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Effect of a live attenuated intranasal vaccine on latency and shedding of feline herpesvirus 1 in domestic cats

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Summary. A prospective study was conducted that evaluated duration of virus shedding through acute and experimentally-induced recurrent disease episodes in 12 cats, and tissue distribution of latent infections, following intranasal vaccination with a temperature sensitive (*ts*) mutant strain of feline herpesvirus 1 (FHV1). Six of these cats were challenged with a virulent field strain of the agent to assess the extent to which vaccination affected subsequent shedding of virus and the establishment of latent infections. Virus isolation (VI) tests were done in parallel with a polymerase chain reaction (PCR) assay to compare the performance of each diagnostic method. The PCR confirmed that all 12 cats shed virus throughout the periods of vaccination, challenge or mock-challenge, and a cyclophosphamide-dexamethasone stress protocol to reactivate latent infections. Shedding to the *ts*FHV1 was documented by VI for up to 25 days following vaccination and for up to 15 days following challenge, but not after experimental stress. Overall, FHV1 was present in 144 of 300 (48%) cat-days of testing by PCR compared to 32 of 300 (11%) by VI. The frequency and distribution of latent FHV1 detected in neurologic, ophthalmic, and other tissues by PCR were identical for vaccine-only and vaccine-challenge groups, thereby disproving previous hypotheses that *ts*FHV1 mutants administered by this route protect against latency.

Introduction

Feline herpesvirus 1 (FHV1) is a typical member of the *Alphaherpesvirinae* subfamily within the *Herpesviridae* and is the etiologic agent of viral rhinotracheitis, a severe upper respiratory disease of cats. In addition, it can cause abortions in breeding queens, high mortality among infected kittens, and is associated with several important acute and chronic ocular and respiratory

conditions in that species [10, 15]. The virus consists of a glycoprotein-lipid enveloped nucleocapsid core containing the linear double-stranded DNA genome of approximately 134 kbp in size [25]. Most infections occur via the ocular and oronasal route, resulting in viral replication and shedding from the mucosal epithelium of those tissues. In at least 80% of cases [10], this is followed by lifelong latency, whereby the agent persists indefinitely and provides an epidemiological source for infection of susceptible cats. Similar to other members within this virus subfamily, the primary site of FHV1 latency is thought to be the trigeminal ganglia [8, 9]. FHV1 may reactivate in response to various stress stimuli and be shed anew in oral and ocular secretions, usually without overt evidence of disease [10].

Historically, detection of FHV1 shedding has been based upon traditional virus isolation (VI) methods in tissue culture or with somewhat less sensitivity, immunofluorescent antibody assays [15]. In addition, explant co-cultivation with a permissive cell line has been valuable for study of FHV1 latency [8, 9, 14]. The identification of a thymidine kinase (TK) gene homolog for FHV1 [16] characteristic of other alphaherpesviruses has recently allowed for development of at least four polymerase chain reaction (PCR) tests for the agent [23, 29, 31, 32], substantially improving upon the ability to detect it under either circumstance.

In response to the recognized importance of FHV1 as a threat to feline health, numerous inactivated, live attenuated, and genetically modified vaccine strains have been produced for disease prevention. Many of these vaccines also contain an attenuated strain of feline calicivirus (FCV), which shares a similar epidemiology and significance in feline respiratory disease. One recent report [35] described a recombinant vaccine in which the FHV1 TK gene had been disrupted by inserting a gene coding for an immunogenic capsid protein of FCV, generating duel protection against both agents. To date, the primary benefits of vaccination have been to reduce disease severity upon FHV1 challenge without completely eliminating virus infection or latency, even by the vaccinal strains themselves [19, 28, 30, 34, 35].

One exception to this conclusion has been the observation from studies in the United Kingdom of a FHV1 temperature sensitive (*ts*) mutant developed for intranasal administration that provided rapid immunity following challenge [2, 3], did not establish latency, and fully prevented virulent challenge strains from doing so as well [20]. Intranasal vaccination of cats using a single dose of attenuated strains of FHV1 has offered a practical disease control strategy, even under the endemic disease conditions that commonly occur in catteries and in the presence of circulating maternal antibodies [10, 28, 33]. Herein we report the results of experiments that re-evaluate the biology of intranasal vaccination with a *ts*FHV1 product [4] on the dynamics of virus shedding during acute and recurrent phases of infection, with and without challenge, using highly sensitive PCR diagnostics in parallel with traditional virus culture assays. We also report on the distribution of latent FHV1 within tissues of vaccinated cats and further explore aspects of FHV1 biology and prevention.

