

THESIS

SEROANALYSIS OF FELIS CATUS GAMMAHERPESVIRUS 1 INFECTION IN DOMESTIC CATS

Submitted by

Kathryn Stutzman-Rodriguez

Department of Microbiology, Immunology, and Pathology

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Master's Committee:

Advisor: Susan VandeWoude

Joel Rovnak

Dawn Duval

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ABSTRACT

SEROANALYSIS OF FELIS CATUS GAMMAHERPESVIRUS 1 INFECTION IN DOMESTIC CATS

We recently described a novel herpesvirus of domestic cats, *Felis catus* gammaherpesvirus 1 (FcaGHV1). FcaGHV1 is a member of the gammaherpesvirus subfamily, which also includes the human cancer-associated herpesviruses, Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). To determine FcaGHV1 antigens that elicit a detectable humoral-immune response in naturally-infected domestic cats, I chose to evaluate seven FcaGHV1 proteins. These proteins are conserved across the subfamily and antigenic in other gammaherpesvirus infections. I amplified and cloned each of the seven FcaGHV1 genes into a mammalian expression vector and transfected intact clones into Crandell Rees feline kidney (CRFK) cells. I developed an immunofluorescent antibody test using transfected cells exposed to sera from nine shelter cats diagnosed as FcaGHV1-positive by quantitative PCR (qPCR) of blood-cell DNA. This analysis indicated that tegument proteins ORF52 and ORF38 reacted most consistently with serum from cats with positive FcaGHV1-qPCR reactions. Based on these results, recombinant antigens were used to develop two optimized indirect ELISAs. Genes for ORF52 and ORF38 were cloned into a mammalian expression vector. Antigens were produced in a transient transfection system and purified using immunoprecipitation. Indirect ELISA conditions were optimized using known positive and negative controls. Using the two optimized ELISAs, I screened sera from 133 shelter cats that had been previously tested by FcaGHV1-qPCR. Seroprevalence of FcaGHV1 reactive antibodies was 32%, compared to the

previously published 16% prevalence evaluated by qPCR. Nineteen of twenty qPCR positive cats were also seroreactive against one or both antigens on ELISA. Sera from 24 cats were seropositive based upon ELISA testing, but negative using qPCR analysis. Risk factors identified in previous publications were confirmed by ELISA, namely geographic location, male sex, adult age, and association of FcaGHV1 with several co-infections. Based on our knowledge of gammaherpesvirus latency, this ELISA provides evidence of viral exposure, while qPCR viral DNA detection likely represents reactivation from latency or primary infection. The addition of serologic analysis as a measurement of FcaGHV1 exposure will aid in determining association of FcaGHV1 with disease and routes of transmission.

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TABLE OF CONTENTS

Abstract.....	ii
Acknowledgements.....	iv
Chapter 1: Overview of study.....	1
Introduction.....	1
Specific aims.....	2
Chapter 2: Seroanalysis of <i>Felis catus</i> gammaherpesvirus 1 infection in domestic cats.....	5
2.1 Introduction.....	5
2.2 Materials and methods.....	8
2.3 Results.....	18
2.4 Discussion.....	33
Chapter 3: Conclusion.....	43
References.....	45

CHAPTER 1: OVERVIEW OF STUDY

The herpesviruses have 3 subfamilies: alpha, beta, and gamma. Alphaherpesviruses have more notoriety, causing mild to moderate disease and with potential to cause severe disease in the face of immune suppression^{2,3}. Many alphaherpesviruses also establish latency in sensory ganglia³. The beta and gammaherpes viruses are not as well characterized; however they can still be powerful agents of disease^{4,5}. The gammaherpesviruses are known for being highly host specific and often target B or T lymphocytes³. Latent gammaherpesvirus is commonly found in lymphoid tissue^{3,4}.

Epstein-Barr virus (EBV) and Kaposi's sarcoma associated herpes virus (KSHV) are examples of human gammaherpesviruses^{3,6,7}. EBV causes mononucleosis upon initial infection and retreats into latency^{3,7}. Reactivation is associated with immunosuppression such as HIV infection^{3,4}. At this stage, EBV is associated with a range of lymphoproliferative disorders^{3,7,8}. Not as much is known about KSHV pathogenesis. KSHV causes mild disease, if at all, during primary infection⁹; instead it seems to require a cofactor to develop disease¹⁰. Immune suppression appears to play a role as cofactor; under these circumstances KSHV causes Kaposi sarcoma^{6,10,11}, primary effusion lymphoma¹², multicentric Castleman's disease, and other non-Hodgkin lymphomas¹³.

Prior to the discovery of *Felis catus* gammaherpesvirus 1 (FcaGHV1), there were no described gammaherpesviruses of the domestic cat. There is an available real time PCR (qPCR) assay that quantitates FcaGHV1 viral DNA in whole blood samples¹. This assay aided

identification of risk factors for FcaGHV1 infection. However, many questions remained unanswered about virus transmission, and disease association that could be addressed by development of serodiagnostics to detect viral exposure and test associations with disease states.

Studies of antigens for EBV and KSHV have revealed a variety of antigens with a varied response¹⁴⁻¹⁷. In one KSHV study to evaluate seroprevalence of patients with KS, western blot was conducted for a variety of KSHV antigens as a prelude to ELISA development. This study led to successful creation of a sensitive KS ELISA, and discovered a varied serologic response to the ELISAs they created for 5 antigens¹⁴.

Thus for my thesis, I sought to develop a high-throughput, sensitive, and specific serologic assay that was capable of detecting antibodies to FcaGHV1. During herpes viral latency, intact viral genomes are maintained in certain host cells, even though the virus is not actively replicating¹⁸. For several gammaherpesviruses including EBV, qPCR of peripheral blood is used to quantitate viral load and document viral activation. EBV negative qPCR assay indicates latency or no infection¹⁹. My hypothesis is that an FcaGHV1 antibody detection assay will show a higher disease prevalence in the cat population than the qPCR assay, because it will be able to detect cats infected with latent FcaGHV1 as well as cats with actively replicating virus. Secondly, qPCR positive cats should also be seropositive, indicating that animals are mounting an active humoral immune response to FcaGHV1 infection.

My specific aims and hypotheses were as follows:

Aim 1: To identify FcaGHV1 antigens that elicit a humoral antibody response in naturally infected domestic cats

Hypothesis: FcaGHV1 antigens that elicit a detectable immune response are analogues of immunodominant antigens identified in gammaherpesvirus infections of other species.

Approach: Select genes from FcaGHV1 partially annotated genome based on homology to KSHV, EBV, and OvHV-2. Clone selected genes into a mammalian expression vector containing a human influenza hemagglutinin (HA) tag. Verify sequence of the clone, and transfect Crandell Rees feline kidney cell line (CRFK). Develop an immunofluorescence antigen screening assay (IFA). Positive controls include anti-HA antibody to confirm transfection, and cells transfected with the FIV capsid antigen tested with serum from an FIV-positive cat. Negative controls include serum from naïve, specific pathogen-free (SPF) cats, vector-only control transfections, and untransfected cell controls. Then, I will test serum from 9 cats that were FcaGHV1 positive by qPCR to validate the IFA and identify FcaGHV1 antigens that react to cat serum in a sensitive and specific way. IFA antibody response will be additionally validated by western blot using transfected cell lysates as immobilized antigen.

Aim 2: To design a sensitive, specific, reproducible serologic assay using one or more FcaGHV1 antigens.

Hypothesis: Immunodominant proteins of FcaGHV1 can be used to develop a serologic assay for testing feline seroprevalence to FcaGHV1.

Approach: Express selected proteins in 293T cells using the similar methods employed during IFA development. Purify FcaGHV1 antigens using magnetic anti-HA immunoprecipitation.

Develop an indirect immunoassay to evaluate antibody reactivity in domestic cat serum banks to one or more FcaGHV1 antigens. Establish controls and optimize assays.

Aim 3: To compare FcaGHV1 prevalence in selected cat cohorts using qPCR and serologic assay.

Hypothesis: FcaGHV1 serologic assay will reveal a higher FcaGHV1 prevalence than detected using qPCR, and qPCR positivity will be highly correlated with seropositivity.

Approach: Test serum samples from 133 cats from three geographic areas of the US with known FcaGHV1 qPCR status. Compare the results of the qPCR assay and serologic assay using appropriate statistical comparisons. Analyze and compare results of the two assays along with predictor variables: sex, age, geographic location, and co-infection status using appropriate statistical tests to provide further depth of knowledge about these previously identified risk factors.

CHAPTER 2: SEROANALYSIS OF FELIS CATUS GAMMAHERPESVIRUS 1 INFECTION IN DOMESTIC CATS

INTRODUCTION

Felis catus gammaherpesvirus 1 (FcaGHV1) was recently discovered in the domestic cat and has a worldwide distribution¹. While only preliminary information has been established relating FcaGHV1 infection to demographic factors, other species of gammaherpesvirus (GHV) subfamily of Herpesviridae have been studied in-depth. Most GHVs are highly specific to their host species^{20,21} making it especially challenging to learn more about human GHVs. Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpes virus (KSHV) are human GHVs that pose important health risks. Diseases of EBV, KSHV and other GHVs appear to be more severe in immunocompromised individuals^{4,10,18}. For example, KSHV causes primary effusion lymphoma (PEL) in immune suppressed patients^{22,23}. PEL is a serious condition with median survival of 6 months even in patients undergoing currently accepted therapies²⁴. Further research on FcaGHV1 will not only benefit cat health, but could also provide a model of naturally occurring infection for better understanding of EBV and KSHV infection in humans.

All known herpes viruses establish a latent, life-long infection¹⁸. GHVs often cause lymphoproliferative disease as well as non-lymphoid cancers with re-activation from latency^{8,18}. For example, EBV often manifests as infectious mononucleosis upon initial infection, then persists in memory B-cells as a latent infection for the life of the individual^{18,7}. When the immune system is suppressed by old age, transplant surgery, or other infections, EBV can re-activate. EBV is strongly associated with Hodgkin lymphoma, non-Hodgkin lymphoma, Burkitt

lymphoma, B-cell lymphoma of immunosuppression, and nasopharyngeal carcinoma⁷. In contrast to EBV, initial infection of KSHV often does not cause disease⁹. However, it is considered a necessary factor in the direct cause of several diseases: Kaposi's sarcoma, PEL, and multicentric Castleman's disease in immune suppressed patients^{9,22,23}.

Herpes virion structure is similar across the family. Virion diameter ranges from 120-300nm. It consists of a DNA core, followed by a capsid layer, tegument layer and envelope. The core of the herpesvirus is usually torus-shaped.³ The capsid has 162 capsomers including both pentameric and hexameric capsomers. The tegument is an ordered structure between the capsid and envelope. Layer thickness may vary across the virion and from one particle to another. Herpes viral envelopes are trilaminar with numerous short glycoprotein protrusions.³

The relationship of FIV and FcaGHV1 may provide a parallel opportunity to study HIV and human GHVs²⁵. FIV is already an established model for HIV because of the disease similarities^{26,27,28}. Patients co-infected with AIDS and either KSHV or EBV are at a greater risk of developing disorders related to these gammaherpesviruses^{4,10,29}. Treatment for GHV-related lymphoproliferative disease provides many challenges for the AIDS patient¹⁰. There is a striking similarity between viral loads in EBV/HIV co-infection patients and cats with FcaGHV1/FIV co-infection. Cats that are co-infected with FIV and FcaGHV1 have higher viral loads of FcaGHV1²⁵. This pattern closely resembles HIV and EBV co-infection²¹. Human viruses of the gammaherpesvirus subfamily are difficult to study because of the wide range of proteins that are expressed during lytic and latent phases of viral infection^{11,30}. Small animal model limitations have compounded this problem¹¹. There is a clear deficit of EBV animal models and

a call for more work in this area ³¹. KSHV *in vivo* models are arguably even less robust¹⁰. Much of the current knowledge base on EBV from studying murine herpes virus 68 (MHV-68)³². One of the major obstacles to progress in the field using this model is that the virus biology for MHV-68 is in some ways more similar to the alphaherpesviruses ³². The humanized mouse model is promising, but it remains to be seen whether this model can truly address virus pathology given the significant physiologic differences between mice and humans^{33,34}. Using FcaGHV1 in cats to model this relationship could provide a unique, cost-effective opportunity to explore the relationship between HIV and EBV in a host that is genetically more closely related to humans, affords a greater opportunity to assess temporal disease, and is easily maintained. However, additional diagnostics are needed to explore this potential model of disease.

Our lab developed a real time PCR (qPCR) assay that detected FcaGHV1 in 16% of US shelter cats¹ and domestic cats infected with FcaGHV1 across three continents²⁵. Results of our qPCR assay revealed several risk factors for FcaGHV1, including being male, adult, and having evidence of co-pathogens. This suggests aggressive male encounters as a potential factor for FcaGHV1 transmission. Older age and a veterinary evaluation of “poor health” were also associated with FcaGHV1^{25,1}. The relationship to FcaGHV1 infection and co-infection status potentially implicates reactivation of FcaGHV1 due to immune suppression.

