of the meal.²⁶⁴ The authors suggest that the rapidity of the onset of symptoms arises from a combination of 1.) a rapid depletion of free arginine in the liver and therefore of ornithine in the postabsorbtive state, and 2.) by inhibition of arginine release from protein catabolism after feeding as a result of the anabolic response of incoming amino acids.

Maggs et al.²⁶⁵ examined the effect of various concentrations of L-lysine and L-arginine on *in vitro* replication of feline herpesvirus-1. Results showed that arginine had a substantial growth promoting effect on FHV-1 and supplementation of the viral culture medium with lysine attenuated this effect. Data indicated that high concentrations of L-lysine reduce *in vitro* replication of FHV-1 in media containing low concentrations of arginine.²⁶⁵

Cats that received oral administration of L-lysine 500 mg every 12 hours beginning 6 hours prior to inoculation of FHV-1 developed less severe conjunctivitis after inoculation than cats receiving placebo.²⁶⁶ Plasma L-lysine and arginine levels were measured prior to the study and on days 3, 14, and 22, which was 1 day after cessation of oral administration of L-lysine. Mean plasma L-lysine concentrations were significantly higher than in untreated cats. Arginine plasma concentrations did not differ significantly. The authors did note that if food was withheld prior to oral administration of 500 mg or 1000 mg L-lysine, 50% of the cats vomited within 1 hour.²⁶⁶ Therefore L-lysine should be given with a small amount of food.

Another study examined the effect of orally administered L-lysine 400 mg once daily on clinical signs of FHV-1 and ocular shedding of virus in latently infected cats.²⁶⁷ The authors found decreased viral shedding but no significant change in clinical signs during reactivation. Plasma L-lysine and arginine concentrations were

assessed on day 30 at 3 and 24 hours following L-lysine administration. Three hours following administration, the mean plasma lysine concentration was significantly increased compared to the control group. Mean plasma arginine concentration did not vary significantly between treated and untreated cats. No significant difference in mean plasma lysine or arginine concentrations were detected between the treatment and the control group 24 hours after administration of L-lysine. These data suggest that twice-daily administration of L-lysine would be more beneficial.

1.13.3 Interferons

Interferons (IFNs) are glycoproteins produced by cells in response to viral infection.²⁶⁸ They are members of the cytokine family and are important mediators of the initial, innate and adaptive immune system.¹ In addition to their antiviral properties, interferons possess anti-proliferative and immune modulating properties.¹ Although the protein was described in 1954,²⁶⁹ it was named interferon in 1957 due to its viral interference.²⁷⁰ Human interferons are reported to have antiviral efficacy against herpes simplex virus as sole agents²⁷¹⁻²⁷³ or administered in combination with nucleoside analogues.²⁷⁴⁻²⁷⁶ Topical application early in the course of HSV-1 infection reduces the severity and duration of oral or genital herpes²⁷⁷ and shortens the period of viral shedding.²⁷⁸ Using topical human leucocyte interferon 3x10⁻⁶ units/ml twice daily in humans with HSV, dendritic ulcers healed more rapidly and viral shedding was significantly inhibited.²⁷⁹ A study comparing the *in vitro* effect of an undefined feline interferon and two recombinant human alpha-interferons on feline herpesvirus-1, feline calicivirus, and vesicular stomatitis virus found that treatment with these interferons reduced the viral yield for each of these three viruses as compared with that of the control cultures.²⁸⁰

Sandmeyer et al.²⁸¹ investigated the effect of interferon-alpha on viability of feline corneal epithelial cells, replication of feline herpesvirus-1, and virus-induced cytopathic changes in primary cultures of feline corneal epithelial cells. Interferonalpha did not have cytotoxic effects and at a concentration of 10^5 IU/ml was moderately effective against FHV-1 infection in primary cell cultures of feline corneal epithelial cells.²⁸¹ In another study, the effect of recombinant feline interferon omega (rFeIFN- ω) administered topically and orally prior to subsequent FHV-1 infection in cats was investigated.²⁸² No beneficial effects of pre-treatment with rFeIFN- ω in the course of primary FHV-1 infection in cats was observed.²⁸² A cat with a longstanding feline herpesvirus-induced facial dermatitis was successfully treated with rFeIFN- ω .²⁸³ The cat received 1.5 million units (MU)/kg on day 0. Half of the dose was injected perilesionally and intradermally, the other half subcutaneously on the lateral thorax. Great improvement was noted after only 2 days. The cat received consecutive injections on days 2, 19, 21, and 23. Four months later at the last recheck examination, the lesion had significantly regressed.

Considering the different results of the various studies or case reports, further investigations and controlled clinical trials would be helpful to assess the *in vivo* therapeutic effect of interferons against FHV-1 infection. Route of application and time point of application in relation to viral infection as well as type of interferon may play an important role for its efficacy.

1.13.4 Bovine lactoferrin

Lactoferrin is an iron binding glycoprotein that has been shown to possess antibacterial,²⁸⁴⁻²⁸⁷ antifungal,²⁸⁸⁻²⁹⁰ antiprotozoal,²⁹¹ and antiviral²⁹²⁻²⁹⁵ properties. Mucosal cells of many mammalian species produce lactoferrin. Fujihara et al.²⁹⁵ reported a significant decrease in HSV-1 titers following topical administration of 1% lactoferrin using a mouse corneal infection model. Another study also found bovine lactoferrin to have potent antiviral activity against HSV-1 infection by competing with the virus for cellular binding sites.²⁹⁶

Bovine lactoferrin was also effective against FHV-1 in cell culture.²⁹⁷ A further study found bovine lactoferrin to have an inhibitory effect on the *in vitro* replication of FHV-1 prior to and during, but not following viral adsorption.²⁹⁸ When CRFK cells were exposed to lactoferrin prior to or during viral adsorption, FHV-1 replication was inhibited by 87-96%. No cytotoxic effect of lactoferrin was observed. The authors concluded that lactoferrin may inhibit FHV-1 adsorption to the cell surface and/or the penetration of the virus into the cell. Controlled clinical studies to evaluate the *in vivo* therapeutic effect against FHV-1 infections are warranted.

1.13.5 Vaccination

Vaccines against FHV-1 have been available for a number of years. There are several types of FHV-1 vaccines commercially available, which are invariably given in association with feline calicivirus (FCV) vaccines.⁶⁶ Modified live virus (MLV), and adjuvanted inactivated virus vaccines are available for parental administration. In some countries, intranasal MLV vaccines are available.⁶⁶ Currently available vaccines induce reasonable protection against disease in previously unexposed cats.^{66,197} However, although severe disease is uncommon in a vaccinated cat, milder forms of FHV-1 disease can occur, especially given a high challenge dose.¹⁹⁷

None of the currently available vaccines appear to completely protect against FHV-1 infection or the development of the carrier state. However, both viral shedding and latency load of wild type virus after viral challenge may be reduced in vaccinated cats compared to unvaccinated controls.^{197,299-301}

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Cocker et al.³⁰² concluded from their study that intranasal vaccination with a cold adapted strain of FHV-1 two days prior to challenge gave partial protection, and vaccination four days before challenge gave complete protection against feline viral rhinotracheitis. Weigler et al.³⁰¹ evaluated the biology of intranasal vaccination with a temperature sensitive FHV-1 mutant. Twelve cats were vaccinated with a temperature sensitive mutant strain of FHV-1 intranasally. Four weeks post vaccination, 6 cats were challenged with a virulent FHV-1 field strain. Eight weeks post vaccination, all 12 cats were subjected to a stress protocol (intravenous injection of 30 mg cyclophosphamide followed by 10 mg dexamethasone 24 hours later) in order to reactivate latent infections. Cats were euthanized 13 weeks post vaccination. Utilizing PCR and VI it was shown that all 12 cats shed virus throughout the 4 weeks following vaccination. Although no clinical signs were observed in the 6 cats that were challenged, FHV-1 was recovered 4 weeks post challenge. Following euthanasia FHV-1 during latency was detected in trigeminal ganglia, optic nerves, corneas, optic chiasma, olfactory bulbs, palatine tonsils and nasal turbinates. The frequency distribution of FHV-1 detected by PCR was identical for vaccine-only and vaccinechallenge groups. Therefore, latent infection with vaccine virus can be established following intranasal vaccination. The authors were not able to differentiate vaccine strain from challenge strain of FHV-1 by the methods utilized in this study.