Materials and methods

Cats

Twelve female 18-week old specific pathogen free domestic shorthair cats (Harlan) were randomly allocated into two treatment groups of 6 cats each. This sample size was sufficient to detect any meaningful ($> 85\%$) difference in latency rates between the groups at a Type I error rate of 0.05 and a Type II error rate of 0.20 [6]. Cats in each group were housed in separate isolation rooms 50 m apart following arrival, caged individually, and maintained in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Husbandry was provided by separate animal care staff for each area and strict hygienic measures were used to avoid any potential for virus transmission between rooms.

Animal experiment

Serum samples collected from cats 1 week after their arrival were examined by indirect fluorescent antibody tests [15] to confirm that cats were initially free of FHV1. After 2 weeks of acclimation post-transport, all cats were given a single dose of vaccine containing the *ts*FHV1 and an attenuated strain of FCV (Felomune CVR, SmithKline Beecham) by instilling one drop of the reconstituted product into each eye and the remainder into the nostrils, according to the manufacturer's directions. Plaque reduction neutralization (PRN) tests [13] done on serum collected two weeks after vaccination were used to confirm a response to vaccination, using one of the pre-vaccination specimens as negative control.

To test for shedding of FHV1, swab specimens were taken with sterile cotton-tipped applicators from the left eye, right eye, and oral mucosa every 3–4 days following vaccination for 13 weeks (25 test days per cat) and placed immediately into 1 ml of virus transport fluid. This fluid consisted of Dulbecco's modified Eagle's medium with 25 mM Hepes buffer, 1% fetal calf serum, 0.25 mg/ml gentamicin, 1000 units/ml penicillin G, and 5 µg/ml amphotericin B. Samples were transported immediately to the laboratory on wet ice and split into two aliquots (0.5 ml each) for FHV1 assay in parallel via VI and PCR.

One treatment group ($n = 6$) was challenged by instilling 100 µl (10^6 plaque forming units/ml) of FHV1 (NCSU727) into the nostrils and conjunctival sacs (300 µl total) at 4 weeks post-vaccination. This isolate is a virulent wild-type strain which has been well characterized through previous studies of FHV1 diagnosis and pathogenesis [13, 32]. The other treatment group ($n = 6$) was mock-challenged in the same manner using virus-free growth medium. Cats in both groups were subjected to a stress protocol at 8 weeks post-vaccination that involved intravenous injections of cyclophosphamide (30 mg) followed by dexamethasone (10 mg) 24 h later in order to reactivate latent infections [14, 32]. Cats were anesthetized with ketamine hydrochloride and then euthanized by pentobarbital overdose at approximately 13 weeks post-vaccination after their final set of ocular and oral swab specimens. Sterile instruments and gloves were used to collect the olfactory bulb, corneas, uveas, optic nerves, optic chiasma, trigeminal ganglia, and sections (approx 1 gm each) of the palatine tonsils and nasal turbinates from each cat. Tissue harvests were placed into 1 ml of virus transport fluid and stored at $-70\,^{\circ}C$ until tested by PCR. Euthanasia and processing of each treatment group was done 3 days apart to further reduce the potential for cross-contamination.

Virus isolation assay

Crandell-Reese feline kidney (CRFK) cell monolayers were used according to reported methods [13, 22] for isolation of infectious virus. Fluid extracts from swab specimens were

plated within 24 h of collection, maintained for 14 days at 35 °C in 5% CO2, and observed daily for characteristic cytopathic effect (CPE). Specific antisera was used in immunofluorescent assays [13] to confirm the identity of isolates. The same methods, using appropriate antisera, also enabled us to identify shedding of the FCV component of the vaccine. For the virus-challenged group of cats, shedding of the *ts*FHV1 vaccine strain was distinguished from FHV1(NCSU727) by its failure to induce CPE when subcultured in CRFK cells at 40 °C [21, 28] as validated in our laboratory.