We hypothesized that the measure of prevalence from our qPCR assay underestimates exposure to FcaGHV1 by not accounting for animals with latent infection. FcaGHV1 qPCR measures peripheral FcaGHV1 DNA viral load which is a measure of active viral replication and, in gammaherpesviruses, can be a good marker of primary infection or re-activation. An ELISA can assess humoral antibody status, which can indicate exposure but not necessarily an active

infection. Due to similarities within the GHV subfamily, it is likely that FcaGHV1 viral-load variation mimics patterns of EBV variation. If so, asymptomatic cats infected with FcaGHV1 would be seropositive but would have low or undetectable viral loads¹⁹. The serologic assays we developed in this project helped to resolve this question of exposure rate.

Here, we developed an indirect ELISA to answer the question of feline exposure to FcaGHV1. We evaluated serum from 133 shelter cats that had previously been tested for FcaGHV1 by blood-viral load qPCR^{1,25}. We compared the data we have on age, sex, and co-infection status to evaluate differences between qPCR results and seroprevalence. Results identified seroprevalence as a potential measure of FcaGHV1 exposure and qPCR as a measure for active FcaGHV1 infection. This information provides a valuable contribution to the growing knowledge of FcaGHV1 and further information about its potential as a model of human GHVs.

MATERIALS AND METHODS

Gene selection for identification of immunodominant antigens:

In a corollary study, our laboratory has obtained partial FcaGHV1 genome data to map FcaGHV1 genes to other GHVs genes (unpublished data). We selected 7 conserved genes from this dataset to evaluate for potential to elicit humoral immunity in naturally infected cats. These included ORF38, ORF42, ORF59, ORF26, ORF52, ORF17.5, and ORF65 which code for proteins analogous to antigenic proteins of KSHV, EBV, and OvHV-2. All 7 proteins are virion-associated (Table 1). The initial list of genes also included ORF8 (glycoprotein B). The glycoproteins are classic antibody targets because of their location on the outside of the virion³.

Attempts at cloning ORF8 provided quite a few technical challenges and initially were all unsuccessful. We postponed work with ORF8 in the interest of the time frame of this study.

Table 1. FcaGHV1 protein analogs are antigenic in other gammaherpesviruses. Malignant catarrhal fever-causing viruses (MCFVs) include Ovine herpes virus 2 and Alcelaphine herpes virus 1. EBV has distinct gene nomenclature listed in parentheses.

Gene	Type of protein	Analog to protein from	Possible function
ORF17.5	Scaffold protein	MCFVs ³⁵	Involved in capsid assembly ³⁶
ORF38	Tegument	KSHV ¹⁷	Support virion maturation in the cytoplasm ³⁷
ORF52	Tegument	EBV (BLRF2) ^{38,39}	Assist virion egress and secondary envelopment ³⁸
ORF65	Capsid	EBV (BFRF3) ³⁹ , KSHV ^{14,40} , MCFVs ³⁵	Small capsid protein ⁴¹ , late gene ⁴²
ORF26	Capsid	EBV (BDLF1) ⁴³	Capsid protein ⁴¹ , late gene ⁴²
ORF59	Phosphoprotein	KSHV ¹⁴	Binds DNA polymerase and dsDNA to promote DNA synthesis by acting as a sliding clamp ⁴⁴
ORF42	Tegument	EBV (BBRF2) ⁴⁵	May contribute to the regulation of mitochondrial function ⁴⁶

Plasmids

Specific primers that incorporated restriction sites were designed for each gene of interest based on our FcaGHV1 genome map (Table 2). All genes were PCR-amplified using a High Fidelity Platinum Taq PCR protocol from template DNA extracted from a cat infected with FcaGHV1 of confirmed sequence. PCR products were run on a 1% agarose gel to confirm expected nucleotide length. Bands were excised and DNA extracted using the manufacturer's standard protocol with a Qiagen gel extraction kit. Concentration of amplicon DNA was evaluated with a NanoDrop spectrophotometer.

Reactions for restriction digest of amplicons were designed exactly as outlined by the New England Biolab NEBcloner online protocol development tool for digests in 50 μ L reactions. BamHI HF and EcoRI HF were used to cut all gene amplicons and the pKH3 vector except ORF17.5, BclI was used in place of BamHI for ORF17.5 restriction digest and the coordinating vector digest. After digest, the reactions were run on a 1% agarose gel and bands of the expected nucleotide length were extracted and purified with the Qiagen gel extraction kit. The NanoDrop spectrophotometer was again used to evaluate concentration.

These restriction enzyme digested amplicons were then ligated into the pKH3 vector using corresponding cloning sites and New England Biolab T4 DNA ligase and coordinating manufacturer protocol for cohesive ends. Reactions were each 20 μ L and ligation performed using a molar ratio of 1:3, vector to insert. The pKH3 vector which contains an HA tag, was a gift from Joel Rovnak and Sandra Quackenbush.

Plasmid constructs were transformed in OneShot Chemically Competent cells using suggested protocols by the manufacturer. Ten colonies from each transformation were screened by PCR targeting each insert and products were run on a gel to ensure that the insert was the correct nucleotide length. Three colonies of appropriate insert size were selected to propagate overnight in Lysogeny broth (LB) + ampicillin. Inserts from selected colonies were sequenced to confirm identity at Colorado State University Proteomics and Metabolics Facility. One clone with perfect sequence was selected for each gene insert. A glycerol stock was created for the selected colony for long term storage. Plasmid was purified from this selected colony using the Qiagen plasmid mini and midi spin prep protocols.

The FIV Gag capsid protein (p27), used as a control for the immunofluorescence assay, was acquired from a previous study⁴⁷. It had been cloned into a pGEX2T vector with the same reading frame as pKH3. Restriction digest was performed with the FIVCA-pGEX2T using BamHI HF and EcoRI HF with the same protocols as described earlier for the other constructs. The product was run on a 1% agarose gel to select the correct insert size and purified using the Qiagen gel purification kit. All the following steps described earlier were performed identically to the other constructs, that is: ligation of FIVCA into pKH3, transformation, storage, and plasmid purification.

Table 2. Primers used for assay development. Open reading frame (ORF) primers were used to amplify the respective FcaGHV1 genes. The pKH3 primers were used to confirm sequence of plasmid constructs.

Gene	Primers
ORF17.5	5'- AGACGCTGATCAATGGCCACTAATGCCAG -3' 5'- CCTGAATTCCTATTAGTTTTTTAGAAAGTTCTTCACAAAATA -3'
ORF38	5'- CGCGGATCCATGGGTATCATCTGCTCTATTT -3' 5'- CCGGAATTCCTATCAGATACTAAATACAACCTTTCTTTTTTATC -3'
ORF52	5'- CGCGGATCCATGGCTTCTAAAAAAGGAACACC -3' 5'- CCTGAATTCCTATTAGGTTGGTTGTTTGGATCTAC -3'
ORF65	5'- CGCGGATCCATGAGTAGGGAACAGTTAAGAG -3' 5'- CCGGAATTCCTATTATTTTTTTTTGCTTCCACTAGTTG -3'
ORF26	5'- CGCGGATCCATGCAAGTTGATAAAAAATCATAGTC -3' 5'- CCGGAATTCCTACTAGATGGACAACCAGCCT -3'
ORF59	5'- CGCGGATCCATGGAAGCTAAAATTACTACTACTA -3' 5'- CCGGAATTCCTATTATGTTGTAATCTTGAATGTTTTG -3'
ORF42	5'- CGCGGATCCATGGACTCTGTAATCCGAACC -3' 5'- CCTGAATTCCTATTATGAAAAGATTTGTACTCTAGGCTT -3'
pKH3	5'- CAACTGCACCTCGGTTCTA -3' 5'- CCATTATAAGCTGCAATAAACAAGT -3'

Immunofluorescence assay

CRFK cell cultures were maintained with CRFK media made from low glucose DMEM + glutamax (Life Technologies brand), with added 10% fetal bovine serum (FBS), 1% sodium

bicarbonate, 1% Penicillin/Streptomycin (Pen-strep) and 1% non-essential amino acids. For additional culture maintenance, they were passaged when confluent (bi-weekly) using trypsin.

For the immunofluorescence assay (IFA), CRFKs were grown on glass slides with twelve 0.4mm wells with the CRFK media mentioned earlier except for no added antibiotic. CRFKs were plated at 5,000 cells per well and incubated for 2 days to allow adherence to the slide. Slides were housed in petri dishes with sterile water to maintain humidity. Transfections with plasmid constructs were performed using Lipofectamine 2000 protocols in 0.4mm well, using 0.15 μ L Lipofectamine and 60ng plasmid per well. Cells were incubated for 21 hours. This time length was selected as optimal for protein expression of FIVCA. This time was kept the same for all transfections and no further experiments were done to optimize time length to specific genes used in the transfection.

After incubation, slides were gently washed with PBS to remove excess media. Cells were then fixed using 2% paraformaldehyde and then 50:50 ethanol/methanol followed by air-drying. The experimental serum used was obtained from nine cats sampled at shelters in CO, CA, or FL previously shown to have high peripheral FcaGHV1 DNA viral loads¹. Each set of cells transfected to produce the selected proteins were tested with serum from all nine FcaGHV1-positive cats. Transfected cells were also tested with negative control serum from three specific pathogen-free (SPF) cats from the CSU SPF retrovirus research cat colony. This colony has been extensively screened and shown to be FcaGHV1 negative. An FIV-antigen capsid protein was used to transfect CRFK cells as described above; exposure to banked FIV-positive serum was used to demonstrate successful transfection and appropriate IFA parameters. Additionally, to

test for protein expression by evaluating the presence of the N-terminal HA tag included in each construct, cells were incubated with Covance rabbit-anti-HA at a 1:500 dilution. Slides were incubated for 1hr at 37C with primary antibodies followed by three more washes with PBS in preparation for secondary antibody exposure.

The secondary antibody used for the rabbit-anti-HA was Covance anti-rabbit-IgG conjugated to fluorescein isothiocyanate (FITC) diluted to 1:500. Secondary antibody to detect feline serum antibodies (Covance anti-cat-IgG FITC) was used at 1:50 and incubated 1hr at 37C followed by 3 PBS washes and a 2 min 4', 6-diamidino-2-phenylindole (DAPI) nuclear stain. All sera described earlier and commercial antibody was diluted in 2% bovine serum albumin (BSA) in PBS. All sera were diluted at 1:20 in BSA/PBS and commercial antibodies at the listed ratios in BSA/PBS. Slide covers were sealed with ProLong Gold Antifade prior to light microscopic viewing (with an Olympus BX60) for immunofluorescence.

Positive results were recorded if there was at least one cell per well that had a stronger immunofluorescence than the strongest background visible on the (negative) wells exposed to SPF naïve serum. This immunofluorescence was also compared to surrounding negative cells within the well when evaluating positive versus negative. Images were recorded with the microscope-connected Olympus DD71 digital capture system. Uniform adjustments were made with Photoshop to improve overall brightness of images for presentation.

Western blot for confirmation of immunodominant antigens

Immunoblot analysis was performed to confirm IFA observations on two candidate antigens demonstrating the most consistent immunofluorescence against qPCR positive

FcaGHV1 cat sera. ORF38 and ORF52 were semi-purified from cell lysates (transfection conditions using Lipofectamine 2000 described earlier scaled-up for 6-well tissue culture plates) using the Pierce magnetic anti-HA IP/ co-IP kit and following manufacturer instructions. After immunoprecipitation, protein concentration was determined using the Pierce BCA kit. NuPage 1.5mm, 4-12% Bis-Tris 10 well gels were used in the NuPage gel box with associated 20x NuPage MES Running Buffer (1M Tris-Base, 1M MES, 2.0%SDS, 2mM EDTA) diluted to 1x working buffer. The power supply was set at 160V for 40min. For each protein being evaluated, 1µg of protein per well was used, with enough wells to evaluate the different samples. The Bio-Rad Precision Plus Blue and Magic Mark standards were run for each gel.

Gels were transferred to PVDF membrane using 1x working stock of the 20x NuPage transfer buffer with added 10% methanol. The gel and PVDF membrane were sandwiched between 9 layers of blotting paper. Transfers were done in a Trans-Blot Turbo Transfer System at 15V for 30min. The PVDF membrane was blocked for 1 hour with 5% non-fat dry milk protein in PBS, and then cut into strips for exposure to serum from 9 qPCR positive cats and 3 SPF naïve cats at 1:20 dilution in PBS for 1 hour. The strips were then washed in PBS plus 2% tween (PBST). The strips were incubated with goat anti-cat phosphatase labeled IgG for 1 hour at 1:2000 in PBS. After an additional wash, strips were incubated with BCIP-NBT phosphatase substrate to visualize bands.