Sussman et al.³⁰⁰ performed quantitative PCR to detect latent wild-type FHV-1 in the trigeminal ganglia, olfactory bulbs, and brain stems of cats either unvaccinated or subcutaneously vaccinated with FHV-1CV, FHV β -galglgE Δ , or FHV-1SA prior to challenge with a virulent FHV-1 field strain. Latent FHV-1 could be detected at significantly higher concentrations in trigeminal ganglia from cats that were either not vaccinated or vaccinated with FHV β -galglgE Δ than in ganglia from cats that were vaccinated with either FHV-1SA or FHV-1CV. In the olfactory bulbs or brain stems only extremely low levels of FHV-1 DNA could be detected. Another study found that administration of a single dose of a commercially available FHV-1 intranasal vaccine within several days prior to exposure lessened clinical signs of disease and FHV-1 shedding compared to unvaccinated cats.²⁹⁹

Vaccines against FHV-1 are generally safe, and although occasional mild transient clinical signs can follow their use, it is unclear if this is due to the FHV-1 or the FCV component.³⁰³ Kruger et al.³⁰⁴ reported that cats vaccinated subcutaneously or intranasally with high doses of a recombinant FHV-1 strain responded with only mild clinical signs and developed strong immunity against subsequent virulent virus challenge. Care should be taken to avoid inadvertent oronasal administration of attenuated FHV-1 vaccines labelled for intramuscular or subcutaneous administration as disease may be induced.^{71,304-306} This occurs because vaccines labelled for intramuscular or subcutaneous administration are truly virulent strains. When administered intramuscularly or subcutaneously, virulence is completely masked. When this commercially available vaccine is administered by the oronasal route to cats disease is induced as seen with natural infection.³⁰⁴

A number of genes have been targeted for the development of recombinant FHV-1 vaccines. These targeted genes mainly encode enzymes involved in DNA synthesis, such as thymidine kinase and ribonucleotide reductase genes, and a number of glycoprotein genes.¹⁹⁷ Yokoyama et al.³⁰⁷ developed a recombinant FHV-1 attenuated live vaccine, which was deleted in a defined region within the thymidine kinase gene. This attenuated live vaccine caused only mild symptoms and provided significant protection against challenge with the parent FHV-1 strain. The deletion also served as a genetic marker for PCR assays and allowed for easy detection of and

differentiation of the vaccinal strain. The level of protection was later improved through the addition of a late promoter gC in the construct.³⁰⁸

Glycoproteins that are essential can be developed for use as subunit vaccines; whereas genes of non-essential glycoproteins can be targeted for the construction of deletion mutants.¹⁹⁷ Recombinant poxviruses and baculoviruses that express the FHV-1 glycoprotein D have been constructed to characterize its properties and potential as an immunogen.^{309,310} Also, multiple FHV-1 deletion/insertional mutants have been developed including gE-gI deletion mutants,^{300,304,311} a gI insertional mutant,³¹² and an insertional mutant of ORF2, downstream of glycoprotein C.³¹³ These mutants are generally less virulent and are reasonably protective against disease, especially when administered via the oro-nasal route.⁶⁶ However, since the protection offered by these new mutants is not superior to conventionally attenuated vaccines, none has been marketed so far.⁶⁶

1.14 Conclusions

Herpesviruses are of major medical importance across all species. Despite extensive research in the past 150 years, many details of the biological nature of the different herpesviruses still have to be elucidated. FHV-1 remains a widespread pathogen within the feline population worldwide. Expansion of our current knowledge and understanding will potentially offer new perspectives for prevention and therapy of the multiple manifestations of feline herpetic disease.

Expanded knowledge of the herpesvirinae also becomes critical because of their potential new applications. A large area of current research focuses on the utilization of HSV-1 as a viral vector for gene therapy and its potential as an oncolytic virus. If cats and humans will be purposely inoculated with the herpesvirinae as therapeutic agents, we must ensure that all ramifications of that infection are known. Through genetic engineering and recombinant DNA techniques, the herpesvirinae are being utilized in the creation of novel vaccines which function as delivery agents. The delivery vaccine consists of an attenuated live vector in which foreign genetic information from one or more other pathogens has been incorporated in order to induce protective immunity against those pathogens and potentially against the field type variants of the vector.³¹⁴ Recombinant FHV-1 has been evaluated as a vector for immunization against feline immunodeficiency virus,³¹⁵⁻³¹⁸ calicivirus,^{308,319,320} and toxoplasmosis.³²¹

Viral oncolysis, the destruction of neoplastic cells by replicating viruses is a promising strategy for cancer treatment. While numerous viruses may be used for this type of therapy, HSV-1 is an attractive vector due to its high infectivity, ease of genetic engineering, and a large transgene capacity.³²² Positron emission tomography (PET) has become a useful tool for tracking sites of viral replication.³²³ HSV thymidine kinase serves as the PET reporter gene. The PET scan provides a novel, non-invasive, easily repeatable instrument to ascertain the location and magnitude of viral replication and tumor lysis.³²²

2. CHAPTER – ANATOMY AND HISTOLOGY OF TISSUES RELEVANT TO THIS STUDY

2.1 Cornea

The cornea is the anterior continuation of the sclera, the fibrous tunic of the globe. The transition zone between the cornea and the sclera is called the limbus. The careful arrangement of the fibrous structure and the absence of blood vessels and pigment allow transmission of light. The cat cornea is slightly conical and has a rather steep curvature with a radius of 8-9 mm.³²⁴ The shape of the cornea is almost circular with the horizontal diameter (16 mm) slightly greater than the vertical diameter (15.5 mm).³²⁴ This shape allows 40-42 diopters of light refraction.³²⁵ In the cat the central thickness of the cornea varies only slightly from the periphery. The average overall thickness is $0.62 \text{ mm} - 0.68 \text{ mm}.^{324,325}$

The most anterior of the corneal layers, the **corneal epithelium**, is a continuation of the conjunctiva, and is composed of nonkeratinized, stratified squamous epithelium.^{324,326} The epithelium has one basal layer of columnar cells, two or three layers of polyhedral cells, and 7-10 layers of squamous cells.³²⁴ The most superficial layer of squamous cells is in direct contact with the precorneal tear film and has a microplicated surface.³²⁷

The **corneal stroma** (substantia propria) comprises 90% of the thickness of the cornea – approximately 0.55 mm in the cat.^{324,325} Parallel bundles of collagen fibrils form lamellae, with each lamella running the entire diameter of the cornea.³²⁵ The collagen fibrils within a lamella are parallel, but between lamellae, they vary in direction.³²⁵ The fixed fibrocytes of the cornea, the keratocytes, lie flattened between the lamellae.³²⁵ These cells and their extensions form and maintain the stromal

lamellae.³²⁵ The precise organization of the corneal stroma is the most important factor in maintaining corneal clarity.^{325,327}

Descemet's membrane, a homogeneous, acellular membrane, is the exaggerated basement membrane of the corneal endothelium.³²⁵ It is produced throughout life, becoming thicker as the animal ages.³²⁵ In cats, the thickness of Descemet's membrane ranges from 8-12 μ m.³²⁴

The innermost layer of the cornea, the endothelium, is a single layer of flattened hexagonal cells.^{325,327} Its thickness in cats is 3-4 μ m.³²⁴

The avascular cornea is nourished by the capillary loops at the limbus, the precorneal tear film, and the aqueous humor.³²⁷ Branches of the ciliary nerves that arise from the ophthalmic nerve, a branch of the trigeminal nerve innervate the cornea.³²⁷

2.2 Conjunctiva

The anterior corneal epithelium is continuous with the bulbar conjunctiva at the corneoscleral junction.³²⁷ The epithelium of the lower nasal and middle fornix and the lower tarsal portion of the palpebral conjunctiva contain the highest density of goblet cells.³²⁵ The bulbar conjunctiva essentially lacks goblet cells.³²⁵ Two layers are distinguished within the substantia propria of the conjunctiva, a superficial adenoid layer, which contains lymphatic follicles and glands, and a deep, fibrous layer. The fibrous layer contains the nerves and vessels.³²⁵ A superficial and a deep plexus of lymphatics are present in the conjunctiva.³²⁵ Drainage extends to the parotid lymph nodes and the submaxillary lymph nodes.³²⁵

