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Short communication

# The domestic cat antibody response to feline herpesvirus-1 increases with age

Michael W. Munks[a,*,1], Alana M. Montoya[a], Cameron M. Pywell[a], Garrick Talmage[a], Anna Forssen[b], Teresa L. Campbell[c], Donald D. Dodge[c], John W. Kappler[a,d], Philippa Marrack[a,d]

[a] Department of Biomedical Research, National Jewish Health, Denver, CO, United States
[b] Division of Biostatistics and Bioinformatics, National Jewish Health, Denver, CO, United States
[c] Jasper Animal Hospital, Lafayette, CO, United States
[d] Howard Hughes Medical Institute, National Jewish Health, Denver, CO, United States

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ABSTRACT

Herpesviruses establish lifelong infections, normally characterized by prolonged periods of latency with intermittent episodes of viral reactivation. Feline herpesvirus-1 (FHV-1) infects domestic cats, and epidemiological studies indicate that many or most domestic cats are exposed to FHV-1, but the strength and longevity of the antibody response to FHV-1 is not fully characterized. Here we describe development of an ELISA, using lysates of cat cells infected with FHV-1, that measure feline antibodies against FHV-1. The assay is sensitive, quantitative and has a large dynamic range. We found that serum anti-FHV-1 antibodies primarily recognize FHV-1 proteins of the Late (L) class and are primarily of the IgG isotype. We then analyzed serum from a cross-sectional cohort of 100 client-owned cats that differed in age, sex and vaccination history. While there was no difference in FHV-1 antibody responses between females and males, antibody levels were significantly increased in older cats in comparison with younger animals ($p = 0.01$). Surprisingly, as the length of time since the most recent vaccination increased, there was no corresponding drop in serum anti-FHV-1 antibody. These data suggest that FHV-1 immunity is very long-lived and support the current recommendation that many cats do not require revaccination against FHV-1 annually.

## 1. Introduction

Feline herpesvirus-1 (FHV-1) is a species-specific herpesvirus that causes rhinotracheitis, including sneezing, oronasal and ocular discharge, fever and lethargy, in infected domestic cats (Gaskell et al., 2007). Although disease can be more severe in young or immunocompromised cats, FHV-1 disease is normally self-limiting. Once the acute infection resolves, FHV-1 undergoes lifelong latency. FHV-1 can be induced to reactivate, via corticosteroid immunosuppression, in experimentally infected cats. Because FHV-1 can be cultured from 70% of such immunosuppressed cats, it is believed that most or all exposed cats become latently infected for life (Gaskell et al., 2007). Thus immunity may be maintained and boosted with time in these cats because of latent gene expression and because of transient reactivation of the virus. Despite the possibility that FHV-1 reactivation may boost the immune response, the strength and longevity of the antibody response following natural or experimental FHV-1 infection is incompletely understood.

In contrast to the context of FHV-1 infection, the antibody response following FHV-1 vaccination has been studied in greater detail, due to the importance of understanding whether giving booster vaccinations is necessary for long-term protection from disease. However, it's unknown whether the live-attenuated FHV-1 F-2 strain is able to partially and/or fully reactivate from latency, which also has the potential to boost immunity.

Three studies have examined the duration of immunity with live-attenuated FHV-1 vaccines and found long-lived immunity (Lappin et al., 2002; Gore et al., 2006; Mouzin et al., 2004). These studies, and indeed most FHV-1 studies, assessed anti-FHV-1 antibodies with the virus neutralization (VN) assay, where dilutions of cat serum are tested for inhibition of FHV-1 infection *in vitro* (Povey and Johnson, 1969). The strengths of the VN assay are that it is specific, quantitative, and

*Abbreviations:* FHV-1, feline herpesvirus-1; VN, virus neutralization; IE, immediate-early; E, early; L, late; CDC, complement dependent cytotoxicity; ADCC, antibody dependent cellular cytotoxicity; CRFK, Crandell-Rees feline kidney; SPF, specific pathogen free

* Corresponding author at: Oregon Health & Science University, 3181 SW Sam Jackson Pk Rd, RJH 6564, Portland, OR 97239, United States.
*E-mail address:* munksm@gmail.com (M.W. Munks).
[1] Present address: Department of Molecular Microbiology and Immunology, Oregon Health & Science University, Portland, OR, United States.

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measures antibody function. However, this test is unable to detect the existence of antibodies that do not neutralize FHV-1, such as antibodies that activate complement-dependent cytotoxicity (CDC) and/or antibody-dependent cellular cytotoxicity (ADCC), despite evidence that these functions contribute to FHV-1 control (Wardley et al., 1976).

At least four FHV-1 ELISAs have been described in the literature (Lappin et al., 2002; Digangi et al., 2011; Satoh et al., 1999; Dawson et al., 1998). One study does not describe the nature of the FHV-1 antigen (Digangi et al., 2011), two studies utilize only nucleocapsid protein (Lappin et al., 2002; Satoh et al., 1999), and the final ELISA uses only a single denatured FHV-1 protein (Dawson et al., 1998). Therefore it appears likely that each of these ELISAs, like the VN assay, may ignore the majority of FHV-1 antibodies.