To ensure the correct protein was on the membrane, one strip with each protein was incubated with Covance HA.11 mouse anti-HA antibody at 1:5000 and secondary goat anti-mouse horse radish peroxidase (HRP) at 1:10000. Dilutions were in PBS and washes were

performed as described earlier with serum incubated strips. Equal amounts of peroxide solution and luminol (Millipore Immobilon Western HRP substrates) were applied to the strip and then visualized using chemiluminescent detection imaging (ImageQuant LAS 4000).

Protein expression for ELISAs

ORF 38 and ORF52 were chosen for larger-scale production in 293T cells. 293T cells were maintained in 293T media (high glucose DMEM with added 2% Glutamax, 10% FBS, and 1% Pen/Strep). Cells were maintained by passaging when confluent on a biweekly basis.

Eight million 293T cells were plated onto 100mm tissue culture plates in antibiotic free media and incubated overnight at 37C. Plasmid constructs that were developed for the IFA were transfected the following day with 45µL Lipofectamine 2000 and 18µg plasmid per plate for ORF52 and 60µL Lipofectamine 2000 and 15µg plasmid for ORF38. Optimem was used in the quantities recommended by Lipofectamine 2000 manufacturer protocols. Transfection length was found to be optimal at 20 hours. Cells were harvested and purified using the Pierce anti-HA magnetic bead kit and protein concentration determined by BCA assay, as described earlier, and stored at 4C.

ELISAs

ELISAs were performed in 96-well plates. Each protein was diluted in a 50mM carbonate buffer, 40ng ORF52 in 100µL buffer per well and 100ng ORF38 in 100µL buffer per well. Plates were incubated overnight at 4C. Contents were discarded and 300µL of 2% BSA in imidazole-buffered saline (IBS) was used to block plates for 2 hours at room temperature. Contents of

block were discarded and wells were incubated at room temperature for 2-hours with 100 μ L serum per well, diluted 1:100 in ELISA diluent made with IBS. Plates were washed 5 times in a plate washer with IBS + 0.2% Tween (IBST).

The secondary incubation was 1-hour at room temperature with 5% mouse sera, ELISA diluent and 1:5000 Cappel goat anti-cat IgG peroxidase conjugate. Wells were again washed in a plate washer 5 times with IBST. TMB-peroxidase detection solution was added to each well (100 μ L) and incubated for 10 min at room temperature. The reaction was stopped using 50 μ L per well of 2.5N H₂SO₄. Absorbances were read at 450nm. Serum from each cat and controls were run in triplicate. Controls run on every plate included serum from 3 different SPF naïve cats, no-antigen control wells, and diluent-only wells.

Optimization was performed to identify the most appropriate dilutions of feline serum, secondary antibody, and concentration of coated antigen. Feline serum from an FcaGHV1 qPCR-positive animal was used for the optimizations. A range of dilutions were initially attempted above and below the ones ultimately selected to identify the largest signal-to-background ratio. Only in the case of the ORF38, a coating concentration was selected that was suboptimal. This compromise was made because of the low protein yield from ORF38 transfections. All other dilutions were selected to have the highest signal-to-background ratio.

Prior to initiation of sample testing each ELISA was tested with serum from 10 SPF naïve cats to ensure that there was no non-specific reaction occurring. Positive threshold was evaluated on a plate by plate basis: the mean absorbance of the replicates of the 3 SPF naïve cats was calculated with the addition of 3 standard deviations. Additionally, if this calculation

yielded a number <0.2 , then 0.2 was considered the threshold for positive for that 96-well plate.

Collection of samples

Domestic cat blood samples were obtained from archived samples of ostensibly healthy animals. These samples were taken from cats upon admission to shelters Florida, California, and Colorado as previously described¹. FcaGHV1 qPCR assay prevalence was performed on DNA extracted from blood cells as previously described¹. Demographic data were recorded for each cat, also described previously^{1,25,48}. Each cat was also previously evaluated for Calicivirus, Feline herpesvirus-1 (FHV1), *Mycoplasma spp.*, *Bartonella spp.* and FIV. Calicivirus and FHV1 were evaluated by oral swab PCR, *Bartonella spp.* was tested both by IgG and PCR. *Mycoplasma spp.* was evaluated using PCR and FIV was by presence of antibody^{1,25,48}.

Statistical analysis

Logistic regression was used to make statistical analysis of geographic data and FcaGHV1 qPCR or ELISA result. Shelter and state were each modeled as categorical independent-variables along with sex and age as binary co-variables. In each model, FcaGHV1 ELISA result or qPCR result was the dependent (response) variable. Binary logistic regression was used to model each co-infection. FcaGHV1 ELISA or qPCR result was the binary dependent-variable and the co-infection, sex, and age as independent binary co-variables. For sex and age, male was recorded as 1 and female as 0, similarly adult as 1 and young as 0. Odds ratios were calculated within the respective logistic models. Linear regression was used to evaluate correlation of absorbance and qPCR viral load. Logistic and linear regression modeling were performed in SAS.

Sensitivity and specificity was calculated using modeling techniques described by Liu et al⁴⁹. This Bayesian model requires a gold standard test used to evaluate only the positive responses from two dependent screening tests along with positive and negative results from those assays. This was adapted to our study by considering the two ELISAs (ORF38 and ORF52) to be the dependent assays. The qPCR assay was considered a gold standard only for the sake of specificity (100%). As with the model designed by Liu et al., two sensitivities were calculated for the ELISAs based on the association to qPCR specificity, only cats testing positive on qPCR were considered in this evaluation. This information was then used as informative priors for a second model to calculate specificity and sensitivity for each ELISA assay as well as prevalence. We used the open access WinBugs program written by Liu et al. to make these calculations⁴⁹.

Linear regression models were also created in SAS to evaluate the relationship between mean triplicate absorbances of qPCR-positive cats and their measured FcaGHV1 viral load. Separate analyses were done for ORF38 ELISA results and ORF52 ELISA. Viral load was modeled as the dependent variable (y). One cat with a viral load greater than 4 standard deviations outside the mean was removed from these analyses. T-tests were used to evaluate the relationship of the variables.

A calculation of the rate of animals infected per year was performed similar to an incidence calculation. The following equation was used: $100 \times (\text{\#qPCR positive cats} / \text{time span of captures in years}/n)$.

RESULTS

Expression plasmid development and controls

Seven FcaGHV1 genes were selected from the FcaGHV1 genome (unpublished data) based on genome alignment to determine homologous genes that code for antigens of KSHV, EBV and malignant catarrhal fever causing viruses (MCFVs) (Table 1). Successful cloning of predicted targeted amplicons was verified by sequencing each construct. The pKH3 vector used for all plasmid constructs contains an HA tag that was expressed at the N-terminus of the protein of interest. The HA tag was used as a control measure to evaluate protein expression in CRFK cells with both IFA and western blot (Figs. 1, 2). As a positive control, we transfected CRFK cells with the antigenic, FIV-capsid protein (FIVCA) and exposed antigen to FIV-positive serum in both the IFA (Fig.1) and western blot (data not shown). FIVCA was inserted in the same pKH3 vector and treated under the same conditions as other proteins. Quantity of DNA, quantity of transfection reagent, and incubation time for transfections used to produce all proteins in both IFA and western blot were optimized to yield the highest quantity of FIVCA protein.

IFA

To determine which proteins of FcaGHV1 elicited IgG antibody during a natural infection, we developed an immunofluorescence assay to screen and evaluate seven FcaGHV1 proteins. Cells were fixed with paraformaldehyde and methanol/acetone. This exposed the transfected intracellular proteins to subsequently added antibodies. All CRFKs were also stained with DAPI. We were able to visualize immunofluorescent cells for all protein transfections after incubation with FITC-tagged anti-HA antibodies (Figs. 1, S1). All cells exposed to cat sera were subsequently incubated with an anti-cat-IgG FITC to visualize immunofluorescence. We

confirmed assay controls: FIVCA transfections incubated with FIV-positive serum antibodies and negative control SPF cat serum (Fig 1).

We screened each of the seven FcaGHV1 antigens following transfection with serum from nine FcaGHV1-positive cats with high peripheral-DNA viral loads (Tables 3, S1, Figs. 1, S1). We assumed these animals would have detectable antibodies against FcaGHV1 antigens. ORF38, ORF65, ORF17.5, and ORF52 all had measurable serum antibody reactivity against one or more of the FcaGHV1-positive cat sera (Tables 3, S1, Fig. 1). ORF38 and ORF52 antigens reacted against more individual FcaGHV1 positive cat samples (Table 3, S1). No immunofluorescent antibody response was detected against ORF26, ORF59, or ORF42 (Table S1, Fig. S1). ORF59, analogue to a phosphoprotein that assists DNA polymerase⁴⁴, was the only protein that localized only to the nucleus. All other proteins had visible fluorescence in the cytoplasm after anti-HA staining (Figs. 1, S1).

Western blot analysis

Western blots were performed with crude lysate for all proteins screened on IFA: ORF42, ORF26, ORF52, ORF65, ORF17.5, ORF38, and ORF59. However, background was too high to make conclusions except for FIVCA reactivity with FIV+ serum (data not shown). Figure S2 shows a comparison of ORF38 crude lysate and semi-purified western blots (remaining crude lysate western blots are not shown). We then confirmed IFA results for ORF52, ORF38, and ORF65 via western blot by immobilizing semi-purified viral antigens versus sera from the same 9 FcaGHV1 positive individuals (Fig. 2, Table 3, S1). Serum from 3 SPF cats was run against all proteins and was consistently negative (Fig. 2).

Indirect ELISA optimization

Given preliminary seroreactivity against ORF38 and ORF 52, we proceeded to develop two indirect ELISAs with these antigens. We scaled-up production of protein in 293T cells using similar techniques employed for CRFK transfection in IFA and western blot. ORF52 had a consistently higher protein yield after purification of approximately 0.5µg/million cells transfected. Despite transfection optimization experiments, maximal ORF38 yield was 0.2µg protein/million cells transfected.

Both ORF38 ELISA and ORF52 ELISA were screened with 10 SPF naïve cats revealing all negative results. Additionally every 96-well plate included 3 SPF naïve cats to define threshold calculations. Triplicate average absorbance for SPF cats ranged from 0.06-0.18 units for ORF52 and 0.07-0.12 units for ORF38. Since some SPF cat absorbances occasionally had sporadic higher or lower results, threshold minimum was established at 0.2 absorbance units for both ORF38 and ORF52 ELISAs to ensure specificity. However, if the average plus 3 standard deviations of the SPF cat absorbances on a particular plate was higher than 0.2, then this became the established threshold for the plate. All three triplicates had to be higher than the plate threshold to be considered positive. We tested serum from 9 cats evaluated on IFA and western blot (Table 3) during assay development. Once assays were optimized, we repeated ELISA on serum from these cats and additional animals representing shelter animals across the US, for a total of 133 cats. Average absorbances for animals defined as FcaGHV1 ELISA positive ranged from 0.21-1.5 units for ORF52 ELISA and 0.27-1.4 units for ORF38 ELISA.

Comparison of seroprevalence and qPCR results

To compare viral loads and immune responses, we surveyed 133 cats previously tested for FcaGHV1 prevalence via qPCR assay of peripheral whole-blood FcaGHV1 DNA^{1,25}. These cats represented animals presented to 8 different shelters in three different states over a period of 2-3 years depending on the shelter. To determine how many cats have an active infection in a given year, an equation was developed in the same format used for calculating incidence. The qPCR results were used in this equation and predicted an annual infection rate of 5 to 8 cats per 100. This estimate was used to further evaluate seroprevalence as a measure of exposure.

The original cohort contained 135 cats; however we did not have serum samples archived for 2 cats, so they were removed from the sample set. This modified sample set included 15% (20/133) qPCR positive animals. Thirty two percent (43/133) overall seroprevalence was calculated after combining results of the two antigens for the ELISAs (Fig. 3D). Of the 20 cats that tested positive on qPCR, 19 also tested positive on serologic assay; this represented 44% of FcaGHV1 seropositive animals. Thus there were 22 cats testing negative on qPCR and positive on the combined serologic assay (Fig. 3).

Figure 3 displays the categorical data results of each assay in comparison to the qPCR results from our previous publication¹. ELISA results generally supported each other; only 1 cat was positive only by qPCR (ELISA negative), 1 cat was positive only with the ORF38 ELISA (ORF52 and qPCR negative) and 11 cats were only positive by ORF52 ELISA (ORF38 and qPCR negative). All other cats were confirmed by at least 2 assays. To further support specificity of ELISAs, there was a non-significant positive trend ($p=0.0705$) with qPCR measured viral load vs

ELISA absorbances for ORF52 (Fig 6). The same analysis of ORF38 absorbances did not reveal a similar trend however ($p= 0.902$) (Fig.7).