2.3 Lacrimal gland

The lacrimal gland lies dorsolaterally enclosed within the periorbita.^{324,325} The gland is 1 mm thick with an equal width and a length of 14 mm.³²⁴ The thickness is

maximal centrally and tapers towards the periphery.³²⁴ Its surface is slightly lobulated, and its anterior edge lies very close to the limbus.³²⁴ The lacrimal gland is tubuloalveolar and of serous quality.³²⁴ It is composed of columnar cells and the secreting acini are arranged in lobules of different sizes, supported by a relatively sparse stroma of connective tissue.³²⁴ The lumen of the interlobular ducts is approximately 30 µm in diameter.³²⁴ The lateral artery, a branch of the internal maxillary artery serves the lacrimal gland.³²⁴ Two lacrimal veins enter into the external rete, which is drained by the ophthalmic vein from which it drains into the trigeminal nerve, receives sympathetic fibers via the internal carotic plexus and parasympathetic fibers via the pterygopalatine ganglion.³²⁸

2.4 Nictitans gland

The gland of the nictitans surrounds the caudal end of the shaft of the cartilagenous plate of the third eyelid.³²⁵ It is thin, and has a flat roughly triangular shape with its apex pointing caudally towards the apex of the orbit.³²⁴ Most of the gland lies on the palpebral side of the cartilage, where it is 1 to 1.5 mm thick, whereas on the bulbar side the gland is very thin.³²⁴ It is a serous gland in the cat.³²⁵ Although the acini are positive for PAS suggesting a seromucoid type.³²⁴ The nictitans gland is much more richly endowed with septa than the lacrimal gland.³²⁴ These septa carry many prominent interlobular ducts and blood vessels.³²⁴ The ducts within the gland have diameters up to 60 μ m.³²⁴ Many fat cells are found within the septa.³²⁴ The nictitans gland receives its parasympathetic innervation via the infratrochlear nerve, a branch of the nasociliary nerve which is a branch of the ophthalmic division of the trigeminal nerve.³²⁸

2.5 Trigeminal ganglion

The trigeminal nerve is the 5th cranial nerve.³²⁹ It has both motor and sensory components.³²⁹ The *Radix sensoria* is much larger than the *Radix motoria*.^{328,329} The trigeminal nerve enters the trigeminal canal on the rostromedial aspect of the petrosal bone.³²⁹ Within this canal is the large trigeminal ganglion. It contains sensory cell bodies of the afferent, sensory fibers.^{328,329} The three branches of the trigeminal nerve emerge from the trigeminal ganglion: the ophthalmic nerve, the maxillary nerve, and the mandibular nerve.^{328,329} The *Radix motoria*, which also contains afferent, proprioceptive fibers, crosses the trigeminal ganglion medially and unites rostrally with one sensory branch forming the mandibular nerve, which is a mixed nerve.^{328,329}

The ophthalmic nerve consists of sensory axons from the eyelids, the globe and the nose.³²⁹ The nerve passes through the orbital fissure of the skull. Within the periorbita, it divides into three branches: the frontal nerve, lacrimal nerve, and nasociliary nerve.³²⁹ These branches innervate the skin of the upper eyelid, lacrimal gland, cornea, conjunctiva, nasal cavity, and the skin of the nostril.³²⁹

The maxillary nerve is sensory to the lower eyelids, nasal mucosa, upper teeth, upper lip, and nose, and its distal branches contain postganglionic parasympathetic axons (via fibers from the pterygopalatine ganglion) that innervate the lacrimal, nasal, and palatine glands.^{328,329} The nerve leaves the cranial cavity through the round foramen, alar canal, and rostral alar foramen and courses rostrally to the maxillary foramen.³²⁹ In the pterygopalatine fossa the maxillary nerve gives rise to the zygomatic nerve, pterygopalatine nerve, and infraorbital nerve.³²⁹

The mandibular nerve is both motor and sensory. It exits the cranial cavity through the oval foramen.³²⁸ The nerve divides into multiple branches: masseteric nerve, lateral and medial pterygoid nerves, tensor tympani nerve, tensor veli palatine

nerve, auriculotemporal nerve, the inferior alveolar nerve, the mylohyoid nerve, and the lingual nerve.^{328,329} The mandibular nerve is sensory to the buccal cavity, tongue, teeth of the lower jaw, the lower lip, in part the skin of the head, and mucosa of the intraosseous part of the external ear canal.³²⁹

2.6 Ciliary ganglion

The oculomotor nerve is the 3rd cranial nerve. It consists of somatic efferent neurons that innervate several of the striated extraocular muscles (dorsal rectus muscle, medial rectus muscle, ventral rectus muscle, ventral oblique muscle, and parts of the retractor bulbi muscle) and visceral efferent neurons (parasympathetic) that innervate ocular smooth muscle.^{328,329} The nerve exits the cranial cavity through the orbital fissue.^{328,329} Upon entering the orbit, the nerve divides into a smaller dorsal branch and a ventral branch.^{328,329} The ventral branch continues rostrally passing lateral and slightly ventral to the optic nerve, where it terminates in a number of branches to the medial rectus, ventral rectus, and ventral oblique muscles.^{328,329} At its termination is the ciliary ganglion where the preganglionic parasympathetic fibers synapse with postganglionic neurons.³²⁹ The two short ciliary nerves contain the postganglionic axons, innervating the ciliary muscle and the iris sphincter muscle of the eye.³²⁸⁻³³⁰

3. CHAPTER - HYPOTHESES AND SPECIFIC AIMS

This investigation was designed to follow infection with FHV-1 through the acute phase and into latency. The viral quantity, location, replicative status, and local tissue effect of the virus were assessed through virus isolation, quantitative PCR, *in situ* hybridization, and routine histopathologic examination of corneas, conjunctiva, lacrimal glands, nictitans glands, trigeminal ganglia, and ciliary ganglia at various time points during the infectious process.

3.1 Hypotheses

- 1. FHV-1 DNA is present during both the acute and latent stages within the corneas, conjunctiva, lacrimal glands, nictitans glands, trigeminal ganglia, and cilary ganglia of experimentally infected cats.
- 2. FHV-1 infects lacrimal tissue and leads to inflammation within the lacrimal gland and nictitans gland.

3.2 Specific aims

- 1. Localization of FHV-1 within the cornea, conjunctiva, lacrimal gland, and nictitans gland by *in situ* hybridization.
- 2. Quantification of FHV-1 within cornea, conjunctiva, lacrimal gland, nictitans gland, trigeminal ganglion, and cilary ganglion during the acute and latent stages of infection by use of a real-time PCR assay.
- 3. Use of virus isolation to determine whether the FHV-1 is active or latent when detected in the tissues using the real-time PCR assay.

4. Assessment of local tissue effect of the virus within cornea, conjunctiva, lacrimal gland, nictitans gland, trigeminal ganglion, and cilary ganglion during the acute and latent stages of infection.

4. CHAPTER – RESEARCH PROTOCOL, RESULTS, AND DISCUSSION

4.1 Introduction

Feline herpes virus 1 (FHV-1) infection is widespread within the cat population.⁴ Herpesviral ocular disease can be quite severe. Typical acute manifestations include ocular discharge, blepharospasm, conjunctivitis, keratitis, and corneal ulceration.^{4,79} Chronic ocular manifestations and sequelae may include persistent conjunctivitis, symblepharon, stromal keratitis and possibly corneal sequestration and eosinophilic keratitis.^{77,80,163}

Decreased tear production in cats has long been suspected to be a potential sequela to FHV-1 infection.³³¹ The cause remains speculative, but it has been assumed that either excretory duct occlusion or lacrimal adenitis is responsible.^{4,80} To the authors knowledge, histopathologic examination of the lacrimal tissues has not been performed to support either of these theories. Experimentally, a temporary keratoconjunctivitis sicca developed in 5 of 10 cats treated pre-inoculation with three consecutive daily subconjunctival injections of betamethasone.⁸⁰ However, aqueous tear production returned to normal in all cats by day 60 post-infection.⁸⁰ Bistner, et al.¹²¹ failed to demonstrate the presence of FHV-1 within the lacrimal gland utilizing specific fluorescent antibody staining or virus isolation. Interestingly, fluorescein-labelled antibody has been used to examine human lacrimal glands and revealed HSV-1 viral antigen in 2/3 patients with acute herpetic keratitis and in 4/7 asymptomatic patients with a history of previous herpetic infection.¹⁴⁵