To gain a better understanding of the strength and longevity of the antibody response to FHV-1, we developed a sensitive FHV-1 ELISA that could potentially detect antibodies specific for any FHV-1 protein, either neutralizing or non-neutralizing. With serum collected from client-owned cats, we then analyzed the major antibody isotype and the major class of viral proteins recognized. Finally, we analyzed serum from a cross-sectional cohort of 100 cats to determine how the FHV-1 antibody response was related to sex, age and vaccine history.

## 2. Materials and methods

### 2.1. Serum and vaccination history

All experiments on live vertebrates were performed in accordance with relevant institutional and national guidelines and regulations. IACUC approval was obtained for acquiring leftover blood samples, collected at Jasper Animal Hospital (Lafayette, Colorado) from client-owned cats for diagnostic procedures, that otherwise would have been disposed of. Most samples were serum but others were blood or plasma. In the cases of blood or plasma, the sample was mixed with calcium and bovine thrombin (Sigma) to induce coagulation and the serum was then collected. The sex of each cat, its known or estimated age, and its known vaccine history were recorded. Pooled specific pathogen free (SPF) cat serum was from Bethyl Laboratories, Inc.

### 2.2. Cells and virus stocks

Crandell-Rees Feline Kidney (CRFK) cells (ATCC; CCL-94) were grown in DMEM with 10% fetal bovine serum, antibiotics and L-glutamine, at 37 °C with 10% $CO_2$. FHV-1 strain C-27 (ATCC; VR-636) was propagated on CRFK cells. Cytopathic effect was usually first evident 24–36 h after infection. When cytopathic effect was complete, after 48–72 h of infection, supernatants were collected, centrifuged at 500*g* for 5 min to remove cells and cellular debris, then stored at −80 °C.

### 2.3. FHV-1 lysates

CRFK cells were infected with FHV-1 at a multiplicity of infection of 5 for 16–20 h. Supernatants were centrifuged at 500*g* for 5 min at 4 °C to collect non-adherent cells. The non-adherent cells, and the adherent cells still attached to the plate, were washed twice with PBS and lysed at $15 \times 10^6$ cells/ml for 10 min with ice cold Lysis Buffer. Lysis Buffer consisted of 10 mM Tris, 150 mM NaCl, 1 mM EDTA and 1% NP-40 detergent at pH 7.2, containing 2 mM phenylmethanesulfonyl fluoride (PMSF, from Sigma) and a complete mini protease inhibitor cocktail tablet (Roche). The plates were scraped and combined with cells that pelleted from the supernatant. The mixture was transferred to a 1.7 ml tube and centrifuged at 14,000*g* for 10 min at 4 °C. The supernatant was transferred and stored at −80 °C until use.

For some experiments, CRFK cells were treated to restrict FHV-1 protein expression as previously reported (Honess and Roizman, 1974; Jones and Roizman, 1979). To enrich for IE proteins, cells were first infected in the presence of 50 ug/ml of cycloheximide (Sigma) to allow expression of IE transcripts but to prevent their translation. Three hours later, the cells were washed and media containing 5 ug/ml actinomycin D (Sigma) was added to allow translation of the IE transcripts into protein, but to prevent expression of E transcripts. After an additional 2 h, cells were lysed. To enrich for E proteins, cells were infected in the presence of 300 ug/ml phosphonoacetic acid (Sigma), to prevent replication of the viral genome. Cells were lysed after 16–20 h. To allow L protein expression, cells were infected for 16–20 h without inhibitors. Uninfected (mock) cells were cultured without inhibitors before lysis.

### 2.4. ELISAs

NP-40 lysates of CRFK cells were diluted 1:2000 in ELISA binding buffer (50 mM Tris, 150 mM NaCl, 0.1% sodium azide, pH 7.4) and added to High Binding 96 well ELISA plates (Thermo-Fisher #14-245-78), sealed, then incubated overnight at 4 °C. After overnight binding, plates were washed three times with ELISA wash buffer (50 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.4) using an ELx405 Auto Plate Washer (BioTek Instruments, Inc.). Plates were then blocked with an ELISA blocking buffer (50 mM Tris, 150 mM NaCl, 0.1% sodium azide, with 1% IgG-free bovine serum albumin (Sigma)) for 1 h at room temperature, then washed three times.