Sensitivity and Specificity

A two stage Bayesian method⁴⁹ was used to evaluate sensitivity and specificity of the ORF38 ELISA and the ORF52 ELISA. Sensitivity of ORF52 ELISA was estimated as 74.3% (95% CI: 61.0 to 92.6), while specificity was calculated as 96.4% (95% CI: 90.7 to 99.8). ORF38 ELISA had a sensitivity of 57.9% (95% CI: 50.3 to 73.8) and specificity of 97.9% (95% CI: 93.5 to 99.9). Using this same Bayesian model accounting for sensitivity and specificity of the ELISAs, seroprevalence of FcaGHV1 was estimated at 30.6% (95% CI: 21.6 to 41.1).

FcaGHV1 predictors and risk factor analysis

For the rest of the analysis, we combined the ELISA results so that if a cat was positive with one or more antigens it was considered ELISA positive for comparison of regional data, risk factors, and co-infection status. We compared assay results by qPCR and ELISA with demographic information including location of the shelter cat, sex and age (Fig. 5). We noted a uniform increase from qPCR prevalence to ELISA prevalence by location, sex, and age categories reflective of the overall increase in prevalence with the ELISA assay (Figs. 4, 5). Evidence of exposure to FcaGHV1 increased when seroprevalence was compared to qPCR prevalence. Detectable exposures increased from 4% (2/52) to 15% (8/52) in Colorado, 13% (4/31) to 29% (9/31) in Florida, and 28% (14/50) to 52% (26/50) in California (Fig. 4). Infection as measured by qPCR was 0% (0/54) for females, while seroprevalence was 13% (7/54). Male cats increased from 29% (20/70) qPCR prevalence to 49% (34/70) seroprevalence. Young cat qPCR prevalence

and seroprevalence remained at 0% (0/30) in stark contrast to 21% (20/95) qPCR prevalence in adults with a 42% (40/95) seroprevalence (Fig. 5).

We used logistic regression to find associations with the ELISA results for risk factors previously evaluated by qPCR (Tables 4-5). The risk factors of adult vs young ($p=0.011$) and male vs female ($p=0.0001$) were corroborated as risk factors identified with qPCR testing¹ (Table 4). Male cats were 7.11 (CI: 2.623, 19.29) times more likely than females to be FcaGHV1 seropositive. The odds ratio for FcaGHV1 seropositivity in adult cats was 43.7 (CI: 2.379, 800.8). The odds for a male cat, compared to female, to be qPCR positive was 43.975 (2.581, 749.1) and adult vs young 9.867 (CI: 1.951, ∞) (Table 5). It should be noted that wide confidence intervals are a reflection of the statistical modeling limits. There were no young cats that were either seropositive or qPCR positive for FcaGHV1 and no female cats that were qPCR positive. The result of this is quasi-complete separation of the data set which was accounted for using Firth's penalized likelihood. This allows calculation of odds ratios and p values but with wide confidence intervals.

There was a significant difference in ELISA result between locations capture by state $p=0.0176$ (Table 4). Similar differences also exist in the qPCR data, $p=0.052$. The widest separation of odds was California vs Colorado for either FcaGHV1 qPCR or ELISA test. Cats from California were 4.650 (CI: 1.601, 13.50) times more likely to be ELISA positive and 4.916 (CI: 1.286, 18.79) times more likely to be qPCR positive than cats from Colorado. There wasn't a significant difference in odds between California and Florida when comparing either qPCR or ELISA FcaGHV1 results (Table 4). There was no significant difference between shelter within the

states with our qPCR analysis ($p=0.402$), we did demonstrate significant differences among shelters when comparing seroprevalence ($p=0.0429$). Odds ratios were calculated to compare shelters within states only (Table 4). There were no significant differences between shelters in Colorado or Florida (data not shown). However in California, cats captured by Ventura Animal Services were 8.613 (CI: 1.257 to 59.02) times more likely to be seropositive when compared to cats captured by San Diego Feral Cat Coalition (Table 4). Although the locations of capture are within 250 miles of each other, cats from Ventura Animal Services were in areas of high human population density while the cats from San Diego were captured in rural areas of the Peninsular mountain range.

We further evaluated associations of FcaGHV1 serology with exposure to other pathogens. Samples from these animals have previously been tested for 6 other infections: FIV, *Bartonella spp.*, *Mycoplasma haemominutum* (Mhm), *Mycoplasma haemofelis* (Mhf), Calicivirus, and Feline herpesvirus 1 (FHV-1)⁴⁸. Odds ratios and p values are summarized in Table S2. *Bartonella spp.* IgG was similarly associated to both FcaGHV1 ELISA and qPCR positivity. There was a near significant association with positive *Bartonella spp.* IgG response and FcaGHV1 seropositive response ($p=0.0516$) and a significant association with FcaGHV1 qPCR positive cats ($p=0.0404$). In contrast, *Bartonella spp.* PCR response differed between the two FcaGHV1 test groups. There was a clear absence of significance with a *Bartonella spp.* PCR positive result and FcaGHV1 seropositivity ($p=0.4921$) while qPCR positivity and positive *Bartonella spp.* PCR had a near significant association ($p=0.0547$). There was a similar dynamic as with *Bartonella spp.* PCR to associations with FIV antibody response. Positive result of FcaGHV1 ELISA showed no association with FIV antibody positive results ($p=0.1242$), while

FcaGHV1 qPCR and FIV association was nearly significant ($p=0.057$). *Mycoplasma haemominutum* (Mhm) and *Mycoplasma haemofelis* (Mhf) were also previously tested for by PCR. Mhm was significant for both FcaGHV1 qPCR positive ($p=0.0338$) and ELISA positive cats ($p < 0.0001$). Mhf was not significant for either FcaGHV1 assay result, but much closer to significance for FcaGHV1 ELISA ($p=0.0995$) than qPCR ($p=0.2656$). Calicivirus and FHV1 positive results were not associated with FcaGHV1 positive assay results.

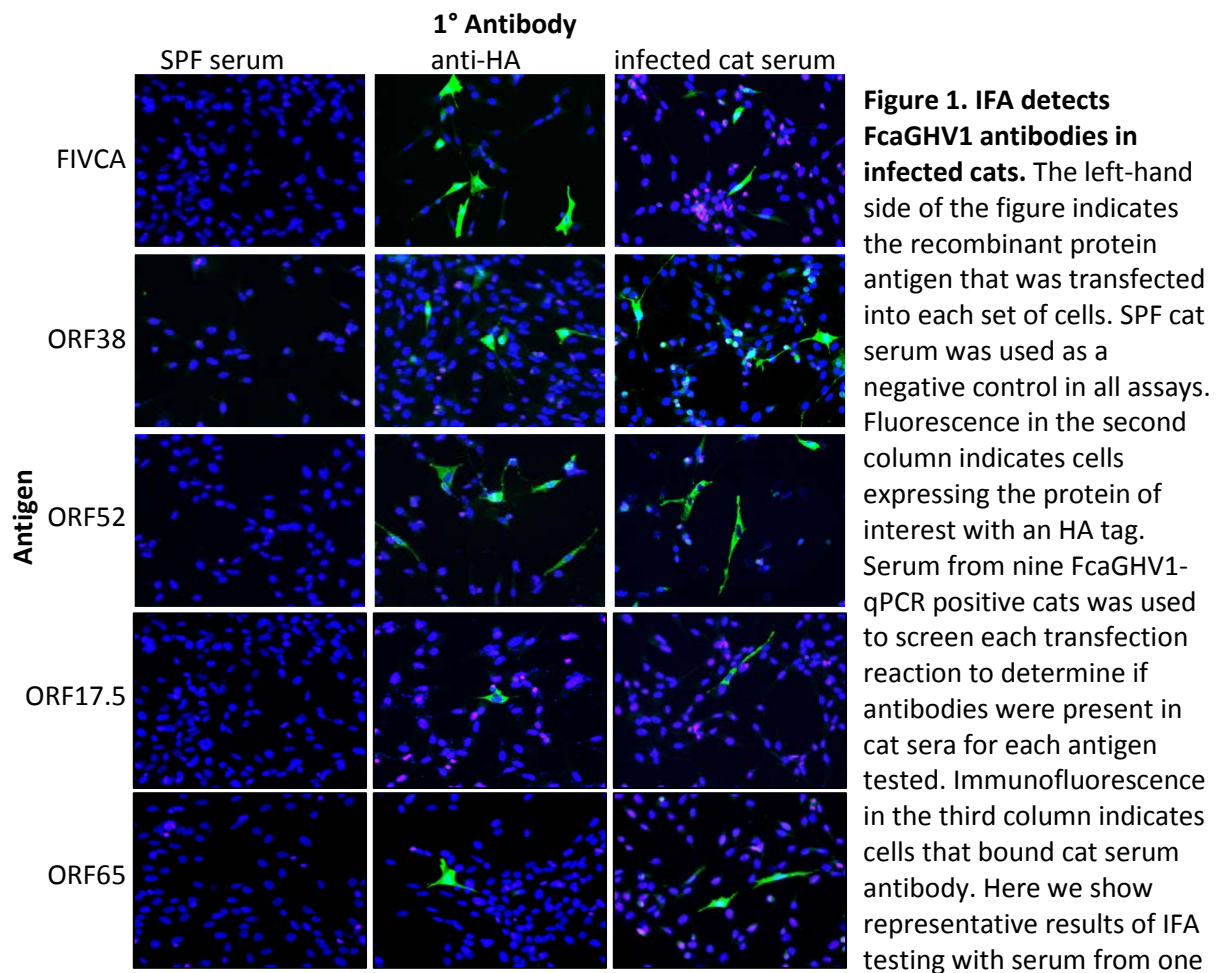


Figure 1. IFA detects FcaGHV1 antibodies in infected cats. The left-hand side of the figure indicates the recombinant protein antigen that was transfected into each set of cells. SPF cat serum was used as a negative control in all assays. Fluorescence in the second column indicates cells expressing the protein of interest with an HA tag. Serum from nine FcaGHV1-qPCR positive cats was used to screen each transfection reaction to determine if antibodies were present in cat sera for each antigen tested. Immunofluorescence in the third column indicates cells that bound cat serum antibody. Here we show representative results of IFA testing with serum from one

FcaGHV1 qPCR-positive cat using proteins ORF38, ORF52, ORF65, and ORF17.5. The FIV capsid (FIVCA) was used as a positive control for detection of viral antigen when exposed to sera from FIV+ cats. A vector-only negative control was also run with each transfection (Fig S1).

FIVCA= FIV capsid protein, ORF38 = tegument protein of FcaGHV1, ORF52= tegument protein of FcaGHV1, ORF17.5= scaffold protein of FcaGHV1, ORF65= capsid protein of FcaGHV1, anti-HA= antibody to HA

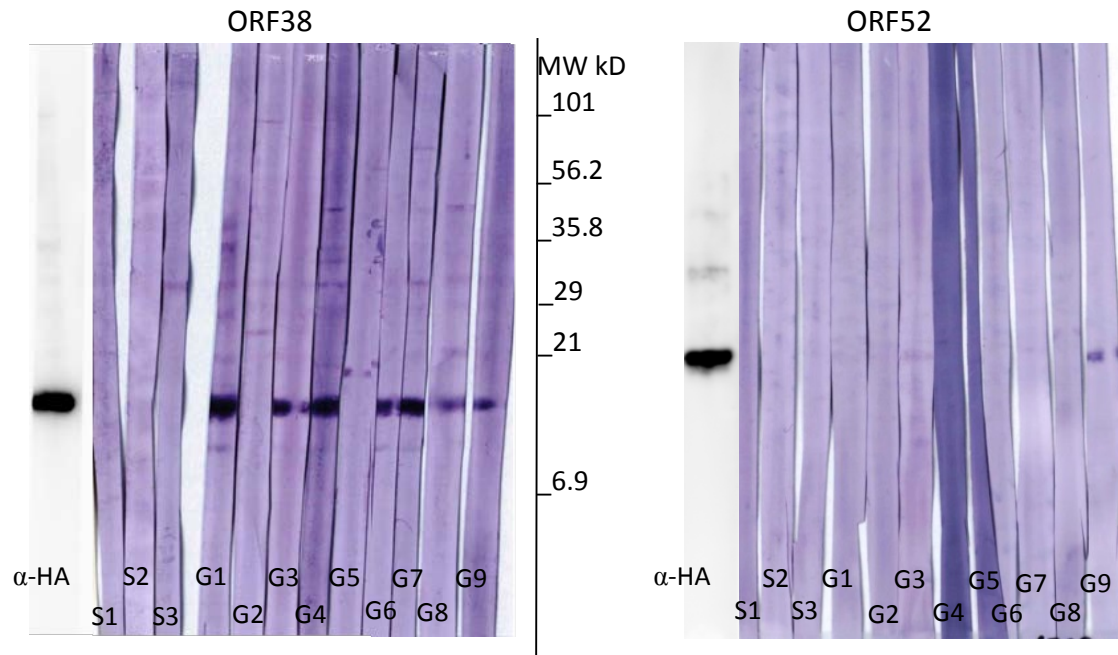


Figure 2. Western blot results confirm IFA. Immobilized protein indicated at top. Each strip was incubated with the serum or anti-HA as primary antibody (listed below). Tabulated results are listed in Table 3. SPF cats (S1-3), GHV+ cats (G1-G9)

Table 3. ELISA assays increase detection of infection with FcaGHV1. Nine cat samples positive for FcaGHV1 by qPCR were used for antigen identification screening. Table shows which cats displayed a serum antibody response to ORF52 and ORF38 proteins on each respective immunofluorescence assay (IFA), western blot (wb), and ELISA.