The potential for recrudescent infection is high because the virus remains latent in approximately 80% of infected cats and 50% of FHV-1 recovered cats will shed virus spontaneously or in response to stress.⁶⁵ During the latent period clinical signs are absent and the virus cannot be isolated using standard cell culture

techniques.³⁸ FHV-1 DNA has been detected utilizing PCR assays in the nasal turbinates, oral fauces, tonsils, salivary glands, mesenteric lymph nodes, conjunctiva, corneas, lacrimal glands, optic nerves, trigeminal ganglia, olfactory bulbs, and cerebra of latently infected cats.^{85,144} Latency was confirmed by concurrent negative virus isolation.³⁸

Furthermore, using *in situ* hybridization and northern blot analysis of trigeminal ganglia, Ohmura et al. detected transcripts of the strand opposite to the immediate-early mRNA in cats latently infected with FHV-1.¹⁹³ These transcripts likely represent latency associated transcripts (LATs) which are the only transcripts present during latency and may therefore serve as markers of latency.^{30,38} Townsend et al.¹⁹⁸ also detected LATs utilizing a reverse transcriptase PCR assay in 4/16 trigeminal ganglia of clinically normal cats. However, to the authors knowledge histologic examination of the latently infected neural tissues to determine the effect of the FHV-1 infection has not been performed.

In humans with a history of previous herpes simplex-1 (HSV-1) infection, the ciliary ganglion has been identified as a site of latency. Bustos et al.¹⁶⁸ identified and detected HSV-1 utilizing PCR in 20/30 ciliary ganglia from human cadavers with no history of active herpetic lesions at the time of death. However, the ciliary ganglion has not been evaluated for presence of FHV-1 during either active or latent infections in cats.

The objectives of this study are to utilize virus isolation, a quantitative realtime polymerase chain reaction assay, histopathologic examination, and *in situ* hybridization to localize and quantify the amount of FHV-1 present and evaluate the associated morphological changes within the corneas, conjunctiva, lacrimal glands, nictitans glands, trigeminal ganglia, and ciliary ganglia of experimentally inoculated specific pathogen free cats during both the acute and latent phases of infection.

4.2 Materials and Methods

4.2.1 Animals

Fifteen 6-month-old female SPF cats were obtained from a commercial vendor. Cats were housed in individual cages with treatment and control groups in separate rooms (Biocontainment Level-2) with controlled temperature, humidity, and lighting. All personnel donned sterile disposable Tyvek coveralls, gloves, head covers, and shoes covers upon entering a room. Cats were fed a combination of dry and moist diets. All cats were acclimated for 7 days before viral inoculation.

All cats were determined to be serologically negative for FHV-1 virus neutralizing antibody prior to inoculation. Blood samples were collected on days 7, 14, and 21 post inoculation from the group 3 cats for evaluation of FHV-1 virus neutralizing antibodies.

4.2.2 Inoculation and clinical scoring

Cats were anesthetized via mask administration of halothane and oxygen. Twelve cats were infected by instilling 1.05 ml $(1x10^5 \text{ TCID}_{50}/\text{ml})$ of the C-27 strain of FHV-1 into the left and right conjunctival sacs (350 µl each), external nares (150 µl each), and oropharynx (50 µl) of each cat. The other 3 cats served as controls. The C27 strain of FHV-1 was obtained from a commercial source (ATCC, Manassas, VA) and was certified to be a purified suspension.

Clinical signs including fever, sneezing, ocular or nasal discharge, anorexia, dyspnea, and dehydration were scored daily for each cat as described (U.S.

Department of Agriculture, Animal and Plant Health Inspection Service, National Animal Veterinary Services Laboratory, 1985) by 3 investigators (Jacobi, Wasserman, Townsend) (Appendix A). Previously published work³⁰⁴ had established that all animals should be exhibiting ocular signs on day 6, all animals should be at the peak of ocular and respiratory signs on day 10, and latency should be established with resolution of clinical signs by day 30. Therefore, on days 6 (group 1), 10 (group 2) and 30 (group 3), four inoculated and one control cats were humanely euthanized and samples were immediately collected. All experiments were performed in full compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with approval from the Michigan State University Animal Care and Use Committee.

4.2.3 Samples

Aseptic, bilateral sample collection (corneas, conjunctiva, lacrimal glands, nictitans glands, trigeminal ganglia, ciliary ganglia) occurred immediately following euthanasia. Particular care was taken to avoid cross contamination between tissue samples by alcohol dipping and flaming of the instruments for each use.

Tissue samples or entire ganglia from the right side were placed in Eppendorf tubes containing one sterile homogenizing bead with 800 μ l of Bovarnick's buffer and homogenized for further virus isolation and PCR analysis. Tissue samples from the left cornea, conjunctiva, lacrimal gland, and nictitans gland were divided. One half was placed immediately in 10% formalin; the other ½ was frozen at -80 °C. Due to their limited size, the entire left trigeminal ganglion and ciliary ganglion were placed immediately in 10% formalin.

4.2.4 Virus isolation

Following homogenization in the Qiagen Mixer mill (QIAGEN Inc., USA) and collection of a 200 μ l aliquot for DNA extraction, the remaining suspension was transferred to a 15 ml conical tube. The volume was brought to 3 ml with Eagle minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS). The homogenate was then filtered (0.45 μ m). Filtered tissue extracts (500 μ l) were inoculated onto monolayers of Crandell feline kidney (CRFK) cells grown in 24-well microtiter plates. All cultures were performed in duplicate.

The cultures were observed daily for five days with an inverted microscope (Nikon Eclipse TS100) for the cytopathic effects (CPE) characteristic of FHV-1. A sample was considered positive for FHV-1 only if it produced CPE within both culture wells. If only one culture was positive, another culture was performed for the sample, again in duplicate. The result was then considered positive if CPE was produced in one or both of the repeat cultures.

4.2.5 Quantitative real time PCR

4.2.5.1 DNA extraction

A mixture of 100 μ l tissue homogenate and 100 μ l phosphate buffered saline (PBS) was utilized for DNA extraction. Extraction controls, performed for each extraction, consisted of 200 μ l PBS. Purification of DNA was performed using a commercially available kit (DNeasy Tissue Kit, QIAGEN, Valencia, CA) in accordance with the manufacturer's protocol. The samples were eluted with 100 μ l Buffer AE (10 mM Tris-Cl, 0.5 mM EDTA; pH 9.0). DNA was quantified using a spectrophotometer (NanoDrop 1000, Thermo scientific, Wilmington, DE).
4.2.5.2 PCR amplification of FHV-1

A real-time SYBR Green-based PCR specific for FHV-1 has been previously described.³³² The primers for this assay target a 478-bp fragment of the FHV-1 gE gene. The sequence of the forward primer is 5'-GGT CAT GTG TAA TGT TGA CG-3', and the reverse primer is 5'- GTC TTT GGT TCT GAT GAG AG-3'.³¹¹ The sensitivity of this assay is 10 viral genome copies. The diagnostic utility of the assay has been confirmed by its ability to amplify the targeted DNA fragment of 48 known clinical isolates of FHV-1. The 50 µl real time PCR reaction mixture consisted of 25 µl 2x SYBR Green mix (Taq DNA polymerase, dNTPs, SYBR Green I, 5.0 mM MgCl₂; QIAGEN QuantiTect SYBR Green PCR Kit, Valencia, CA), 1 µl forward primer (25 pmol/µl), 1 µl reverse primer (25 pmol/µl), 100 ng of template DNA and ddH₂O. PCR amplification was performed on an iCycler[™] iO[®] System (Bio-Rad Laboratories, Hercules, CA) with the following cycling conditions: pre-denaturation at 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 1 min. Amplification was continuously monitored in real time by quantifying the amount of fluorescence emitted at 530 nm at each annealing step. Following the final elongation step at 72 °C for 60 seconds, a post-PCR melt curve analysis was performed, amplicon melt temperatures were determined by raising the temperature in 0.5 °C increments from 55 °C to 95 °C and monitoring changes in emitted fluorescence (Figures 4.1 and 4.2). All reactions were conducted in duplicate. DNA extraction controls and reagent controls were included in each run. A standard curve of threshold cycle (Ct) in relation to starting viral copy number was constructed (Figures 4.3 and 4.4). The standard curve for FHV-1 was generated by a series of 10fold dilutions $(10^1 - 10^6)$ of FHV-1 DNA. Samples for the standard curve were assayed in triplicate for each run and served also as the positive control.