Cat serum was diluted in ELISA blocking buffer, starting with a 1:1000 dilution then proceeding with 1:3 dilutions through a final dilution of 1:729,000, added to the ELISA plates in triplicate, then incubated for 1 h at room temperature. Plates were washed three times and goat anti-cat IgG-Fc fragment specific antibody conjugated to HRP (Bethyl Laboratories, Inc., A20-117P), was diluted 1:10,000 in ELISA wash buffer, then incubated for 1 h at room temperature. In experiments where isotype-specific ELISAs were performed, goat anti-cat IgM-HRP or goat anti-cat IgA-HRP (Bethyl Laboratories, Inc.) were used in parallel to goat anti-cat IgG Fc-HRP. After five washes, plates were developed with One-Step Ultra TMB-ELISA Substrate Solution (Thermo-Pierce). After 20 min, 2 M $H_2SO_4$ stop solution was added. Plates were read at 450 nm with an ELx808 Automated Microplate Reader (BioTek Instruments, Inc.). Standards were fitted to a sigmoidal standard curve using Prism (GraphPad Software, Inc.). Unknowns from each animal that fell within the standard curve's dynamic range were interpolated, then multiplied by their dilution factor to obtain the antibody concentration in undiluted serum. In instances where cats had very low FHV-1 antibody concentrations, the ELISA was repeated starting with 1:100 serum dilutions. When necessary, weak antibody responses were arbitrarily adjusted to 0.1% of the reference serum to facilitate statistical analysis and to allow graphing on a log scale.

### 2.5. Statistical analysis

The data in **Supplemental Table S1** were analyzed by simple linear regression models, using SAS software, with sex, age, time since last vaccination and spay/neuter status as predictors of FHV-1 antibody titer. Comparison of years since last vaccination vs. age was performed by linear regression in Prism. Comparison of FHV-1 antibody titer as a function of number of vaccinations was performed by ANOVA using SAS. The data were also analyzed with age, time since last vaccination, and number of vaccinations as predictors by multiple linear regression.

## 3. Results

### 3.1. Cat antibody responses to FHV-1 are focused on L proteins

To develop a method that could potentially detect any FHV-1 antibody, we infected Crandell-Rees feline kidney (CRFK) cells with wild type FHV-1, then used NP-40 detergent to make cellular lysates that were adsorbed to ELISA plates for use with cat serum. The ELISA

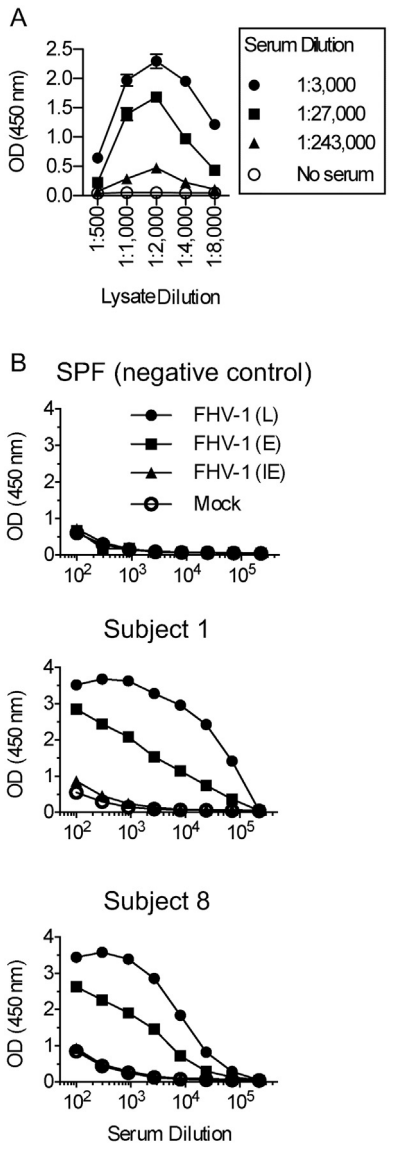


**Fig. 1.** Cats Respond Preferentially to FHV-1 Late Proteins.**A**. NP-40 lysates of CRFK cells, either mock-infected or infected with FHV-1, were used to coat ELISA plates at various dilutions. The ELISA was then performed with serum from subject 1, detected with goat anti-cat IgG-Fc conjugated to HRP.**B**. FHV-1 infection of CRFK cells were performed under conditions that favor IE, E or L protein expression, then the lysates were used in an ELISA with either SPF cat serum or serum from client-owned cats. The last dilution is a no-serum control, which cannot be properly graphed on a log scale. Two representative cats from the same experiment are shown. Similar results were obtained in an independent experiment that included subject 1 and three additional subjects.