PCR+ cat#	ORF38			ORF52		
	IFA	wb	ELISA	IFA	wb	ELISA
G1 (CA)	+	+	+	+	-	+
G2 (CO)	-	-	-	+	-	+
G3 (CA)	+	+	+	+	-	+
G4 (CA)	+	+	+	-	-	+
G5 (FL)	-	-	-	-	-	-
G6 (FL)	+	+	+	-	-	+
G7 (CA)	+	+	+	-	-	+
G8 (FL)	+	+	+	-	-	+
G9 (FL)	+	+	+	+	+	+
Total pos.	7	7	7	4	1	8

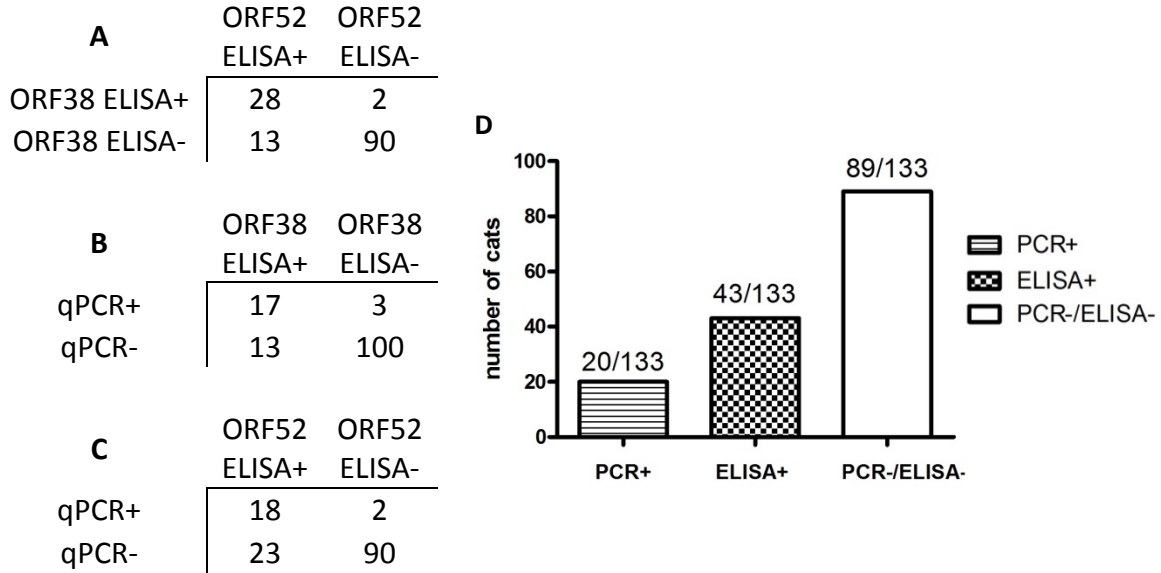


Figure 3. FcaGHV1 ELISA results coincide with qPCR and show additional positive results. A.-C. Categorical data comparison of cats tested for all 3 assays: ORF38 ELISA, ORF52 ELISA, and evidence of qPCR FcaGHV1 viral DNA load. D. Comparison of FcaGHV1 serology to qPCR¹ for 133 shelter cats. One adult male cat from Florida was PCR+/ELISA- (data not shown). All other PCR+ animals were also ELISA+.

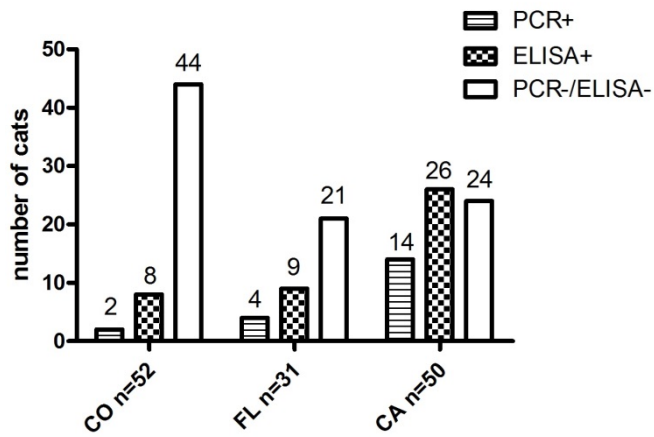


Figure 4. FcaGHV1 ELISA enhances infection detection across geographic locations. Comparison of assay results by location: Colorado, Florida, and California.

Table 4. Geographic risk factors identified by FcaGHV1 qPCR status are supported by FcaGHV1 ELISA result. P values were calculated for differences by state or shelter using categorical logistic regression modeling with sex and age as co-independent variables and qPCR or ELISA result as the response variable (y). Odds ratios (OR) were calculated to compare shelters in California: Ventura Animal Shelter, San Diego Feral Cat Coalition, and Corona Animal Shelter. Odds ratios between Colorado and Florida shelters were not significant (not shown).

	y= ELISA			y= qPCR		
	P (Wald)	OR	95% CI	P (Wald)	OR	95% CI
State	0.0176	-	-	0.0520	-	-
California vs Colorado	-	4.650 (1.601 to 13.50)		-	4.916 (1.286 to 18.79)	
California vs Florida	-	1.567 (0.449 to 5.467)		-	0.941 (0.217 to 4.078)	
Florida vs Colorado	-	2.967 (0.788 to 11.17)		-	5.223 (0.942 to 28.96)	
Shelter	0.0429	-	-	0.402	-	-
Ventura vs San Diego	-	8.613 (1.257 to 59.02)		-	2.189 (0.320 to 14.99)	
Ventura vs Corona	-	1.942 (0.336 to 11.22)		-	0.7 (0.135 to 3.616)	

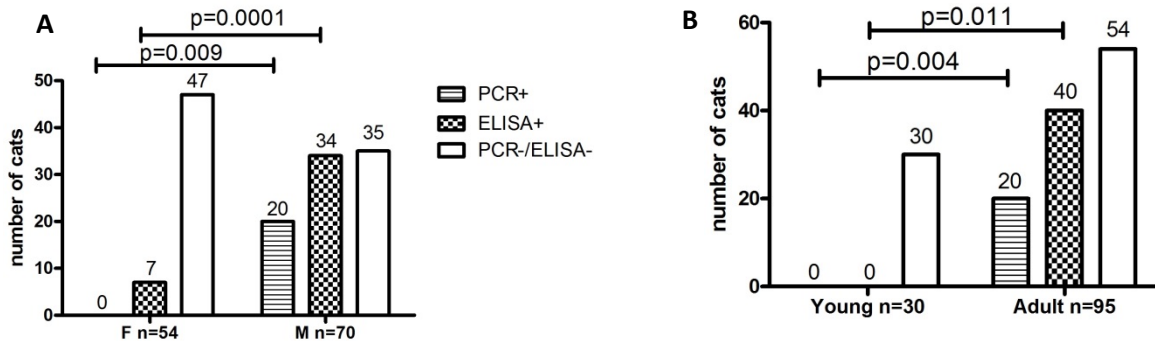


Figure 5. FcaGHV1 ELISA enhances infection detection across sex, and age categories. A. Assay comparison by sex: male (M) and female (F). Unknown sex not shown: n=9. B. Assay comparison by age. Unknown age not shown: n=8. P value from Wald testing shows significance of binary logistic regression modeling with FcaGHV1 ELISA result or qPCR result as the response variable.

Table 5. Sex and age are highly significant risk factors for both FcaGHV1 qPCR and ELISA response. Odds ratios (OR) and confidence intervals (CI) calculated using binomial logistic regression.

	y= ELISA		y= qPCR	
	OR	95% CI	OR	95% CI
Male	7.110	(2.623 to 19.29)	43.98	(2.581 to 749.1)
Adult	43.70	(2.379 to 800.8)	9.867	(1.951 to ∞)

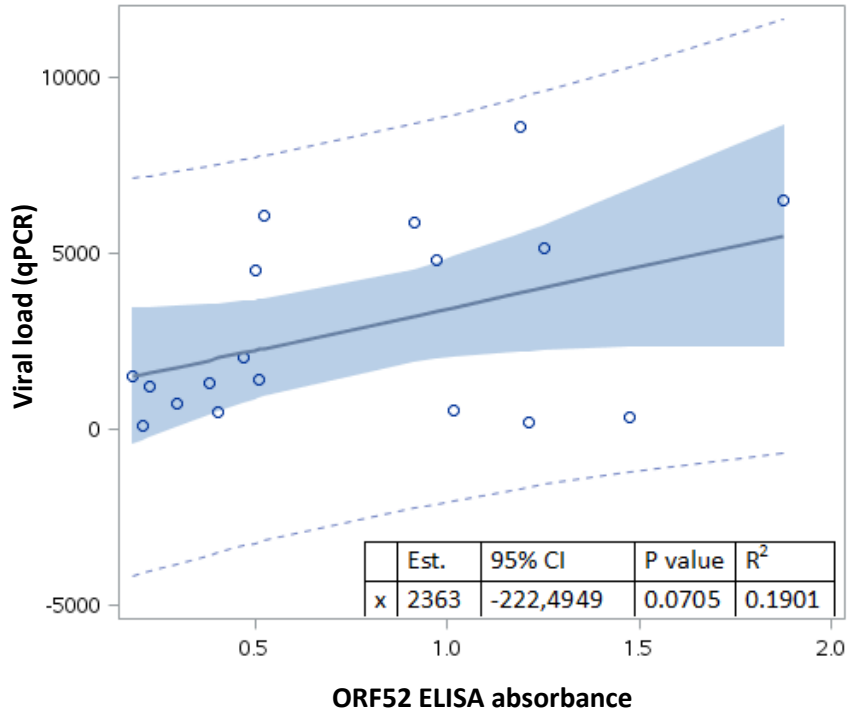


Figure 6. Viral load vs ELISA absorbance for ORF52 trends positively. A t-test of the linear regression model shows a trend towards a significant relationship between the 2 variables, $p=0.0705$. One extreme outlier (viral load 4 standard deviations above the mean) was removed from the data set. Est. = estimated slope (x) with 95% confidence interval.

— Fit □ 95% Confidence Limits - - - - 95% Prediction Limits

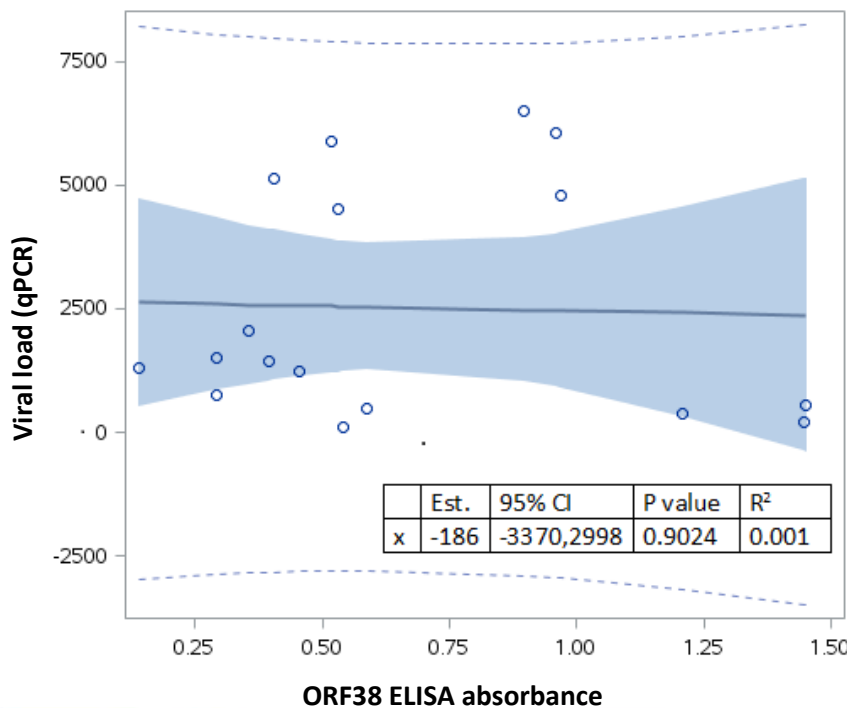


Figure 7. Viral load and ORF38 ELISA absorbance are not significantly correlated. T-test of linear regression indicates there is no relationship between the variables $p=0.9024$. One extreme outlier with a viral load 4 standard deviations above the mean was removed from the data set. Est. = estimated slope (x) with 95% confidence interval.