Figure 4.1. Standard melt curve chart.



Figure 4.2. Standard melt peak chart.



Figure 4.3. Standard amplification chart, 10 fold dilutions of 10^6 genomic copies of FHV-1, triplicate.



PCR Standard Curve: Data 2006-05-15 2150.opd Figure 4.4. PCR standard curve.

4.2.5.3 Statistical analysis

Data were analysed using the Wilcoxon Rank –Sum test for non Gaussian data distribution. Following Bonferroni's adjustment for multiple comparisons, the level of significance was p < 0.017. For all tissues mean copy numbers of the 3 time points (day 6, day 10, and day 30 post inoculation) were compared.

4.2.5.4 Histopathology

Following fixation in 10% formalin, tissues were paraffin-embedded, sectioned at 5 μ m, deparaffinized, and stained with hematoxylin and eosin (HE). All samples were examined with a light microscope.

4.2.5.5 In situ hybridization

In situ hybridization was performed to specifically localize FHV-1 within the cornea, conjunctiva, lacrimal gland, and nictitans gland. The *in situ* protocol was first optimized by evaluating various pretreatment methods: 1.) baking for 30 min at 60 °C prior to deparaffinization of the section followed by enzymatic digestion (100 μ l/ml proteinase K in TES buffer (50 mM Tris HCl, pH 7.4, 10 mM EDTA, 10 mM NaCl)) for 15 minutes at 37 °C; 2.) enzymatic digestion following deparaffinization without previous baking; 3.) microwaving: slides covered with 10 mM Tris HCl, pH 8.1 + 1 mM EDTA buffer were microwaved at full power for 5 or 10 minutes. Also antibody dilutions of 1:250 and 1:500 were evaluated (Figure 4.5). All optimization was performed on FHV-1 pellets. Superior results were obtained by pre-treating via microwaving for 10 minutes and using an antibody dilution of 1:250 (Figure 4.6). This pretreatment protocol was used for all tissue sections.



Figure 4.5. FHV-1 pellet pre-treated with different protocols.

- A. Enzymatic digestion with proteinase K using an antibody dilution of 1:500.
- B. Enzymatic digestion with proteinase K using an antibody dilution of 1:250.
- C. Baking for 30 min at 60 °C prior to deparaffinization of the section followed by enzymatic digestion with proteinase K using an antibody dilution of 1:250.
- D. Micowaving for 10 minutes using an antibody dilution of 1:500.



Figure 4.6. FHV-1 pellet pre-treated via micowaving for 10 minutes using an antibody dilution of 1:250.

Cornea, conjunctiva, lacrimal gland, and nictitans gland were examined in duplicate. The FHV-1 DNA probe used targets a portion of the gE gene. The 30-mer synthetic oligonucleotide probe with the sequence 5' CGA TGA CAG AAG GTG CCG CAC TTT TAG TCG ATG GG 3' was synthesized and labeled with digoxigenin-11-dUTP by a commercial supplier (IDTDNA, Coralville, IA). Positive (FHV-1 pellet) and negative controls (canine nictitating membrane, FHV-1 pellet with no probe applied) were included with each batch of slides.

Paraffin sections were prepared on silane-coated slides. Sections were then dewaxed in xylene (2 x 10 min), taken through a series of graded ethanols (1 x 5 min in 100%, 95%, 75%, and 50%) and washed in nanopure water (2 x 5 min).¹⁹⁵ Sections were then placed in Tris/ethylenediaminetetraacetic acid (EDTA) buffer (10 mM Tris HCl, pH 8.1 + 1 mM EDTA) and microwaved at full power continuously for 10

minutes. Sections were allowed to cool for 15 minutes and nanopure water was gradually added over 5 minutes to reduce the temperature.¹⁹⁵ Tissues were then fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 minutes.³³³ Slides were allowed to air dry and the tissue sections were layered with 75 μ l of prehybridization solution containing 4X saline sodium citrate (SCC); 50 mM NaH₂PO₄/Na₂HPO₄, pH 7.0; 1 mM EDTA; 5X Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone and 1% bovine serum albumin); 10 µl sonicated salmon sperm DNA(500 μ g/ml); formamide to 25%; and nanopure water. The solution was held in place by the use of a liquid blocker (PAP PEN, Invitrogen, Carlsbad, CA). Prehybridization was allowed for 60 minutes at room temperature. Then hybridization solution consisting of the prehybridization solution and 200 ng/ml DIG labeled probe was layered over each section. Slides were heated for 6 minutes at 95 °C and immediately quenched on ice for 1 minute. Hybridization followed at 25 °C overnight in a humidified chamber. Sections were then washed twice in 2X SCC for 5 minutes and 0.1X SCC for 10 minutes at room temperature. Slides were rinsed briefly in buffer 1 (100 mM Tris HCL, 150 mM NaCl, pH 7.5). The tissues were then covered with buffer 2 (100 mM Tris HCL, 150 mM NaCl, pH 7.5, and 0.5% blocking reagent) and cover-slipped with parafilm. Slides were allowed to sit for 15 minutes at room temperature. Following removal of the cover slips, slides were rinsed briefly in buffer 1. Then, 30 µl of 1:250 anti-DIG AP, Fab fragment antibody conjugate was layered onto the tissues, which were cover-slipped with parafilm, and placed in a humidified chamber at 25 °C for 2 hours. Slides were then washed twice for 10 minutes in buffer 1 and allowed to equilibrate for 5 minutes in buffer 3 (0.1 M Tris HCL, pH 9.5, 0.1 M NaCl) at room temperature. Then 20 µl of 1:50 NBT/BCIP solution was layered onto each tissue, cover-slipped with glass cover slips, and covered with aluminum foil to prevent illumination. Color was allowed to develop overnight at 25 °C. Following removal of the cover-slips, the reaction was stopped by rinsing the slides briefly in 10 mM Tris HCl, pH 8.1 + 1 mM EDTA. Slides were then washed in nanopure water. Neutral red counter-stain was layered over each tissue for 1 minute and then rinsed with nanopure water. Slides were then cover-slipped (Immu-mount, Shandon, Pittsburgh, PA) and evaluated under a light microscope.

4.3 Results

4.3.1 Clinical features

All inoculated cats euthanized on day 30 post inoculation developed antibodies against FHV-1 with titers ranging from 1:32 to 1:128. The control cat did not develop antibodies against FHV-1. None of the control cats showed any signs of disease at any time. Sneezing developed in 11/12 inoculated cats with a peak between day 3 and 9 post-inoculation. None of the cats developed a cough, rales, open mouth breathing, or corneal or cutaneous ulceration. Blepharospasm of variable severity and duration was noted in 10/12 inoculated cats. All 12/12 inoculated cats developed serous or in some cases mucopurulent ocular discharge of variable duration and severity between days 3-14 post-inoculation. Conjunctivitis was found in all 12/12 inoculated cats of variable duration and severity starting on day 3 post-inoculation. Other signs noted in variable severity and duration were nasal discharge in 10/12 inoculated cats. Two of the 12 inoculated cats developed mild signs of dehydration, 6/12 cats developed a transient, mild anorexia, and 5/12 cats had a transient pyrexia (>102.5 °F) between days 3 and 7 post-inoculation. None of the cats required or received any treatment

during the study. See clinical scores in Table 4.1. Detailed data are shown in Appendix B.

Day of euthanasia post inoculation	Cat number	Clinical score
Day 6	1	8
	2	7
	12	4
	4	5
	5	0
Day 10	6	24
	7	19
	8	16
	9	31
	10	0
Day 30	11	20
	3	44
	13	27
	14	20
	15	0

Table 4.1. Clinical scores.