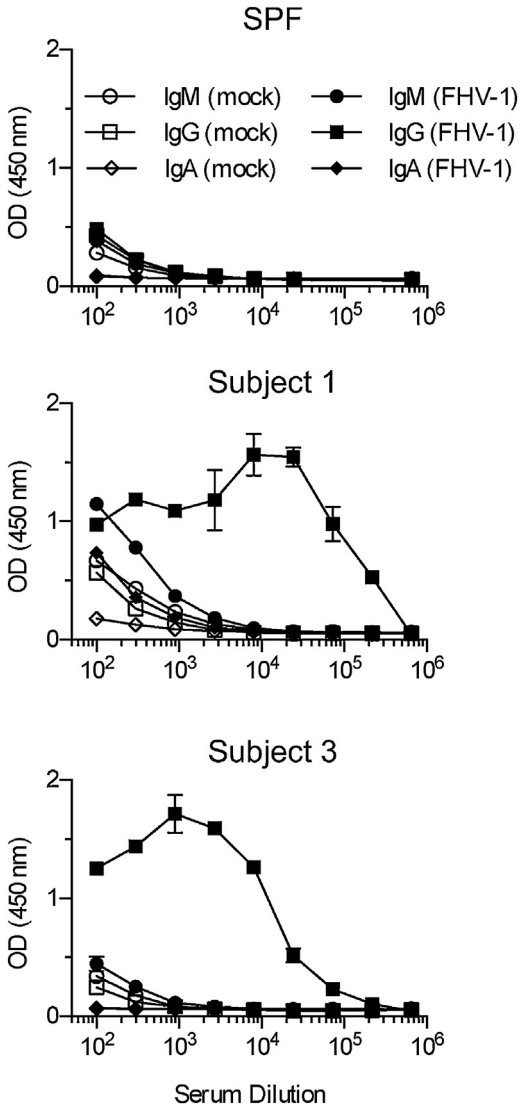


**Fig. 2.** Cats Preferentially Make FHV-1 Antibodies of the IgG Isotype.**A**. Lysates of CRFK cells that were either uninfected, or at the L stage of FHV-1 infection, were used in an ELISA with serum from client-owned cats. Isotype-specific secondary antibodies were used that recognize either cat IgM, cat IgG-Fc or cat IgA. Two representative subjects are shown. Similar results were obtained with three additional subjects. Note that very high HRP activity, seen when low serum dilutions are detected with anti-IgG-Fc, results in secondary substrate oxidation and some loss of absorbance at 450 nm.

worked sub-optimally when the lysate was diluted 1:500 or 1:1000, perhaps because the high detergent concentration prevented the viral antigens from adsorbing efficiently to the ELISA plates (Fig. 1**A**). It also worked poorly with lysate diluted 1:4000 or 1:8000, presumably because the antigen became limiting, but worked effectively at 1:2000, when the final NP-40 concentration was 0.0005% (Fig. 1**A**).

Herpesvirus infections have three distinct classes of protein expression, referred to as immediate-early, early or late (IE, E or L) (Honess and Roizman, 1974; Jones and Roizman, 1979). Lysates of cells arrested pharmacologically at each stage of infection, were used to determine if serum antibodies recognize all classes similarly. As expected, specific pathogen free (SPF) cat serum did not recognize any of the lysates (Fig. 1**B, top**). In contrast, serum from client-owned cats had weak or undetectable reactions to IE proteins, moderate responses to E proteins, and the strongest response to L proteins (Fig. 1**B, middle & bottom**). All further experiments thus used FHV-1 lysates from the L stage of infection.

### 3.2. FHV-1 elicits primarily IgG antibodies

To determine the primary antibody isotype(s) that recognize FHV-1, we performed ELISAs with secondary antibodies specific for feline IgM, IgG or IgA. Again SPF cat serum had no antibody reactivity to FHV-1 (Fig. 2, **top**). Subject 1, which was immunized to FHV-1 multiple times, had only a weak IgM and IgA response to FHV-1, which titrated to baseline by the 1:2700 serum dilution, while the IgG response to FHV-1 was clearly detected even at 1:218,700 (Fig. 2, **middle**). The response of subject 3 was qualitatively similar to that of subject 1, with IgM and IgA responses that were detectable only at low serum dilutions, while its IgG response was detected at 1:72,900 (Fig. 2, **bottom**). These data indicate that anti-FHV-1 antibodies were primarily class-switched to IgG, with minimal IgM and IgA responses.