— Fit □ 95% Confidence Limits - - - - 95% Prediction Limits

SUPPLEMENTARY FIGURES

Table S1. Feline serum antibody responses to other prospective antigens tested by IFA were not routinely reactive. FcaGHV1 positive cats (G1-9, qPCR-tested) showed no antibody response to ORF59, ORF26, or ORF42. ORF65 and ORF17.5 had fewer cats with an antibody response than ORF38 and ORF52. A semi-purified western blot (wb) was also run for ORF65.

	ORF59	ORF65		ORF17.5	ORF26	ORF42
	IFA	IFA	wb	IFA	IFA	IFA
G1 (CA)	-	-	+	-	-	-
G2 (CO)	-	-	-	-	-	-
G3 (CA)	-	+	+	+	-	-
G4 (CA)	-	-	-	-	-	-
G5 (FL)	-	-	-	-	-	-
G6 (FL)	-	-	-	-	-	-
G7 (CA)	-	-	-	-	-	-
G8 (FL)	-	-	-	-	-	-
G9 (FL)	-	+	-	-	-	-
total pos.	0	2	2	1	0	0

Table S2. FcaGHV1 ELISA and qPCR associate with coinfections in differing patterns. Predictor variables and coinfection data for FcaGHV1 positive serology. P value from Wald testing shows significance of binary logistic regression modeling with FcaGHV1 ELISA result or qPCR result as the response variable (y). Separate models were created with each co-infection as independent variables (x) along with sex and age as co-independent variables. Odds (OR) with 95% confidence interval (CI) of a positive FcaGHV1 ELISA or qPCR result were calculated for each independent variable. This table also shows the number of animals in each model (n) and the number of positive animals for each independent disease variable within that sample (pos. x_n). Bart. = *Bartonella* spp. Mhm = *Mycoplasma haemominutum*, Mhf = *Mycoplasma haemofelis*, FHV1= Feline herpesvirus 1. Both FHV1 and Calicivirus were tested by oral swab PCR. FIV, Mhm, and Mhf were evaluated with PCR testing.

	y= ELISA					y= qPCR				
	OR	95% CI	P (Wald)	n	pos. x _n	OR	95% CI	P (Wald)	n	pos. x _n
Bart. IgG	2.720	0.993 to 7.468	0.0516	117	30	3.148	1.051 to 9.425	0.0404	119	30
Bart. PCR	1.570	0.432 to 5.726	0.4921	117	17	3.988	0.973 to 16.35	0.0547	119	17
FIV	3.070	0.734 to 12.869	0.1242	120	15	3.983	0.959 to 16.53	0.0570	121	15
Mhm	11.30	3.736 to 33.90	<.0001	115	35	3.369	1.098 to 10.34	0.0338	117	36
Mhf	4.900	0.740 to 32.396	0.0995	115	11	2.271	0.536 to 9.620	0.2656	117	11
Calicivirus	1.070	0.279 to 4.121	0.9184	118	15	0.865	0.201 to 3.713	0.8453	120	15
FHV1	9.610	0.484 to 190.7	0.1377	118	6	2.552	0.414 to 15.72	0.3124	120	6

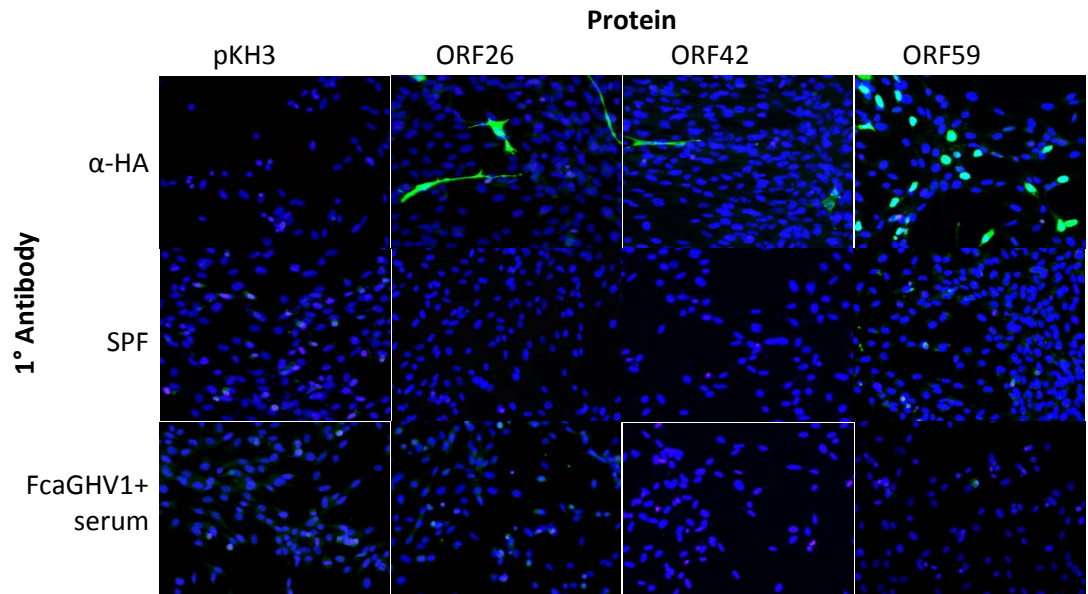


Figure S1. IFA detects no antibody response to remaining FcaGHV1 proteins. The top of the figure indicates the recombinant protein that was transfected into each set of cells. SPF cat serum was used as a negative control in all assays. Fluorescence in the first row indicates cells expressing the protein of interest with an HA tag. Serum from nine FcaGHV1-qPCR positive cats was used to screen each transfection reaction to determine if antibodies were present in cat sera for each protein tested. Immunofluorescence in the third row indicates cells no antibody response to incubation with FcaGHV1+ cat serum. A vector-only negative control (pKH3) was also run with each transfection (Fig S1). ORF26 = capsid protein of FcaGHV1, ORF59= phosphoprotein of FcaGHV1, ORF42= tegument protein, anti-HA= antibody to HA

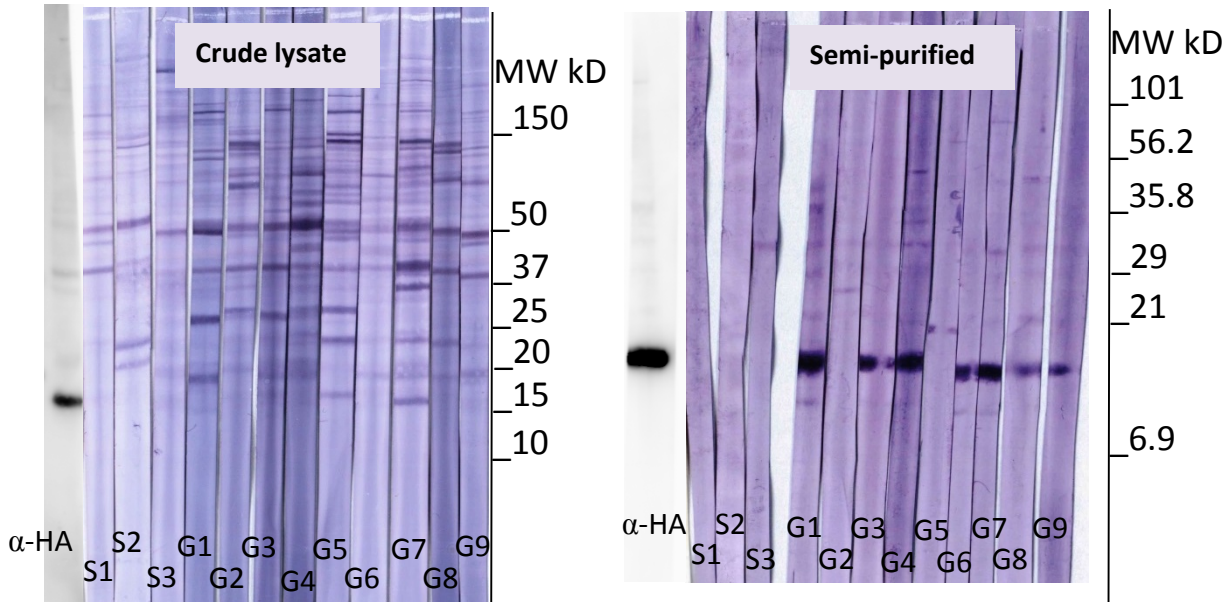


Figure S2. Crude lysate western blots are inconclusive. The left panel shows immobilized crude ORF38 protein immobilized, the right panel shows immobilized semi-purified ORF38. Each strip was incubated with the serum or anti-HA as primary antibody (listed below). SPF cats (S1-3), GHV+ cats (G1-G9)

DISCUSSION

As hypothesized, we detected a higher prevalence of FcaGHV1 in a population of 133 shelter cats via ELISA (32%) compared to qPCR positive individuals (15%). Antibody response reflects exposure to the virus while qPCR assay more likely measures an active or reactivated viral infection. From this analysis we conclude that close to half (44%) of FcaGHV1 seropositive cats have a qPCR detectable FcaGHV1 viral load. Our measured FcaGHV1 seroprevalence is much lower than the seroprevalence of EBV in humans, which has a global seroprevalence of greater than 95% ⁵⁰. The seroprevalence of FcaGHV1 in cats more closely resembles the seroprevalence of KSHV in humans. There is wide variation in KSHV seroprevalence rates partly because there is no gold standard assay⁹. Furthermore, reported KSHV exposures range dramatically by region. North America, Asia, and Europe are considered low seroprevalence areas and most studies have indicated exposure rates of <5%⁹. However, one study which tested routine pediatric patients in southern Texas by immunofluorescence assay, ELISA, and immunoblot found a seroprevalence of 26%⁵¹. Higher KSHV seroprevalence, approximately 50%, is found in regions of Africa and also the Brazilian Amazon⁹.

Studies to date have suggested FcaGHV1 has a closer phylogenetic relationship to KSHV than EBV¹, and it has genes homologous to KSHV that are not conserved in other gammaherpesviruses, such as latency-associated nuclear antigen (LANA) (unpublished data). Assuming that the FcaGHV1 ELISA assays reported in these studies are sensitive enough to identify most of the cats exposed to FcaGHV1, seroprevalence is below 60%, similar to KSHV. KSHV's lower seroprevalence and high regional variation might be due to factors involved with

virus shedding⁵². Twenty to thirty percent of healthy adults with latent EBV infection intermittently shed virus in oral secretions⁵⁰. High rates of KSHV shedding in healthy adults is individual specific and highly sporadic. Variation in KSHV shedding plays a role in regions with high rates of mother-to-child transmission through direct contact with saliva. There is evidence to show that this KSHV persistent shedding is related to host genetic factors⁵². FcaGHV1 may also have similar host genetic factors to KSHV that result in some infected cats being more likely to shed virus. Since an increase in KSHV replication is associated with development of KS⁵², future studies should evaluate relationships between FcaGHV1 exposure and feline neoplastic disorders.

The results of this ELISA data strongly corroborate age and sex as risk factors for FcaGHV1 that were identified previously^{1,25}. Being male and adult appears to be strongly associated both with qPCR-positive FcaGHV1 infection ($p=0.009$, $p=0.004$) and ELISA-positive results ($p=0.0001$, $p=0.011$) (Fig. 5, Table 5). Interestingly, no young cats ($n=30$) were seropositive for FcaGHV1, and while some female cats in this population were seropositive (7/54), none were qPCR-positive. This suggests that mother-to-kitten, vertical transmission, or vector borne disease transmission are unlikely modes of spread of FcaGHV1 in the US feral cat populations. Aggressive contacts between males is a more probable mode of transmission given the strong association of infection with older male attributes. An adult cat may have more aggressive encounters as well as a longer time to contract infection than a young animal.

FcaGHV1 ELISA seroprevalence and qPCR data also demonstrated a potential correlation between exposure to FcaGHV1 and co-infection with other pathogens²⁵. This might be related

to pathogenesis of FcaGHV1 and also to similar routes of transmission and risk factors between agents. *Mycoplasma* spp. and FIV are thought to be transmitted via antagonistic encounters^{53,54}. Comparisons of FcaGHV1 ELISA and qPCR showed some associative trends with detection of FIV antibodies and FcaGHV1 qPCR-positive response ($p=0.057$). There was also a significant association with *Mycoplasma haemofelis* PCR-positive animals and both ELISA-positive ($p<0.0001$) and qPCR-positive FcaGHV1 results ($p=0.0338$) (Table S2).

Human gammaherpesviruses can be transmitted through direct contact with saliva. EBV is transmitted mostly through salivary secretions⁵⁰. The most current information suggests saliva is the major route for KSHV infection as well⁵⁰. Initially it was thought that KSHV was transmitted sexually⁵⁵. More recent studies have shown that KSHV viral secretions are highest in oral mucosa⁵⁶, with further evidence of high seroprevalence estimates from populations of children^{51,57} where vertical transmission probably plays a minor role⁵⁸.