4.3.2 Virus isolation

None of the tissues were positive on virus isolation for the control cats. Virus isolation from the four inoculated cats euthanized on day 6 and 10 post-inoculation

demonstrated 2/4 positive trigeminal ganglia, 2/4 positive ciliary ganglia, and 4/4 positive lacrimal glands, nictitans glands, corneas, and conjunctiva respectively. Virus isolation from the four inoculated cats euthanized on day 30 post-inoculation demonstrated only 1 positive sample; a corneal specimen. All other tissues from the group 3 cats were negative for virus isolation of FHV-1. Results are summarized in the Table 4.2. Data are shown in detail in Appendix C.

Sample	Day 6	Day 10	Day 30
Cornea	4/4	4/4	1/4
Conjunctiva	4/4	4/4	0/4
Trigeminal ganglion	2/4	2/4	0/4
Ciliary ganglion	2/4	2/4	0/4

Table 4.2. Summarized results of virus isolation (positive/total)

4.3.3 Quantitative real time PCR

FHV-1 DNA could not be detected in any of the tissue samples from the control cats. In all infected animals FHV-1 DNA could be detected in cornea, conjunctiva, trigeminal ganglia and ciliary ganglia on days 6 and 10 post inoculation. All inoculated cats had positive trigeminal ganglia on day 30. In addition, one cat was positive for FHV-1 DNA in the corneal, conjunctival, and ciliary ganglion samples on day 30. The three remaining inoculated cats were negative for those tissue samples on day 30. (Table 4.3)

Copy numbers per 100 ng DNA were similar for the corneal samples in all cats on day 6 and day 10. In conjunctival samples, copy numbers tended to be lower on day 10 than on day 6 post-inoculation. For the trigeminal ganglion and the ciliary ganglion, copy numbers tended to be increased on day 10 compared to day 6. However, statistical analysis using the Wilcoxon Rank-Sum test with a level of significance of p < 0.017 following Bonferroni's adjustment for multiple comparisons, showed that none of the comparisons between the 3 different time points were significant.

PCR results for the cornea, conjunctiva, lacrimal gland and the nictitans gland tissues for cats # 4, 7, and 12 revealed positive extraction controls. PCR for these samples was not repeated for the glandular tissues due to the fact that upon histologic examination, conjunctival tissue was found to be adherent to the glandular material and could have resulted in false positive results because of possible presence of FHV-1 in the conjunctiva rather than in the gland itself. PCR was repeated for the corneal and conjunctival tissues of cats # 4, 7, and 12 in duplicate. Results are summarized in the Table 4.3. Data are shown in detail in Appendix D.

Sample	Day 6	Day 10	Day 30
Cornea	4/4	4/4	1/4
Conjunctiva	4/4	4/4	2/4
Trigeminal ganglion	4/4	4/4	4/4
Ciliary ganglion	4/4	4/4	1/4

Table 4.3. Summarized results of quantitative real time PCR (positive/total)

4.3.4 Histopathology

None of the control cats showed any histomorphologic abnormalities in any of the tissues examined. Conjunctival changes characterized by epithelial cell attenuation or loss and lymphoplasmacytic inflammation were seen in all 12 inoculated cats (Figure 4.7), although the changes were milder in cats euthanized on day 30 postinoculation. In these cats areas of conjunctival re-epithelialization were present. All corneal sections were normal except for those from one cat (cat # 9) euthanized on day 10 post-inoculation. Multiple, solitary, degenerate epithelial cells and a mild lymphoplasmacytic infiltrate characterized the changes within the corneal section (Figure 4.8). The glandular tissues within all sections of lacrimal and nictitans glands appeared normal (Figure 4.9). However, histopathologic examination revealed that multiple specimens of lacrimal and nictitans gland contained the adjacent conjunctiva. The conjunctiva in these samples exhibited changes consistent with those noted in all other conjunctival samples.

All 4 trigeminal ganglion samples obtained on day 6, 3 samples obtained on day 10, and 1 sample obtained on day 30 post-inoculation revealed histopathologic changes including ganglion cell necrosis, drop out or loss of ganglion cells, increased numbers of glial cells, and lymphoplasmacytic inflammation (Figure 4.10). All ciliary ganglia evaluated appeared normal. Due to the small size of the ganglia from one cat euthanized on day 10, no appropriate section of the trigeminal ganglion could be obtained. From 1 cat (day 10) and 2 cats (day 30 post inoculation) no appropriate section of the ciliary ganglion could be obtained.



Figure 4.7. Conjunctival sample from cat # 12. (HE)



Figure 4.8. Corneal sample from cat # 9. (HE)



Figure 4.9. Lacrimal gland sample from cat # 2. (HE)



Figure 4.10. Trigeminal ganglion sample from cat # 1. (HE)

4.3.5 In situ hybridization

Results of *in situ* hybridization are summarized in Table 4.4. A blue to brown reaction product confined to the nuclei of intact epithelial cells characterized a positive hybridization signal. None of the control cats showed positive results in any of the tissue samples. In conjunctival samples the positive cells were found adjacent to areas of inflammation (Figure 4.11). No inflammatory changes were noted adjacent to the positive superficial corneal epithelial cells (Figure 4.12) Hybridization signals were not detected within the lacrimal or nictitans glands. However, positive hybridization signals were detected within the overlying conjunctiva. Corneal samples of cat # 2 were not available for evaluation via *in situ* hybridization. The tissue quality of all 4 samples from the group 3 (day 30) cats was very poor. One cat appeared to have a positive hybridization signal in the cornea, but this result may be spurious due to the poor tissue morphology.

		ISH					
	Cat No.	Cornea	Conjunctiva	Lacrimal Gland	Nictitans Gland		
	1	+	+	-	-		
Day 6	2	Not avail.	+	-	-		
	12	-	+	-	-		
	4	+	+	-	-		
	5 (control)	-	-	-	-		
	6	-	-	-	-		
	7	-	-	-	-		
Day 10	8	-	-	-	-		
	9	-	-	-	-		
	10 (control)	-	-	-	-		
	11						
	3]					
Day 30	13	Poor tissue	morphology				
	14						
	15 (control)	7					

Table 4.4. Summarized results of in situ hybridization



Figure 4.11. In situ hybridization of the conjunctival sample from cat # 12.

50 µm

Figure 4.12. In situ hybridization of the corneal sample from cat # 4.

4.4 Discussion

Clinical signs of natural as well as experimentally induced FHV-1 infection can be quite severe.^{70,79} In the present study infected cats generally exhibited mild signs of upper respiratory and ocular disease. A possible explanation for the mild clinical signs compared to natural infection or experimental infection with other viral strains could be viral attenuation over time leading to a reduced pathogenicity of the C27 strain of FHV-1.

Similar to observations by Bistner et al.¹²¹ no clinical signs of keratitis were observed in any of the cats at any point in time during this primary infection. However, fluorescein staining and Rose Bengal staining of the cornea were not performed daily. Therefore very subtle lesions may not have been detected.

Oral ulcers are reported to occur rarely in association with feline herpes virus 1 infection and are more commonly associated with feline calicivirus infection.^{69,106} Although the areas of oral ulceration noted were generally small and transient, 75% of inoculated cats were affected. It is possible those small ulcers are overlooked in clinical cases or that field strains do not induce development of oral ulceration. The oropharyngeal administration of 50 μ l of viral suspension may also represent a greater viral inoculum than generally reaches those tissues during natural infection.

At 30 days post-inoculation, latency should be established with resolution of clinical signs.³⁰⁴ Latency has been previously defined as the absence of detectable FHV-1 as assessed by virus isolation, but detectable viral DNA using PCR assays.^{85,144} In this study, virus isolation was performed immediately following tissue harvest. Infectious virus was isolated from all ocular tissues and 50% of the ganglia on day 6 and day 10 post-inoculation. This is consistent with virus shedding as expected with active disease. On day 30 all tissue samples from 3 of the inoculated

cats were negative on VI. This is consistent with establishment of latency, which has previously been shown to occur by this time post inoculation.³⁰⁴ However, FHV-1 could be isolated from the corneal sample of one cat. From the same cat FHV-1 DNA could also be detected using quantitative real-time PCR in the cornea, conjunctiva, and both the ciliary and trigeminal ganglia. Although the cat did not show any clinical signs at the time of euthanasia, these results suggest the cat was either suffering a recrudescent infection or failed to clear the primary infection. A similar scenario has been noted in studies by Stiles et al.¹⁶² and Townsend et al.¹⁹⁸ in which FHV-1 DNA was detected in the corneas of clinically normal cats. In these studies virus isolation was not performed. However, in the study by Townsend et al.¹⁹⁸ LATs could not be detected in the cornea, suggesting the infection was not latent.