Polymerase chain reaction

The PCR assay for swab specimen extracts was done as reported [32] using the upstream primer FHV-B1 (5′-TGTCCGCATTTACATAGATGG-3′) and downstream primer FHV-B2 (5′-GGGGTGTTCCTCACATACAA-3′) which are specific for a 322-bp segment within the TK region of the agent. In titration studies, this assay was shown to reliably detect 240 copies of the viral genome [32]. Swab specimens were processed by the freeze-thaw, centrifugation and heating protocol described previously [32] and evaluated by PCR in duplicate assays. FHV1 infected CRFK cells and template-free mixtures were used as positive and negative controls, respectively. Reaction products were visualized after electrophoresis through a 1.5% agarose gel and staining with ethidium bromide. Through in vitro testing, we confirmed that this assay also functioned appropriately for detecting the reconstituted *ts*FHV1 vaccine strain used in the present investigation. PCR results from swab specimens were scored positive for FHV1 if a clear band of the appropriate size was evident in either replicate. Select swab samples were also examined through hybridization to the internal probe FHV-B3, as described below.

Tissue harvests

DNA was extracted from the feline post-mortem tissue harvests by overnight digestion with proteinase K followed by standard phenol-chloroform-isoamyl alcohol purification and sodium acetate-ethanol precipitation methods [26]. An additional collagenase step was added to aid in the digestion of corneal specimens [17]. The extracted DNA was quantified spectrophotometrically ($A_{260/280}$) through interpolation from known size standards and run by the PCR in duplicate at 1 μg per reaction. PCR products were transferred by Southern blotting onto Zeta-probe nylon membranes (BioRad), hybridized to the specific 25-mer probe FHV-B3 and examined by autoradiography, as reported [32]. Blots from tissue specimens were scored positive for FHV1 if either replicate contained the specific amplicon.

Statistical methods

The total number of FHV1-positive days per cat were compared over treatment groups using the Mann-Whitney test for non-parametric data. These results were further evaluated by stratifying into the post-vaccination period (weeks 1–4), the post-challenge or mock-challenge period (weeks 5–8), and the post-stress period (weeks 9–13). Contingency tables were used for analysis of categorical data, where appropriate [6]. Results were considered statistically significant at $P < 0.05$

Results

None of the cats developed clinically apparent signs of infection following vaccination, but all responded appropriately with significant PRN titers ranging

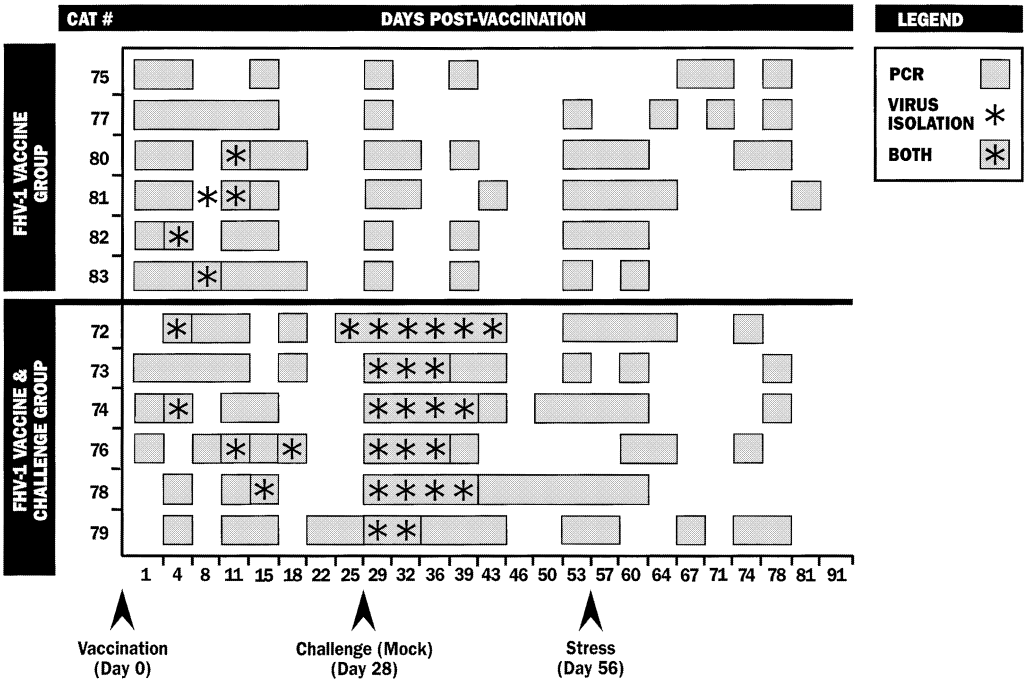

Fig. 1. Results of PCR and VI testing for FHV1 in ocular and oropharyngeal swab specimens from 12 intranasally vaccinated cats, with and without wild-type challenge. Cats were tested on each of the indicated days for a total of 300 tests (12 cats, 25 testing days). All cats were chemically stressed with cyclophosphamide-dexamethasone on day 56 post-vaccination. In this summary, cats were considered to be FHV1-positive for the diagnostic assay if virus was detected in any of the three swab specimens collected on each day