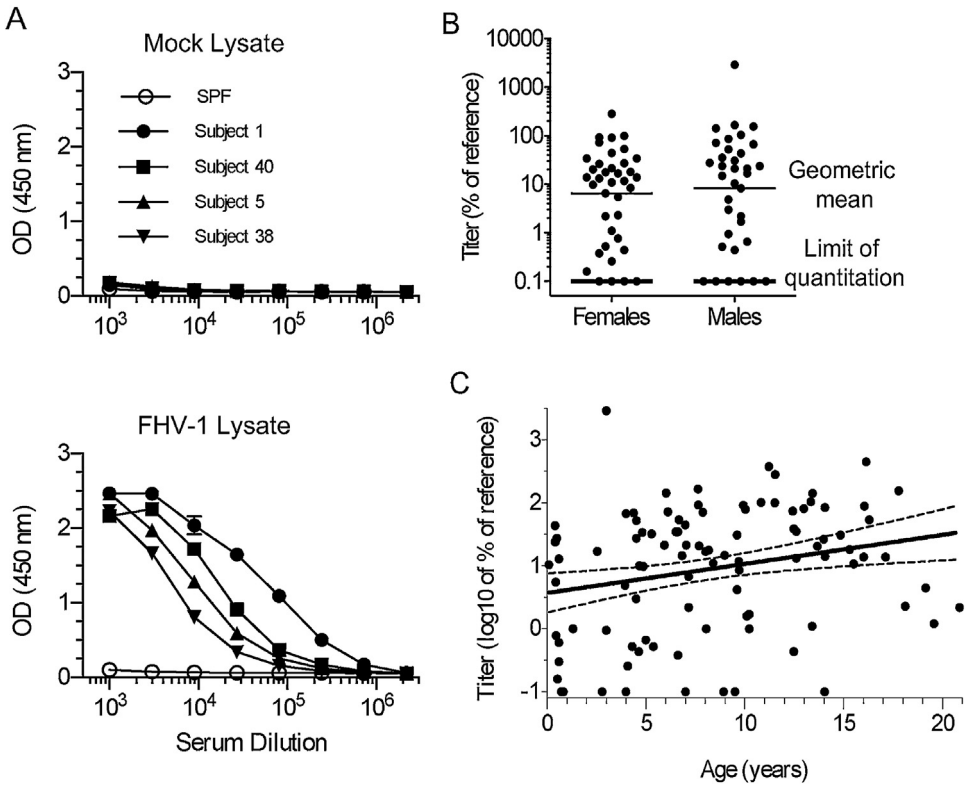


**Fig. 3.** Older Cats Have Stronger FHV-1 Antibody Responses than Younger Cats.**A.** ELISA assays were performed with mock or FHV-1 lysates. IgG antibody responses of individual cats to FHV-1 were normalized to subject 1.**B.** Serum IgG levels to FHV-1, normalized to subject 1, were compared between female and male cats (p = 0.70).**C.** Serum IgG levels to FHV-1, normalized to subject 1, were plotted as a function of age (p = 0.01).

### *3.3. FHV-1 antibody responses are stronger in older cats*

We next quantified the antibody response to FHV-1 in a cross-sectional cohort of 100 serum samples from client-owned cats that varied in age, sex and vaccination history **(Supplemental Table S1, columns 1–5)**. To allow a direct comparison between samples run on different plates and/or different days, the sigmoidal ELISA curve of each sample was normalized to subject 1, which was included on each plate, as shown in Fig. 3**A**. Thus the data generated from all 100 cats could be directly compared to each other **(Supplemental Table S1, Column 6).** These data were then analyzed statistically to determine what effect, if any, the individual characteristics had on antibody titers to FHV-1.

In both humans and mice, there are immunological differences between males and females (Rubtsova et al., 2015). We thus compared FHV-1 antibodies between female cats and male cats, but found that both sexes had similar antibody titers (Fig. 3**B**; **p = 0.70**). We next analyzed the FHV-1 antibody concentration of each cat as a function of age. The current feline vaccine recommendations assume that immunity wanes over time. Thus we were surprised to find a positive correlation between antibody and age (Fig. 3**C**; **p = 0.01**), indicating a stronger antibody response in older cats.

### *3.4. FHV-1 antibodies are not dependent on recent vaccination or total vaccinations*

It was possible that the older cats in our cohort had been vaccinated more recently, which could explain their higher antibody titers. This was not the case, as the time since last vaccination was actually longer in the older cats (Fig. 4**A**, **p < 0.01**). Because it was counter-intuitive that older cats, with on average a longer interval since vaccination, had stronger anti-FHV-1 antibody responses, we looked at the underlying assumption that recent vaccination would be associated with stronger anti-FHV-1 antibody responses. However, the data did not support this assumption, as we found that there was no statistical relationship between recent vaccination and antibody levels (Fig. 4**B**; **p = 0.98**).

We next examined whether there was a correlation between the anti-FHV-1 antibody response and the total number of vaccinations. The power of this analysis was weakened by the fact that there were only four cats with no history of vaccination, only four cats with four or more documented vaccinations, while the remaining 45 cats that could be analyzed had either 1 (n = 11), 2 (n = 20) or 3 (n = 14) documented vaccinations. Thus we decided, somewhat arbitrarily, to group cats into those with 0–1 vaccinations (n = 15), 2 vaccinations (n = 20) or 3+ vaccinations (n = 18). This analysis revealed that cats that had been vaccinated three or more times did have increased anti-FHV-1 antibody titers compared to cats that had been vaccinated only once or not at all (p = 0.02), and compared to cats that had been vaccinated only twice (p = 0.04) (Fig. 4**C**). However, these differences were modest, as the median antibody titer between the highest and lowest group differed by only about 4-fold, and by only about 2-fold between the highest and middle group. When age, time since most recent vaccination, and number of vaccinations were analyzed by multiple linear regression, an $R^2$ of 0.29 was obtained. This indicated that age and vaccine history predicted a substantial amount of, but not most of, the FHV-1 antibody titer.