With the assumption that reactivated FcaGHV1 infection would occur after the onset of an immune suppression event, I expected to see an association with multiple co-infections in cats FcaGHV1-positive in an assay that measures viral load (qPCR) and no association in cats positive in an assay thought to measure FcaGHV1 exposure (ELISA). There were some trends suggesting this with *Bartonella* spp. PCR positivity and FIV antibodies (Table S2), but it is difficult to make any concrete conclusions when looking at associations between multiple variables likely dependent on, not causal to, immune suppression.

Geographic variation was a risk factor for both FcaGHV1 seropositivity and FcaGHV1 qPCR positive result. Cats captured in California were 4.916 (95% CI: 1.286, 18.79) times more

likely to be qPCR positive and 4.65 (95% CI: 1.601, 13.50) times more likely to be ELISA positive than Colorado cats (Table 4). Moreover, a comparison of shelters revealed no significance in qPCR results, however, cats from Ventura Animal Shelter were 8.613 (95% CI: 1.257, 59.02) times more likely to be ELISA positive than cats captured by the San Diego Feral Cat Coalition. Both of these shelters were located in California (Table 4). A closer analysis of the geography reveals that the sample of cats in California likely came from much denser, city populations. Additionally, the majority of the cats captured by the San Diego group were in mountainous areas several hours from San Diego with low human populations, whereas the Ventura Animal Shelter captured cats in urban Ventura. This information suggests that feline population density may play a role in FcaGHV1 transmission. High density may also correspond with increased aggressive encounters further supporting this as a major mode of viral transmission.

Sensitivity and specificity are difficult to measure without a gold standard test. In order to use Bayesian probability, at least 2 tests must be compared⁵⁹. The two assay types in this study, qPCR and ELISA do not evaluate the same thing, active viral infection vs viral exposure. Therefore, sensitivity and specificity cannot be evaluated with this method. However, ORF38 and ORF52 ELISA assays could be evaluated using a Bayesian logistic regression model. From these calculations, the ORF38 ELISA had a sensitivity of 57.9% (95% CI: 50.3 to 73.8) and specificity of 97.9% (95% CI: 93.5 to 99.9) while ORF52 ELISA had (95% CI: 90.7 to 99.8) specificity and 74.3% (95% CI: 61.0 to 92.6) sensitivity. The accuracy of these estimates is only as good as the probability distributions of the known parameters⁵⁹. A better method which could be used with future development of more assays (3 or more) would be to use latent class modeling, modified for sensitivity and specificity calculations^{59,60}.

The trend towards positive correlation between viral load and ORF52 ELISA absorbance provides evidence of ELISA specificity (Fig. 6). We also recorded that 19/20 qPCR positive cats were positive on ELISA. Both of these observations provide assurance that qPCR-/ELISA+ results are accurate. As noted, only one cat (G5 from Florida) was qPCR positive and negative on both ELISAs. Cat G5 had a relatively high whole-blood viral-DNA load recorded initially and during re-testing (4491 copies/million cells). This animal was also negative on both ORF65 and ORF17.5 IFAs. Negative serology and positive qPCR could be explained by a recent infection that had not yet resulted in seroconversion. Other explanations include the individual's inability to produce antibodies due to severity of some other immune problems or a sample identification error.

Most likely the correlative viral load vs ORF52 ELISA absorbance (Fig. 6) also reflects a situation where most of the cats that are qPCR positive in this sample set are cats with a re-activated infection or a late stage primary infection. In both of these situations antibody titer and viral load are high^{61,62}. Reactivation would result in higher levels of viral antigen in circulation, which would boost anamnestic humoral immune response, causing higher antibody loads. ORF38 ELISA absorbance, however, did not show a relationship to FcaGHV1 qPCR viral load (Fig.7). There is likely some complex immune activity occurring with this dynamic. Studies of KSHV sero-reversion in longitudinal studies of HIV patients have demonstrated plasticity of KSHV antibody titers throughout HIV infection and relative to development of KS⁶³. More study into the particular activity of the feline FcaGHV1 antibody response is needed to completely understand these subtleties.

An estimate of how many cats might have an active FcaGHV1 infection in a given year gives more information about potential for virus transmission. The samples used in this study were collected at each site over a 2 to 3 year period depending on exact site. The estimate using qPCR prevalence data is thus 5 to 8 cats out of a 100 cats annually with an active infection. This is not a true measure of incidence since a primary infection cannot be detected by ELISA or qPCR assay. Based on kinetics of EBV serology patterns, an individual who is in late primary infection would have a similar profile as someone with re-activated infection. Additionally, viral-DNA load in the blood could also be similar for both infection stages⁶¹. It is therefore difficult to come to a conclusion about primary versus re-activated infection. However, the rate of 5-8 cats out of 100 annually with an active infection does give some idea about chances of transmission. Considering a cat fight might be required for transmission, this rate of infected animals would also be congruent with interpreting FcaGHV1 seroprevalence (35%) as a measure of exposure. Density is another important factor in considering transmission rate, and could be further evaluated to assess additional risk factors of FcaGHV1 exposure.

The IFA and western blot testing proved to be an effective method of screening immunodominant antigens of FcaGHV1 to target certain proteins for high throughput antibody screening. The combination of these initial tests allowed me to confirm that at least some domestic cats with FcaGHV1 would form antibodies to ORF38 and ORF52. Western blot proved to be a valuable technique in further characterizing immune responses indicated by IFA screening assays. The combination of these assays provided me with more confidence against false negatives on the ELISAs I developed. In the future, I think they would be good

complements to data on new antigen testing, but they could be performed after ELISA development as confirmation.

I encountered background issues on both IFA and western blot. The alkaline phosphatase method of detection had a fairly high level of background that limited western blot diagnostic utility and may have contributed to the lack of sensitivity with ORF52. I noted that there was a higher level of background on IFA when I was looking at samples exposed to cat serum as opposed to the anti-HA antibody. This included SPF naïve cat serum and the background was particularly intense around the nucleus of the CRFKs. This was likely a generalized reaction of feline serum to the particular feline cell line or to ubiquitous cell culture contaminants.

Most of the proteins, including tegument proteins ORF38 and ORF52 appeared to localize to the cytoplasm or a combination of cytoplasm and nucleus of the CRFKs. However ORF59 localized only to the nucleus (see Fig. S1). ORF59's localization to the nucleus during transient transfection may be explained by the function of ORF59 gammaherpesvirus homologues as the DNA polymerase processivity factor^{44,64}. In KSHV, there is some evidence that ORF59 forms a homodimer when it enters the cytoplasm⁶⁴. If this were the case in FcaGHV1 as well, the dimerization may have concealed the HA tag on the recombinant construct making it only visible in the nucleus when anti-HA was used as a primary antibody. The earlier described higher background with nuclear staining could have also masked ORF59 positive results if the case were that feline antibodies only bind to the non-complexed ORF59 protein.

There was variation in individual cat antibody response with respect to each antigen of FcaGHV1 evaluated in this study. ORF38 and ORF52 proteins both reacted with 65% of all seropositive cats, 5% only reacted with ORF38 and 30% only with ORF52. Of the 15 cat sera samples that only reacted with one antigen, 20% were also confirmed by qPCR-positive results. Variation in humoral immune response is certainly not uncommon but it may be more pronounced during gammaherpesvirus infection. Katano et al. evaluated seroprevalence in patients with KS for a variety of KSHV antigens. They performed initial testing with a smaller set of KS patients using western blot, and noted that seroreactivity to antigens was not uniform. Because of the wide range in response, they created a more sensitive ELISA for 5 antigens¹⁴. The results for cats tested in this assay reflect a similar varied antibody response. Variation may be a due to a combination of the complexity of gammaherpesvirus life cycle, variations in host immune response, and technicalities of assay development with ORF38 as noted below.

ORF38 and ORF52 did not have uniform performance across IFA, western blot and ELISA (Table 3). The ORF38 ELISA detected 2 individuals positive that were ORF52 ELISA negative while ORF52 detected 13 individuals that were ORF38 ELISA negative. There were 28 cats that were positive on both ELISAs (Fig. 3). This difference between IFA, western blot, and ELISA could be explained by some factor in the conditions of the IFA or the western blot that did not allow proper expression of ORF52. An alternative explanation may relate to optimization of the ELISA assay. ORF38 had a rather low yield of protein per transfection. I optimized transfection efficiency by adjusting amounts of transfection reagents and harvesting at different time points for ORF38, however despite this, at best I only arrived at half of the protein production per million cells as I did with those exposed to ORF52 transfection. Since we have not yet

synthesized and purified large quantities of protein for ELISA optimization, additional assay development may enhance the sensitivity of ORF38 as a serologic target. Enhancement of antigen production would be a logical next step for standardizing ORF38 and ORF52 ELISAs for large-scale reproducible use.

The antigens identified as immunodominant (ORF38 and ORF52) in this analysis represent tegument proteins in other gammaherpesviruses. The tegument is a layer of the virus between the envelope and the capsid³. In general herpesvirus proteins have multiple highly distinct functions³. Studies of KSHV, Rhesus macaque rhadinovirus, and MHV-68 show that ORF38 and ORF52 also potentially provide more efficient virion maturation and egress^{17,37,38}. These additional roles may mean high production of these proteins contribute to other aspects of their antigenic nature. The proteins that form the tegument are particularly understudied in herpes viruses. Studies of Herpes Simplex Virus (HSV) have revealed that HSV particles are coated with tegument in the cytoplasm of the host cell ^{65 66}. This corresponds to localization of these tegument proteins to the cytoplasm during transient transfection (Fig. 1). HSV tegument proteins are known to have roles beyond structure formation including activation of glycoproteins and capsid proteins⁶⁶. There is also evidence that tegument proteins have roles in binding to cellular transport proteins⁶⁷. These additional functions may increase exposure to the host immune system. Additionally, variation in pathogen recognition factors likely plays an important role.

KSHV data suggests that sensitivity of mixed-antigen ELISAs can increase with use of a combination of latent and lytic protein antigens¹⁴. In this study we did not evaluate proteins

that are considered to be homologous to herpesvirus proteins expressed during latency. There is potential to pursue this in the future with the FcaGHV1 homologue to KSHV's LANA. That said, the ELISAs developed in this study are likely a fairly accurate measure of exposure, it could be a small percentage that are being missed by not evaluating further antigens.

A case-control study of cats that have been co-infected with FIV and FcaGHV1 would allow for in-depth observations of many aspects of FcaGHV1 and would be the ideal setting to evaluate a full panel of screening ELISAs tested sequentially during FcaGHV1 infection. Co-infected cats would be compared to uninfected cats as well as cats with a singular infection. A study of this type has the potential to quickly answer questions discussed earlier about FcaGHV1 transmission, pathogenesis, and viral kinetics. It could provide information about actual clinical disease associations with FcaGHV1 and the value of therapeutic interventions. This could potentially advance understanding of the FcaGHV1 life cycle as well.

Studying EBV and KSHV has been difficult because they are complex viruses with large DNA genomes. Furthermore, the associated lifelong latency periods make study of transmission and reactivation even more difficult and have been a challenge for effective vaccine development. Appropriate animal models allow us to better study these sorts of details rather than relying on human longitudinal studies that attempt to make up for an inability to manipulate study variables. Consequently, one of the most important benefits of feline case-control studies of FcaGHV1 would be to evaluate the potential for a feline model of HIV/GHV co-infection of humans.

CHAPTER 3: CONCLUSIONS

Indirect ELISA based upon FcaGHV1 tegument associated antigens ORF52 and ORF38 ELISA provided an enhanced assay to detect viral exposure compared to FcaGHV1 qPCR. We concluded that approximately half of seropositive cats have a detectable FcaGHV1 viral genomes using qPCR.

This study additionally showed strong support of risk factors of FcaGHV1 infection identified previously. Adult male cats are much more likely to be both seropositive and have peripheral-blood viral load. There was also a relationship between co-infection with other pathogens and detection of FcaGHV1 seropositivity. These factors point strongly toward inter-cat aggression as a mode of transmission.

It may appear from this data set that ORF52 ELISA alone would provide accurate and sensitive seroprevalence information. However, given what we know about individual and regional variations in sero-response of KSHV, I believe the ORF38 ELISA is an important addition to the ELISA. Ideally, antigens could be optimized for use in a multiplex assay. Additions of other antigens to the ELISA could also be explored, preferably in settings of more controlled experimentation such as case-control studies.

IFA and western blot assays proved to be useful tools for bolstering assurance of specificity during ELISA development. In the future they will be helpful techniques that could also be used after ELISA development instead of before. To improve the ELISA assay a more productive method of producing ORF38 and ORF52 will also be important.