from 1:20 to 1:80 (geometric mean = 1 : 34) two weeks later. Of the 900 swab specimens collected during this study (3 tissue sites from 12 cats over 25 test days), 231 (25.7%) were FHV1 positive by PCR versus 70 (7.8%) by VI. The PCR results were almost always unambiguous, requiring confirmation by Southern blotting in only 9 of the 900 (1%) swab replicates. No significant pattern emerged in the likelihood of recovering FHV1 by PCR from conjunctival tissues of the left (38%) or right (33%) eye versus the oropharynx (29%), but cats would have been considered FHV1 negative on 58 occasions had all three sites not been evaluated in this manner. When the PCR results from swab specimens (conjunctival plus oropharyngeal) were pooled to represent any evidence for virus shedding by cats over time, FHV1 was present in 144 of 300 (48%) swab days by PCR compared to 32 of 300 (11%) by VI (Fig. 1).

All cats shed the *ts*FHV1 throughout the 4 weeks following vaccination, with oropharyngeal swabs positive for up to 25 days according to both assays and conjunctival swabs positive for up to 11 days by VI and 18 days by the PCR, respectively. No significant difference was found in the number of virus-

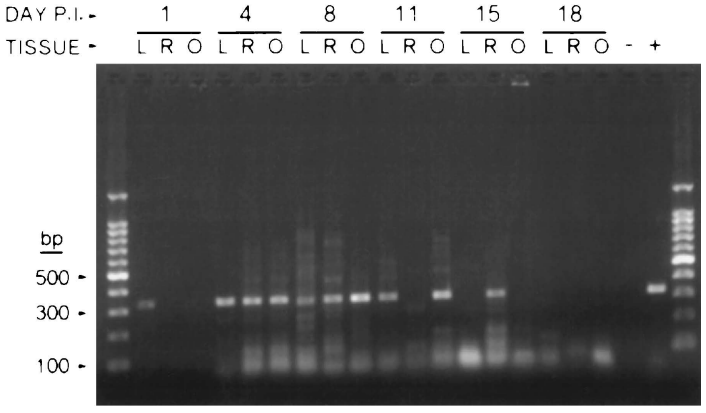

Fig. 2. Representative agarose gel of PCR amplification products from swabs of the left eye (*L*), right eye (*R*), and oropharynx (*O*) of cat no. 77 on days 1 through 18 following intranasal vaccination with a temperature-sensitive mutant strain of FHV1. The specific 322 base pair (bp) amplicon is clearly visible 15 days post-vaccination in this cat. Negative (−; uninfected CRFK cells) and positive (+; FHV1 infected CRFK cells) control specimens are also shown. Molecular size markers $\phi\chi$ 174 RF DNA/*Hae* III (Stratagene) are also shown

positive days between groups during the acute phase of infection, regardless of methods of FHV1 detection. Subsequently, the *ts*FHV1 was not recovered by VI in cats from the vaccine-only group for the duration of the experiment (weeks 5 through 13), but PCR results showed that the agent was present throughout that period (Fig. 1). An example of PCR results from one cat in the vaccine-only group up to 18 days after intranasal dosing with the *ts*FHV1 is shown in Fig. 2.

Although no outward signs of infection occurred in vaccinated cats following challenge, FHV1 was readily recovered from them for up to 15 days according to VI results, and for the entire 4 week post-challenge period according to PCR (Fig. 1). Of 53 recoveries of virus from swab specimens following challenge, 36 (68%) were verified by subculture at 40 °C to be of the wild-type strain FHV1(NCSU727).

Compared to the vaccine-only group, PCR detected more FHV1-positive days among challenged cats during this period ($P = 0.006$). Recurrent FHV1 infections were evident in all cats following the stress protocol, as determined by PCR (Fig. 1), with virus detectable in conjunctival swabs for up to 25 days. No significant difference was found in FHV1-positive days between groups during this period. All swab specimens except a single conjunctival result were negative for evidence of FHV1 shedding during the 8 to 11 days prior to euthanasia.