In this study, we used lysates of cat cells infected with FHV-1 to establish an ELISA capable of detecting both neutralizing and non-neutralizing cat serum antibodies recognizing FHV-1. This ELISA may be more sensitive than the commonly used FHV-1 VN assay, since the antibody titers presented here are much larger than those reported in the literature for the FHV-1 VN assay (Gore et al., 2006; Mouzin et al., 2004; Povey, 1979; Kruger et al., 1996; Willemse et al., 1996), and also differs from previous FHV-1 ELISAs (Lappin et al., 2002; Satoh et al., 1999) in that it could potentially detect almost any FHV-1 antibody. Most antibodies recognized the L class of FHV-1 proteins, consistent

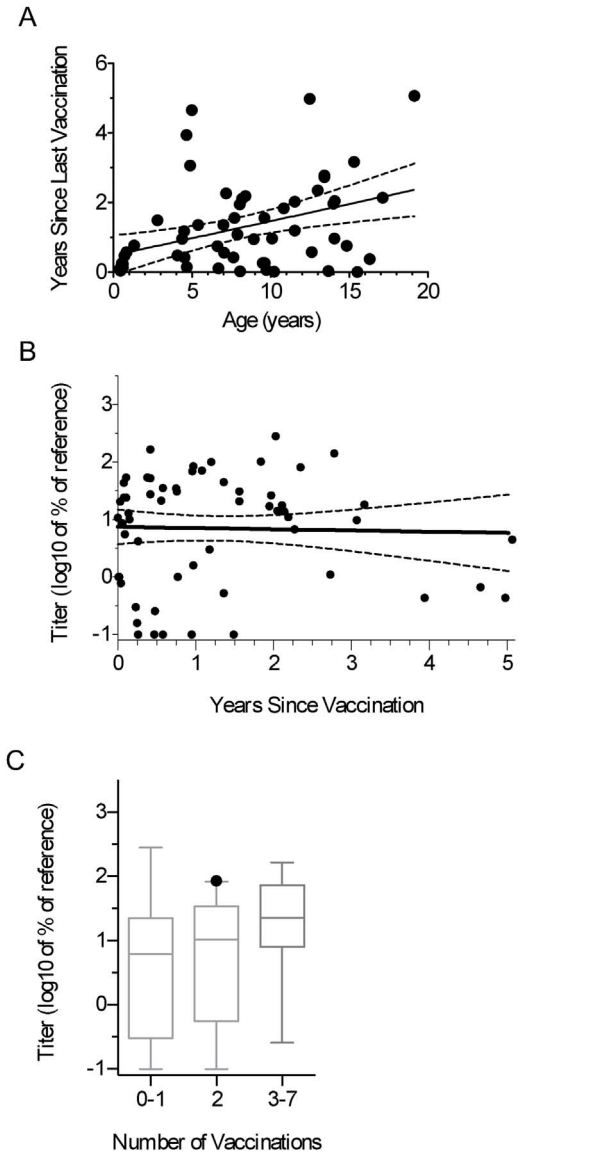


**Fig. 4.** Recent Vaccination Does Not Correlate with Increased FHV-1 Antibodies.**A**. The data in Table S1 were analyzed, with years since last vaccination plotted as a function of age. Solid and dashed lines indicate the linear regression and 95% confidence interval, respectively. $p < 0.01$; slope = 0.10 ± 0.03; $r^2 = 0.15$.**B.** The normalized serum FHV-1 antibody levels, from Table S1, were analyzed as a function of time since most recent vaccination. $p = 0.98$; $r^2 < 0.01$.**C.** The normalized serum FHV-1 antibody levels, from Table S1, were analyzed as a function of known vaccine history by ANOVA to determine if there was any relationship between the number of vaccinations and the FHV-1 antibody levels.

with human herpesvirus studies, where responses to structural glycoproteins, belonging to the L class, are common (Britt et al., 1990; Cairns et al., 2014). We also found that most serum antibodies were IgG, even though a mucosal pathogen such as FHV-1 might be expected to preferentially induce IgA. It may be relevant that commercial FHV-1 vaccines in the U.S. are not intranasal but instead parenteral, which may favor IgG.

But the primary findings of our cross-sectional study were that antibodies were higher in older cats, which was not due to more recent vaccination, and that the relationship between antibody levels and increased number of vaccinations was surprisingly modest. Including this study, there are now four studies examining adaptive immunity more than 2 years after vaccination with live-attenuated FHV-1 (Lappin et al., 2002; Gore et al., 2006; Mouzin et al., 2004). Two of these were challenge studies showing that live-attenuated FHV-1 offered significant protection against virulent FHV-1 challenge for at least 2.5 years (Lappin et al., 2002) or 3 years (Gore et al., 2006). Although neither study compared vaccine-induced protection in the first year after vaccination to protection multiple years after vaccination, three of these studies have now examined the antibody response to FHV-1 over a long period of time. In one study (Gore et al., 2006), FHV-1 VN titers peaked one month after vaccination, declined 6-fold within 9 months, then remained stable near the limit of detection through 3 years. In another study (Mouzin et al., 2004), client-owned cats vaccinated at least 1 year earlier were grouped according to the interval since most recent vaccination, from 12 months to 48 months. In this study, FHV-1 VN titers varied less than 2-fold between any time points (Mouzin et al., 2004). Our own study, using an FVH-1 ELISA rather than an FHV-1 VN assay, did not find a drop in FHV-1 antibodies within the first year of vaccination, or any time later, but instead found a slight increase. It is unclear whether the difference in FHV-1 antibody kinetics in the first year after vaccination is due to differences in the assay type (VN vs. ELISA), the animal populations (SPF cats in references 2 and 3 vs. client-owned cats in reference 4 and the present study) or vaccine formulation, but all of these studies are in agreement that more than 1 year after vaccination, there is durable maintenance of antibodies over the long term.