Histopathological examination revealed typical changes for the conjunctival tissues in all inoculated cats. However, glandular tissues from all lacrimal and nictitans glands were normal. These results do not support the suggestion by Nasisse⁴ and Nasisse et al.⁸⁰ that lacrimal adenitis is responsible for FHV-1 related keratoconjunctivitis sicca (KCS). As inflammatory changes were noted in the overlying conjunctiva, perhaps occlusion of the excretory ductules is the cause of FHV-1 related KCS. Clinically none of the cats demonstrated clinical signs consistent with KCS. However, tear production was not evaluated in this study. Inoculation with a more virulent strain of FHV-1 might cause KCS to develop and better elucidate the cause.

Conjunctival tissue exhibiting changes consistent with conjunctivitis was noted in sections of the lacrimal and nictitans glands. Therefore, the results of the virus isolation for these tissues are suspect, since FHV-1 could be isolated from conjunctiva during the acute phase. Unfortunately lacrimal tissue could not be harvested without contamination from the overlying conjunctiva. For this reason, quantitative real-time PCR was not repeated for the glandular tissue samples with positive extraction controls.

All trigeminal ganglia examined histopathologically during the acute infectious state showed signs of lymphoplasmacytic inflammation. However, only 1 trigeminal ganglion obtained from a cat on day 30 showed histomorphological changes; a drop out of ganglion cells, but no concurrent inflammation. These results suggest that the trigeminal ganglia developed transient inflammation during the acute infection. The absence of lymphocytes during the latent phase is very interesting. Current studies of HSV-1 in man suggest lymphocytes play a key role in preventing reactivation of the virus within neuronal tissue.⁵⁸⁻⁶⁰

In contrast to the inflammation seen within the acute phase trigeminal ganglia, no histomorphologic changes could be detected in any of the ciliary ganglia. This occurred despite a 50% isolation rate on VI and a 100% detection rate on quantitative real time PCR during the acute phase of FHV-1 infection. It is possible that localized inflammatory changes do not occur or they were missed on histopathologic examination due to the limited number of sections obtained from the ganglia.

The FHV-1 DNA copy numbers in corneal samples as determined by quantitative real-time PCR were similar in samples obtained on day 6 to samples collected on day 10 post-inoculation. Except for one cat's corneal sample in which recrudescent or persistent infection is suspected, all other cats euthanized on day 30 had negative tissue samples. DNA copy numbers in conjunctival tissues tended to be lower on day 10 than on day 6. Therefore it is possible that the infection clears from the conjunctiva before it clears from the cornea. For the trigeminal ganglia a transient increase on day 10 was noted before the viral copy number decreased by day 30. A similar phenomenon of detectable viral DNA increase on day 10 was noted in the ciliary ganglia. Therefore we would speculate that these ganglia reach their peak viral load later than the surface ocular tissues. In contrast to findings in a previous study³⁰⁰ FHV-1 DNA copy number within the trigeminal ganglia during latency was only slightly lower than during active infection. In that study quantification was carried out using an automated electrochemiluminescence detector. The higher sensitivity of the quantitative real time PCR assay utilized in the present study could explain the difference in viral copy numbers between the two studies. However, our results are similar to those seen in murine models of HSV-1.³³⁴⁻³³⁶ On day 30 a low copy number of FHV-1 DNA was detected only in the ciliary ganglion from the cat with a suspected recrudescent or persistent infection. All other ciliary ganglia were negative. From these data we cannot conclude that the ciliary ganglion is a site of latency for FHV-1 in cats. Further investigation will be necessary.

All cats euthanized on day 6 post-inoculation were positive on ISH within corneal and/or conjunctival tissues. In contrast none of the cats euthanized on day 10 post-inoculation had a clearly positive result in any of the tissues. The positive control samples processed at the same time demonstrated strong positive hybridization signals. Furthermore, these tissues were processed with other tissues in which positive hybridization signals were detected. These results are somewhat surprising as virus isolation for conjunctival and corneal samples was positive for all inoculated cats euthanized on days 6 and 10. Furthermore, viral load as determined by quantitative real time PCR was similar for ISH-positive and -negative cats euthanized on day 6 and 10.

The strongest positive hybridization signal was detected within conjunctival epithelial cells from cat #12 euthanized on day 6 post-inoculation. This cat also had

the highest viral load within corneal and conjunctival tissue samples based upon the RT-PCR results. However, no positive signal was found within this cat's cornea. FHV-1 positive cells were generally sparse in positive tissues. Therefore it would be possible to have examined sections that did not contain cells harboring sufficient virus to produce a detectable positive hybridization signal. Also, cats with severe conjunctivitis had necrotized or very attenuated epithelium. This leaves less intact epithelial cells in which to detect the virus. The overall sensitivity of the ISH to detect FHV-1 utilizing this protocol was poor when compared to the sensitivity of the RT-PCR assay. A possible explanation could be the localized distribution of FHV-1 infected cells within the tissues.

No virus could be detected with this method within glandular tissue samples examined. This is consistent with the normal tissue morphology shown in standard histopathologic examination. However, FHV-1 in low quantities could be present. Furthermore the clinical signs seen in this study were generally mild. Therefore it is possible that FHV-1 infects lacrimal and nictitans glands in more severe infections.

All tissues of cats euthanized on day 30 showed poor cellular morphology in the sections examined by ISH. The morphology of the same tissues was excellent for standard histology. This is difficult to explain, since these tissues were processed for the ISH in different runs together with other tissues that demonstrated good morphology. It could be possible that fixation of the tissues post euthanasia was either insufficient or excessive and the intense treatment during ISH led to the morphological changes noted. Repeating the ISH for these tissues with varying conditions could help to explain the changes found.

In future studies, additional tissues such as uvea, retina, optic nerve, olfactory bulb, brainstem, cerebellum, cerebrum (visual cortex), optic chiasm, cranial cervical

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ganglion, otic ganglion, pterygopalatine ganglion, mandibular salivary gland, submandibular lymph node, parotid salivary gland, parotid lymph node, nasal turbinates, tonsil, lung, trachea, oral fauces, and retropharyngeal lymph node could be examined to identify other targets of FHV-1 during both the acute and latent stage of infection. Herpesvirinae have been detected within some of these tissues during studies of HSV-1 infection in murine models³¹ and pseudorabies virus infection in piglets.^{337,338} The presence of virus within these tissues could expand our understanding of the pathology induced by FHV-1 infection and the patterns of recrudescence noted clinically.

Modulation of apoptosis may be a key factor allowing herpesvirinae to establish latency.^{339,340} However, the ability of FHV-1 infection to influence cellular apoptosis has not been investigated. If FHV-1 infection can block apoptosis of infected cells, this is a mechanism by which the host defenses are evaded. Apoptosis could be assayed by measuring caspase-3 activity. Caspase activation is thought to be the most specific indicator of apoptosis and caspase 3, the key converging enzyme is ubiquitously distributed making it an excellent choice for a marker of apoptosis.³⁴¹

More sensitive methods to detect FHV-1 could be utilized including *in situ* PCR or laser capture of individual cells for PCR analysis. Furthermore, a different FHV-1 strain exhibiting higher virulence than C27 could be used for inoculation. This could also facilitate the detection rate and help to identify further targets of FHV-1.

In conclusion, this study confirmed the ability of FHV-1 to acutely infect not only conjunctival epithelium, corneal epithelium, and trigeminal ganglion, but also the ciliary ganglion. The ciliary ganglion may serve as an additional site of latency for FHV-1 and could explain the preponderance of ocular lesions noted during recrudescent infections. However additional studies to prove this hypothesis are

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required. The copy number within latent trigeminal ganglia was much higher than those previously reported due to the increased sensitivity of the assay utilized. The copy numbers reported are similar to those found for HSV-1 in murine models of infection. However, despite an initial lymphoplasmacytic inflammatory response and persistence of the virus, the ganglia appeared normal by day 30 post-inoculation. This lack of inflammation is in direct contrast with the current theory that lymphocytes are a key factor in regulation of the latent state. The theory that lacrimal adenitis contributes to the development of KCS was not supported as there was no evidence of FHV-1 infection or secondary inflammation within the glandular tissues at any time.