The VI methods readily distinguished the vaccinal FCV from *ts*FHV1 recovered from swab specimens, even though both grew readily in CRFK cells and were sometime present concurrently. Consistent with the literature [5, 10],

Table 1. FHV1 DNA positive tissues in 12 cats latently infected after receiving an attenuated intranasal vaccine with and without challenge exposure with wild-type virus, as determined by polymerase chain reaction

Tissue sample	Identification # for vaccinated cats						Identification # for vaccinated-challenged cats					
	75	77	80	81	82	83	72	73	74	76	78	79
Left trigeminal ganglia	+	+	+	+	+	+	+	+	+	+	+	+
Right trigeminal ganglia	+	+	nd[a]	+	+	+	+	+	+	+	+	+
Left optic nerve	−	−	nd[a]	−	+	+	−	−	−	−	−	+
Right optic nerve	−	+	nd[a]	−	−	+	−	+	−	−	−	−
Left cornea	−	−	−	+	−	−	−	−	−	+	−	−
Right cornea	−	−	−	+	+	−	−	−	−	−	−	−
Left uvea	−	−	nd[a]	−	−	−	−	−	−	−	−	−
Right uvea	−	−	nd[a]	−	−	−	−	−	−	−	−	−
Optic chiasm	−	−	nd[a]	−	−	−	−	+	−	−	−	−
Olfactory bulb	−	−	nd[a]	−	−	−	−	−	+	−	−	−
Palatine tonsil	−	+	−	−	−	−	−	−	+	−	−	−
Nasal turbinate	−	−	+	−	−	−	−	−	+	−	−	+

[a] Not done

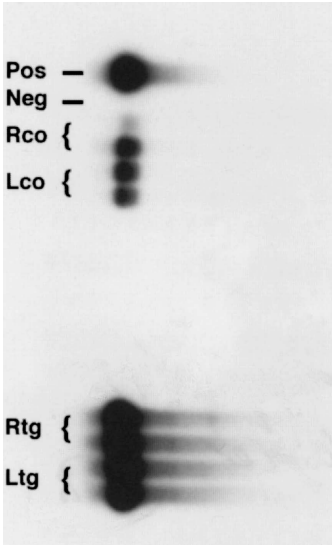

Fig. 3. Southern blot analysis with probe FHV-B3 of PCR products from various tissues of cat no. 81 harvested on day 88 following intranasal vaccination with a temperature-sensitive mutant strain of FHV1. Mock-challenge and drug-induced stress occurred on days 28 and 57, respectively. In this cat, FHV1 DNA was detected in the right (*Rtg*) and left (*Ltg*) trigeminal ganglia and in the right (*Rco*) and left (*Lco*) corneal specimens. Positive (*Pos*) and negative (*Neg*) control specimens are also shown. All products hybridizing with the specific probe were 322 base pairs in size

FCV was shed for 11 to 36 (mean= 25) days following vaccination of both groups, with most (71%) isolations made from oropharyngeal swabs.

PCR testing of tissue harvests collected at necropsy provided evidence that *ts*FHV1 established latent infections in the trigeminal ganglia of vaccinated cats, and in at least four other tissues as well (Table 1). Seven tissues from one cat in the vaccine-only group were unavailable for testing. The frequency distribution of FHV1 DNA positive tissues did not substantively differ among cats in the vaccine-only or vaccine-challenge group for these experiments, and was consistent with previous reports regarding possible sites of latency following exposure to wild-type virus [23, 32]. An example of the findings from one latently infected cat in the vaccine-only group is shown in Fig. 3.

Discussion

Much work has been devoted to improving strategies for vaccination of cats against FHV1, one of the most widespread and significant of all diseases known in that species. The variety of inactivated and live attenuated vaccines strains now in widespread use for FHV1 control and prevention have often met with mixed results under natural conditions [5, 10]. Genetically engineered products that provide simultaneous protection against multiple infectious agents and include molecular markers to distinguish vaccine from wild-type virus [34, 35] could potentially be of great benefit. However, information as to whether this approach can meaningfully temper the dynamics of infection and latency under field conditions of repeated wild-type challenge exposure has yet to be forthcoming. The attenuated product we studied consists of a *ts* strain of FHV1 produced via chemical mutagens and ultraviolet radiation [4] and is the only

intranasal FHV1 vaccine currently licensed for use in the United States. It was selected for our work as potentially representative of the issues facing all replication-competent vaccines administered by this route.