The basis of this very long-lived antibody response to FHV-1 is unknown. Many FHV-1 vaccines contain the live-attenuated FHV-1 F-2 vaccine strain (Bittle and Rubic, 1975), so one possibility is that the vaccine strain establishes asymptomatic latency that boosts antibodies through periodic reactivation or abortive reactivation. In support of this argument, the CD8 T cell response to a mouse herpesvirus, murine cytomegalovirus (MCMV), increases with time through a process referred to as memory inflation (Holtappels et al., 2002; Karrer et al., 2003; Munks et al., 2006). Furthermore, this memory inflation occurs even with extremely attenuated MCMV (Snyder et al., 2011), a situation analogous to the FHV-1 vaccine strain. In contrast, long-lived immunity also has been observed in humans vaccinated with the live smallpox vaccine (Hammarlund et al., 2003), a non-persisting virus. Thus it's unclear whether the FHV-1 antibody response is long-lived because the vaccine is live, because the virus persists, or both.

It has been noted that the FHV-1 F-2 vaccine strain is grown in a feline kidney cell line (CRFK), that most FHV-1 vaccine formulations do not remove CRFK cells from the vaccine (Whittemore et al., 2010), and that commercial FHV-1 vaccines induces antibodies specific for CRFK and feline renal cells (Whittemore et al., 2010; Lappin et al., 2005, 2006), which may contribute to renal disease in domestic cats. We tested antibody reactivity to uninfected CRFK cell lysates as a negative control in every experiment (data not shown). Although our ELISA was not designed to detect low levels of anti-CRFK antibodies, these responses were generally minimal and do not add additional support to this hypothesis.

The current recommendation is that after the primary FHV-1 series, cats at low risk should only be vaccinated every three years (Scherk et al., 2013). In conjunction with the studies described above, our data further support this recommendation and raise the possibility that even longer revaccination intervals may provide adequate protection against FHV-1.

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The funding sources had no involvement in the study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

## Conflicts of interest

The authors declare no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetimm.2017.05.002.