APPENDICES

USDA Scoring	g Method for FHV-1 ^a	······································
Clinical signs	Days	Score
Fever		
103.0 to 103.9 °F		1 each day
104.0 to 104.9 °F		2 each day
≥105 °F		3 each day
Conjunctivitis		•••
Serous discharge only	1 to 3	1
	≥	2
Mucopurulent discharge	1 to 2	2
	3 to 5	4
	ĭ	6
Rhinitis	·	
Serous discharge only	1 to 3	1
	×	2
Mucopurulent discharge	1 to 2	2
	3 to 5	4
	≱	6
Sneezing		1 each day
Dyspnea		
Audible rales		2 each day
Coughing		2 each day
Open mouth breathing		3 each day
Depression		
Anorexia		1 each day
Dehydration	1 to 2	3
	23	4
Hypothermia <99 °F		2 each day
Oral ulcers (linguinal or oral mucosa)		······································
1 ulcer <4 mm	1 to 5	2
	6 to 9	3
	20	4
Multiple ulcers <4 mm	1 to 4	3
-	5 to 8	5
	29	7
Ulcer or ulcers >4 mm	1 to 4	5
	5 to 8	7
	≥9	9
Salivating		1 each day
Extenal ulcers (lip or nares)		······································
Nonbleeding ulcer		4
Bleeding ulcer		6
Death		15

5. APPENDIX A

^a U.S. Department of Agriculture, Animal and Plant Health Inspection Service, National Animal Veterinary Services Laboratory (1985).

Table 5.1. USDA Scoring Method for FHV-1

6. APPENDIX B



Figure 6.1. Results of the clinical examination: sneezing.



Figure 6.2. Results of the clinical examination: elevated body temperature.

elevated body temperature

dehydration



Figure 6.3. Results of the clinical examination: dehydration.

anorexia



Figure 6.4. Results of the clinical examination: anorexia.

ptyalism



Figure 6.5. Results of the clinical examination: ptyalism.

oral ulceration



Figure 6.6. Results of the clinical examination: oral ulceration.

nasal discharge



Figure 6.7. Results of the clinical examination: nasal discharge.

blepharospasm



Figure 6.8. Results of the clinical examination: blepharospasm.

ocular discharge



Figure 6.9. Results of the clinical examination: ocular discharge.



conjunctivitis

Figure 6.10. Results of the clinical examination: conjunctivitis.

7. APPENDIX C

DAY 6

Sample	Control 5	Cat 4	Cat 1	Cat 2	Cat 12
Trigeminal Ganglia	_	-		+	+
Ciliary Ganglia	_	+	_	+	_
Lacrimal Gland	_	+	+	+	+
Cornea	_	+	+	+	+
Conjunctiva	_	+	+	+	+
Nictitating Gland	-	+	+	+	+

 Table 7.1. Virus isolation day 6 post inoculation

DAY 10

Sample	Control 10	Cat 6	Cat 7	Cat 8	Cat 9
Trigeminal Ganglia	-	+	+	_	-
Ciliary Ganglia	_	+	+	_	_
Lacrimal Gland	_	+	+	+	+
Cornea	_	+	+	+	+
Conjunctiva	_	+	+	+	+
Nictitating Gland		+	÷	+	+

Table 7.2. Virus isolation day 10 post inoculation

DAY 30

Sample	Control 15	Cat 3	Cat 11	Cat 13	Cat 14
Trigeminal Ganglia	_		_	_	_
Ciliary Ganglia	-	_	_	_	_
Lacrimal Gland	_	-	_	_	_
Cornea	-	_	+	_	_
Conjunctiva	_	-	_	_	_
Nictitating Gland		_		_	

Table 7.3. Virus isolation day 30 post inoculation

Cat #	Repeat#1	Repeat#2	Average	Std Dev
DAY 6 P.I.				
1	172,000	224,000	198,000	36,770
2	1,370,000	1,510,000	1,440,000	98,995
4	56,500	86,900	71,700	21,496
12	269,000	384,000	326,500	81,317
5	0	0	0	0
DAY 10 P.I.				
6	226,000	298,000	262,000	50,912
7	73,400	74,500	73,950	778
8	177,000	223,000	200,000	32,527
9	189,000	247,000	218,000	41,012
10	0	0	0	0
DAY 30 P.I.				
3	0	0	0	0
11	22,600	28,000	25,300	3,818
13	0	0	0	0
14	0	0	0	0
15	0	0	0	0

8. APPENDIX D

Table 8.1. PCR results of cornea (Copy no. / 100 ng DNA)

.

Cat #	Repeat#1	Repeat#2	Average	Std Dev
DAY 6 P.I.				
1	125,000	174,000	149,500	34,648
2	610,000	648,000	629,000	26,870
4	434,000	317,000	375,500	82,731
12	2,510,000	2,410,000	2,460,000	70,711
5	0	0	0	0
DAY 10 P.I.				
6	4,200	5,510	4,855	926
7	619,000	493,000	556,000	89,095
8	16,500	19,600	18,050	2,192
9	27,500	29,500	28,500	1,414
10	0	0	0	0
DAY 30 P.I.				
3	0	0	0	0
11	105	122	114	12
13	0	0	0	0
14	5	14	9	6
15	0	0	0	0

Table 8.2. PCR results of conjunctiva (Copy no. / 100 ng DNA)

	Repeat#1	Repeat#2	Average	Std Dev
DAY 6 P.I.				
1	2,770	3,620	3,195	601
2	59,500	78,300	68,900	13,294
4				
12	<i>\////////////////////////////////////</i>			
5	0	0	0	0
DAY 10 P.I.				
6	24,000	28,900	26,450	3,465
7				
8	2,260	2,900	2,580	453
9	456	481	469	18
10	0	0	0	0
DAY 30 P.I.				
3	0	0	0	0
11	0	0	0	0
13	0	0	0	0
14	0	0	0	0
15	0	0	0	0

Table 8.3. PCR results of lacrimal gland (Copy no. / 100 ng DNA)Marked fields: contamination of sample, assay not repeated.

Cat #	Repeat#1	Repeat#2	Average	Std Dev
DAY 6 P.I.				
1	103,000	119,000	111,000	11,314
2	90,600	106,000	98,300	10,889
4				
12	<i>````</i>			
5	0	0	0	0
DAY 10 P.I				
6	27,200	33,700	30,450	4,596
7				
8	5,850	7,410	6,630	1,103
9	11,500	12,999	12,250	1,060
10	0	0	0	0
DAY 30 P.I				
3	0	0	0	0
11	10,800	11,700	11,250	636
13	0	0	0	0
14	0	0	0	0
15	0	0	0	0

Table 8.4. PCR results of nictitans gland (Copy no. / 100 ng DNA)Marked fields: contamination of sample, assay not repeated.

.

Cat #	Repeat#1	Repeat#2	Average	Std Dev
DAY 6 P.I.				
1	16,500	16,200	16,350	212
2	12,200	13,000	12,600	566
4	14,100	16,700	15,400	1,838
12	10,800	10,600	10,700	141
5	0	0	0	0
DAY 10 P.I.				
6	17,500	31,800	24,650	10,112
7	57,000	37,700	47,350	13,647
8	13,400	13,200	13,300	141
9	50,400	52,400	51,400	1,414
10	0	0	0	0
DAY 30 P.I.				
3	15,900	34,200	25,050	12,940
11	3,110	3,130	3,120	14
13	5,370	5,790	5,580	297
14	13,500	16,100	14,800	1,838
15	0	0	0	0

Table 8.5. PCR results of trigeminal ganglia (Copy no. / 100 ng DNA)
Cat #	Repeat#1	Repeat#2	Average	Std Dev
DAY 6 P.I.				
1	239	192	216	33
2	2.440	1.630	2.035	573
4	5.660	3.230	4.445	1.718
12	149	65	107	59
5	0	0	0	0
DAY 10 P.I.				
6	5.130	4.560	4.845	403
7	26.000	47.600	36.800	15.274
8	685	512	599	122
9	410	364	387	33
10	0	0	0	0
DAY 30 P.I.				
3	0	0	0	0
11	100	115	108	11
13	0	0	0	0
14	0	0	0	0
15	0	0	0	0

Table 8.6. PCR results of ciliary ganglia (Copy no. / 20 ng DNA)

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