Previous work with *ts* mutant strains of FHV1 given intranasally has validated their ability to protect against symptomatic disease in cats, even during the outbreak conditions frequently recognized in breeding catteries and in the presence of maternal antibodies [10, 28, 33]. Establishment of an appropriate and persistent immune response in mucosal tissues representing the natural portals of virus entry should improve the likelihood of complete protection against this type of agent [24]. In one such study, it was suggested that another intranasal *ts*FHV1 vaccine did not become latent and could also prevent wild-type virus used in challenge experiments from established latency. These finding have clear importance to FHV1 biology and disease control options for cats and were addressed in part through the present investigation. Through sensitive PCR diagnostic technology in parallel with virus culture assays, we showed that the profile of FHV1 shedding during acute and recurrent disease episodes did not substantively differ with respect to intranasal vaccination history. We also demonstrated that this vaccinal *ts*FHV1 strain appeared to establish latent infections in frequencies identical to that for wild-type virus under the same experimental conditions [32], including evidence for non-neuronal sites of virus persistence in either case.

Other workers [30] have used quantitative PCR assays to evaluate colonization of the trigeminal ganglia and other tissues following FHV1 vaccination and challenge. In that report, vaccination of cats with a commercial product licensed only for subcutaneous injection reduced the latency load of wild-type FHV1 infections ten-fold over that of a constructed deletion mutant FHVβ-galgIgEΔ used as vaccine by the same route, but oronasal vaccination with this mutant generated an improved protective immunity. In contrast, virulent disease resulted when the commercial vaccine strain was instead given intranasally [11], substantiating concerns that not all current products are fully attenuated. As for those experiments [30], our PCR assay did not allow for differentiation of wild-type challenge virus from vaccine strain in experimentally infected cats. The *ts* mutation in the product we evaluated has yet to be characterized genotypically, thereby precluding use of strain-specific DNA amplification assays at present. Nonetheless, the remarkably similar patterns of virus shedding between groups of cats, compared to findings from historical challenge-only controls [32], are sufficient to argue against any confounding from that factor.

Vaccine studies of related alphaherpesviruses in other species [7, 12, 27] for which prevention of latency is desirable have often been disappointing. Most studies utilizing appropriately sensitive methods of assay have found that both the vaccinal and challenge agents establish latent infections, although the severity of disease and extent of virus shedding that follow are reduced substantially. To date, this has also held true for the experimental recombinant vaccines for FHV1 [11, 30, 34, 35] and even for nucleic acid vaccines used as

replication-competent plasmids for closely related agents [1]. Likewise, our findings may hold true for other live attenuated FHV1 vaccine products commercially available for veterinary use.

The sensitivity of PCR assays over traditional VI methodology for detection of FHV1 in clinical swab specimens has been previously emphasized [23, 31, 32]. In many instances, cases of FHV1 shedding may have been greatly underestimated, particularly for vaccinated animals [31], and most likely explains our findings in contrast with earlier reports regarding the ability of vaccination to impact the carrier state [2, 9, 20]. Future studies should also employ PCR technology to closely examine the ability of live attenuated and recombinant FHV1 vaccines to be transmitted among cats.

The biological implications of persistent FHV1 infections in tissues other than the trigeminal ganglia adds to traditional viewpoints on the pathogenesis of this disease [8, 9, 14] and reinforces recent findings based upon PCR as the method of testing [23, 32]. Non-ganglionic sites appear to harbor a smaller viral load during latency [30], and may account for the difficulty in identifying the agent even in cases when the benefits of PCR have already been recognized [29]. Because of their documented role as important sites of viral replication with correlates for maintaining a protective immunity [3, 8], our observation of apparently latent FHV1 in tissues of the palatine tonsils and nasal turbinates supports earlier findings [23, 32] and should be considered alongside similar aspects of FCV biology and vaccine development [10].

Recent concerns have been raised regarding possible associations between FHV1 infection and pathogenesis of the chronic feline ocular conditions of eosinophilic keratitis and corneal sequestration [13, 29], while the role of FHV1 in experimentally induced models of stromal keratitis is already well-documented [13]. Therefore, the reports of FHV1 DNA in corneal tissues of lesioned and normal cats by way of different PCR assays [13, 23, 29, 32] is highly consequential and should explored in detail. Future studies of FHV1 pathogenesis should also examine evidence for transcriptional activity in these and other tissues during presumptively latent phases of infection [18], analogous to the latency-associated transcripts characterized from other alphaherpesviruses. Given our findings, it will likewise be important to discover whether any of these chronic conditions are exacerbated by a history of vaccination with certain FHV1 strains or routes of administration and could be prevented by entirely different strategies in immunization technology.

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