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**Supplemental Table S1**

| Subject | Sex | Age (years) | Documented Vaccinations | Years Since Vaccination | Titer (% of Subject 1) |
|---|---|---|---|---|---|
| 1 | F-S | 11.53 | 7 | 1.20 | 100.00 |
| 2 | M-N | 0.42 | 2 | 0.08 | 43.30 |
| 3 | M-N | 0.42 | 2 | 0.08 | 23.80 |
| 4 | M-N | 4.51 | 3 | 1.18 | 3.00 |
| 5 | M-N | 7.64 | 3 | 0.42 | 167.00 |
| 6 | M | 4.52 | 2 | 0.42 | 52.20 |
| 7 | M | 4.52 | 2 | 0.42 | 27.80 |
| 8 | F-S | 7.00 | | 1.36 | 44.20 |
| 9 | F-S | 18.13 | 0 | | 2.30 |
| 10 | M-N | 9.61 | | 1.56 | 30.90 |
| 11 | M-N | 4.99 | 2 | 4.66 | 0.66 |
| 12 | F-S | 17.13 | | 2.14 | 13.90 |
| 13 | M-N | 7.05 | 4 | 0.56 | 21.20 |
| 14 | F-S | 9.73 | 1 | 0.06 | 8.50 |
| 15 | F-S | 9.70 | 0 | | 11.80 |
| 16 | F-S | 8.03 | 3 | 1.95 | 16.90 |
| 17 | M-N | 9.51 | 1 | 0.26 | *0.10* |
| 18 | F-S | 0.54 | 2 | 0.25 | 0.16 |
| 19 | M-N | 0.72 | 2 | 0.47 | *0.10* |
| 20 | F-S | 6.62 | | | 0.38 |
| 21 | M-N | 4.64 | 1 | 3.94 | 0.44 |
| 22 | M-N | 8.99 | | | 14.80 |
| 23 | M-N | 2.80 | 1 | 1.49 | *0.10* |
| 24 | M-N | 13.35 | | | 104.60 |
| 25 | F-S | 13.99 | | 1.97 | 26.50 |
| 26 | F | 4.00 | 0 | | *0.10* |
| 27 | F | 0.44 | | 0.09 | 5.50 |
| 28 | F-S | | | | 72.60 |
| 29 | F-S | 15.30 | | 3.17 | 18.10 |
| 30 | F-S | 6.68 | 4 | 0.11 | 53.70 |
| 31 | M-N | 6.03 | | | 143.50 |
| 32 | M-N | 4.31 | | | 0.52 |
| 33 | F-S | 13.68 | 5 | 0.03 | 20.40 |
| 34 | M-N | 3.01 | | | 0.95 |
| 35 | M-N | 14.06 | 2 | 0.97 | 86.00 |
| 36 | F-S | 14.07 | 1 | 2.05 | 14.00 |
| 37 | F-S | 4.08 | 3 | 0.48 | 0.26 |
| 38 | M-N | 2.54 | | | 16.80 |
| 39 | F-S | 8.19 | 3 | 2.11 | 17.70 |
| 40 | M-N | 0.10 | | | 10.40 |
| 41 | M-N | 12.61 | 2 | 0.58 | 35.50 |
| 42 | F-S | 6.62 | 3 | 0.75 | 34.40 |
| 43 | M-N | 3.94 | | | 4.90 |
| 44 | F-S | 12.48 | | 4.98 | 0.44 |
| 45 | F-S | 4.83 | | | 34.20 |
| 46 | F-S | 11.54 | 1 | 2.03 | 282.40 |
| 47 | M-N | 3.00 | | | 2910.00 |
| 48 | F-S | 4.88 | 2 | 3.07 | 9.80 |
| 49 | F-S | 5.95 | | | 21.40 |
| 50 | F-S | 7.65 | | | 93.00 |
| 51 | M-N | 10.23 | | | 1.70 |
| 52 | F-S | 7.01 | | | *0.10* |
| 53 | F-S | | | | 1.10 |
| 54 | M-N | 14.05 | | | *0.10* |
| 55 | F-S | 5.39 | 2 | 1.36 | 0.53 |
| 56 | M-N | 6.11 | | | 72.00 |
| 57 | F-S | 8.94 | 1 | 0.95 | *0.10* |
| 58 | M-N | 4.00 | | | 67.30 |
| 59 | M-N | 7.16 | | | 2.20 |
| 60 | M | 17.78 | | | 154.90 |
| 61 | F-S | 9.93 | | | 91.10 |
| 62 | M-N | 0.84 | 2 | 0.58 | *0.10* |
| 63 | F-S | 20.87 | | | 2.20 |
| 64 | F-S | 8.41 | 2 | 2.19 | 10.90 |
| 65 | M-N | 7.70 | 1 | 1.56 | 20.90 |
| 66 | F-S | 12.60 | | | 13.20 |
| 67 | M | 0.43 | 3 | 0.11 | 23.90 |
| 68 | F | 0.49 | 0 | | 27.50 |
| 69 | F-S | 0.46 | 2 | 0.04 | 0.77 |
| 70 | M-N | 10.22 | | 0.01 | 1.00 |
| 71 | F-S | 6.84 | | | 14.50 |
| 72 | F-S | 7.17 | | 2.27 | 6.70 |
| 73 | F-S | 16.05 | | | 89.10 |
| 74 | M-N | 13.42 | 2 | 2.73 | 1.10 |
| 75 | F-S | 9.62 | 3 | 0.26 | 4.20 |
| 76 | F-S | 12.48 | | | 38.60 |
| 77 | F-S | 10.83 | 1 | 1.84 | 103.40 |
| 78 | M-N | 16.30 | 2 | 0.38 | 53.40 |
| 79 | M-N | 7.89 | 3 | 1.08 | 71.30 |
| 80 | M-N | 13.00 | 3 | 2.35 | 81.60 |
| 81 | M-N | 10.04 | | | 78.60 |
| 82 | M-N | 10.10 | 2 | 0.97 | 1.60 |
| 83 | F-S | 6.52 | | | 34.60 |
| 84 | F-S | 19.58 | | | 1.20 |
| 85 | M-N | 11.23 | | | 382.40 |
| 86 | M-N | 1.32 | 1 | 0.77 | 1.00 |
| 87 | M-N | 16.14 | | | 447.30 |
| 88 | M-N | 12.43 | | | 74.70 |
| 89 | F-S | 8.06 | 3 | 0.02 | 1.00 |
| 90 | F-S | 5.29 | | | 32.40 |
| 91 | F-S | 4.38 | 3 | 0.96 | 69.00 |
| 92 | M-N | 19.15 | 1 | 5.07 | 4.50 |
| 93 | F-S | 16.02 | | | 13.90 |
| 94 | M-N | 13.43 | 3 | 2.78 | 141.80 |
| 95 | M-N | 0.60 | 2 | 0.14 | 12.90 |
| 96 | M-N | 15.52 | | 0.00 | 10.60 |
| 97 | F-S | 0.60 | | | 0.60 |
| 98 | F-S | 0.60 | 2 | 0.23 | 0.30 |
| 99 | M-N | 4.68 | 3 | 0.15 | 10.00 |
| 100 | M-N | 14.85 | 2 | 0.76 | 31.00 |

Sex: F (female); F-S (female-spayed); M (male); M-N (male-neutered)
Total subjects (n=100); subjects with known or estimated age (n=98); subjects with documented vaccinations (n=59).

Antibody responses less than 0.1% of the reference sample were arbitrarily assigned a value of 0.1% to facilitate statistical analysis and allow graphing on a log scale (shown in *italics*).