Other results of this study suggest the biology of FcaGHV1 is more similar to KSHV than EBV infection in humans, illustrating potential utility of studying FcaGHV1 infection to aid studies of KSHV disease. It would be important to design studies aimed at identifying disease associations and routes of transmission to confirm observations made in this initial study. Ideally, future studies would evaluate FcaGHV1 incidence in populations of cats by both qPCR and ELISA. I would target disease patterns such as chronic lymphocytic leukemia, inflammatory bowel disease, B cell lymphocytosis, and intestinal lymphoma and assess both FcaGHV1 seropositivity and qPCR. Based on our knowledge of gammaherpesviruses and common clinical syndromes in cats that have lymphoproliferative tendencies, these would be the most likely diseases to have an association with FcaGHV1.

REFERENCES

- 1 Troyer, R. M. *et al.* Novel Gammaherpesviruses in North American Domestic Cats, Bobcats, and Pumas: Identification, Prevalence, and Risk Factors. *Journal of virology* **88**, 3914-3924 (2014).
- 2 Reusser, P. Herpesvirus resistance to antiviral drugs: a review of the mechanisms, clinical importance and therapeutic options. *Journal of Hospital Infection* **33**, 235-248 (1996).
- 3 Fields, B. N., Knipe, D. M. & Howley, P. M. *Fields virology*. (Wolters Kluwer Health/Lippincott Williams & Wilkins, 2007).
- 4 Cesarman, E. Gammaherpesvirus and lymphoproliferative disorders in immunocompromised patients. *Cancer Lett* **305**, 163-174, doi:10.1016/j.canlet.2011.03.003 (2011).
- 5 Griffiths, P., Clark, D. & Emery, V. Betaherpesviruses in transplant recipients. *Journal of Antimicrobial Chemotherapy* **45**, 29-34 (2000).
- 6 Boshoff, C. & Weiss, R. Kaposi's sarcoma-associated herpesvirus. *Advances in cancer research* **75**, 57-87 (1998).
- 7 Pietersma, F., Piriou, E. & van Baarle, D. Immune surveillance of EBV-infected B cells and the development of non-Hodgkin lymphomas in immunocompromised patients. *Leukemia & lymphoma* **49**, 1028-1041 (2008).
- 8 Thorley-Lawson, D. A. & Gross, A. Persistence of the Epstein–Barr virus and the origins of associated lymphomas. *New England Journal of Medicine* **350**, 1328-1337 (2004).
- 9 Dow, D. E., Cunningham, C. K. & Buchanan, A. M. A Review of Human Herpesvirus 8, the Kaposi's Sarcoma-Associated Herpesvirus, in the Pediatric Population. *Journal of the Pediatric Infectious Diseases Society* **3**, 66-76 (2014).
- 10 Ganem, D. KSHV infection and the pathogenesis of Kaposi's sarcoma. *Annu. Rev. Pathol. Mech. Dis.* **1**, 273-296 (2006).
- 11 Orzechowska, B. U. *et al.* Rhesus macaque rhadinovirus-associated non-Hodgkin lymphoma: animal model for KSHV-associated malignancies. *Blood* **112**, 4227-4234 (2008).
- 12 Okada, S., Goto, H. & Yotsumoto, M. Current status of treatment for primary effusion lymphoma. *Intractable & rare diseases research* **3**, 65 (2014).
- 13 Oksenhendler, E. *et al.* High incidence of Kaposi sarcoma–associated herpesvirus–related non-Hodgkin lymphoma in patients with HIV infection and multicentric Castleman disease. *Blood* **99**, 2331-2336 (2002).
- 14 Katano, H. *et al.* Identification of antigenic proteins encoded by human herpesvirus 8 and seroprevalence in the general population and among patients with and without Kaposi's sarcoma. *Journal of virology* **74**, 3478-3485 (2000).
- 15 Lang, D. *et al.* Comparison of the immunoglobulin-G-specific seroreactivity of different recombinant antigens of the human herpesvirus 8. *Virology* **260**, 47-54 (1999).
- 16 Klutts, J., Ford, B., Perez, N. & Gronowski, A. Evidence-based approach for interpretation of Epstein-Barr virus serological patterns. *Journal of clinical microbiology* **47**, 3204-3210 (2009).
- 17 Labo, N. *et al.* Heterogeneity and breadth of host antibody response to KSHV infection demonstrated by systematic analysis of the KSHV proteome. *PLoS pathogens* **10**, e1004046 (2014).
- 18 Speck, S. H. & Ganem, D. Viral latency and its regulation: lessons from the γ -herpesviruses. *Cell host & microbe* **8**, 100-115 (2010).
- 19 Stevens, S. J. *et al.* Frequent monitoring of Epstein-Barr virus DNA load in unfractionated whole blood is essential for early detection of posttransplant lymphoproliferative disease in high-risk patients. *Blood* **97**, 1165-1171 (2001).
- 20 Ehlers, B. *et al.* Novel mammalian herpesviruses and lineages within the Gammaherpesvirinae: cospeciation and interspecies transfer. *Journal of virology* **82**, 3509-3516 (2008).

- 21 Rivaller, P. *et al.* Experimental rhesus lymphocryptovirus infection in immunosuppressed macaques: an animal model for Epstein-Barr virus pathogenesis in the immunosuppressed host. *Blood* **104**, 1482-1489 (2004).
- 22 Hu, Z. & Usherwood, E. J. Immune escape of γ -herpesviruses from adaptive immunity. *Reviews in medical virology* **24**, 365-378 (2014).
- 23 Ablashi, D. V., Chatlynne, L. G., Whitman Jr, J. E. & Cesarman, E. Spectrum of Kaposi's sarcoma-associated herpesvirus, or human herpesvirus 8, diseases. *Clinical Microbiology Reviews* **15**, 439-464 (2002).
- 24 Chen, Y.-B., Rahemtullah, A. & Hochberg, E. Primary effusion lymphoma. *The Oncologist* **12**, 569-576 (2007).
- 25 Beatty, J. A. *et al.* *Felis catus* gammaherpesvirus 1; a widely endemic potential pathogen of domestic cats. *Virology* **460-461**, 100-107, doi:10.1016/j.virol.2014.05.007 (2014).
- 26 Stump, D. S. & VandeWoude, S. Animal models for HIV AIDS: a comparative review. *Comparative medicine* **57**, 33-43 (2007).
- 27 Sliva, K. Latest animal models for anti-HIV drug discovery. *Expert opinion on drug discovery*, 1-13 (2014).
- 28 Shelton, G. H. *et al.* Feline immunodeficiency virus and feline leukemia virus infections and their relationships to lymphoid malignancies in cats: a retrospective study (1968-1988). *JAIDS Journal of Acquired Immune Deficiency Syndromes* **3**, 623-630 (1990).
- 29 Gloghini, A., Dolcetti, R. & Carbone, A. in *Seminars in cancer biology*. 457-467 (Elsevier).
- 30 Gramolelli, S. & Schulz, T. F. The role of Kaposi sarcoma-associated herpesvirus in the pathogenesis of Kaposi sarcoma. *The Journal of pathology* **235**, 368-380 (2015).
- 31 Chatterjee, B., Leung, C. S. & Münz, C. Animal models of Epstein Barr virus infection. *Journal of immunological methods* **410**, 80-87 (2014).
- 32 Mrázová, V. *et al.* Murine Gammaherpesvirus (MHV-68) Transforms Cultured Cells in vitro. *Intervirology* **58**, 69-72 (2015).
- 33 Wang, L.-X. *et al.* Humanized-BLT mouse model of Kaposi's sarcoma-associated herpesvirus infection. *Proceedings of the National Academy of Sciences* **111**, 3146-3151 (2014).
- 34 Yajima, M. *et al.* A new humanized mouse model of Epstein-Barr virus infection that reproduces persistent infection, lymphoproliferative disorder, and cell-mediated and humoral immune responses. *Journal of Infectious Diseases* **198**, 673-682 (2008).
- 35 Bartley, K. *et al.* Identification of immuno-reactive capsid proteins of malignant catarrhal fever viruses. *Veterinary microbiology* **173**, 17-26 (2014).
- 36 Dry, I. *et al.* Proteomic analysis of pathogenic and attenuated alcelaphine herpesvirus 1. *Journal of virology* **82**, 5390-5397 (2008).
- 37 Shen, S., Guo, H. & Deng, H. Murine gammaherpesvirus-68 ORF38 encodes a tegument protein and is packaged into virions during secondary envelopment. *Protein & cell* **5**, 141-150 (2014).
- 38 Anderson, M. S., Loftus, M. S. & Kedes, D. H. Maturation and Vesicle-Mediated Egress of Primate Gammaherpesvirus Rhesus Monkey Rhadinovirus Require Inner Tegument Protein ORF52. *Journal of virology* **88**, 9111-9128 (2014).
- 39 Shedd, D., Angeloni, A., Niederman, J. & Miller, G. Detection of human serum antibodies to the BFRF3 Epstein-Barr virus capsid component by means of a DNA-binding assay. *Journal of Infectious Diseases* **172**, 1367-1370 (1995).
- 40 Schulz, T. Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8): epidemiology and pathogenesis. *Journal of Antimicrobial Chemotherapy* **45**, 15-27 (2000).
- 41 Kúdelová, M. *et al.* Recombinant herpesviruses as tools for the study of herpesvirus biology. *Acta virologica* **57**, 149 (2013).

- 42 Wong-Ho, E. *et al.* Unconventional sequence requirement for viral late gene core promoters of murine gammaherpesvirus 68. *Journal of virology* **88**, 3411-3422 (2014).
- 43 Chang, Y. *et al.* Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* **266**, 1865-1869 (1994).
- 44 Massimelli, M. J. *et al.* Multiple Regions of Kaposi's Sarcoma-Associated Herpesvirus ORF59 RNA are Required for Its Expression Mediated by Viral ORF57 and Cellular RBM15. *Viruses* **7**, 496-510 (2015).
- 45 Zheng, D. *et al.* Comparison of Humoral Immune Responses to Epstein-Barr Virus and Kaposi's Sarcoma-Associated Herpesvirus Using a Viral Proteome Microarray. *Journal of Infectious Diseases* **204**, 1683-1691 (2011).
- 46 Kelly, B. J., Fraefel, C., Cunningham, A. L. & Diefenbach, R. J. Functional roles of the tegument proteins of herpes simplex virus type 1. *Virus research* **145**, 173-186 (2009).
- 47 Wood, B. A., Carver, S., Troyer, R. M., Elder, J. H. & VandeWoude, S. Domestic cat microsphere immunoassays: Detection of antibodies during feline immunodeficiency virus infection. *Journal of immunological methods* **396**, 74-86 (2013).
- 48 Bevins, S. N. *et al.* Three pathogens in sympatric populations of pumas, bobcats, and domestic cats: implications for infectious disease transmission. *PLoS One* **7**, e31403 (2012).
- 49 Liu, J., Chen, F., Yu, H., Zeng, P. & Liu, L. A two-stage Bayesian method for estimating accuracy and disease prevalence for two dependent dichotomous screening tests when the status of individuals who are negative on both tests is unverified. *BMC medical research methodology* **14**, 110 (2014).
- 50 Jenson, H. B. Epstein-Barr virus. *Pediatrics in Review-Elk Grove* **32**, 375 (2011).
- 51 Baillargeon, J., Leach, C. T., Deng, J. H., Gao, S. J. & Jenson, H. B. High prevalence of human herpesvirus 8 (HHV-8) infection in south Texas children. *Journal of medical virology* **67**, 542-548 (2002).
- 52 Alkharsah, K. R., Dediccoat, M., Blasczyk, R., Newton, R. & Schulz, T. F. Influence of HLA alleles on shedding of Kaposi sarcoma-associated herpesvirus in saliva in an African population. *Journal of Infectious Diseases* **195**, 809-816 (2007).
- 53 Tasker, S. Haemotropic mycoplasmas: what's their real significance in cats? *Journal of feline medicine and surgery* **12**, 369-381 (2010).
- 54 de Oliveira Medeiros, S., Martins, A. N., Dias, C. G. A., Tanuri, A. & de Moraes Brindeiro, R. Natural transmission of feline immunodeficiency virus from infected queen to kitten. *Virology journal* **9**, 1-7 (2012).
- 55 Martin, J. N. *et al.* Sexual transmission and the natural history of human herpesvirus 8 infection. *The New England journal of medicine* **338**, 948-954, doi:10.1056/NEJM199804023381403 (1998).
- 56 Taylor, M. M. *et al.* Shedding of human herpesvirus 8 in oral and genital secretions from HIV-1-seropositive and -seronegative Kenyan women. *The Journal of infectious diseases* **190**, 484-488, doi:10.1086/421466 (2004).
- 57 Plancoulaine, S. *et al.* Human herpesvirus 8 transmission from mother to child and between siblings in an endemic population. *Lancet* **356**, 1062-1065, doi:10.1016/S0140-6736(00)02729-X (2000).
- 58 Mantina, H. *et al.* Vertical transmission of Kaposi's sarcoma-associated herpesvirus. *International journal of cancer* **94**, 749-752 (2001).
- 59 Hadgu, A., Dendukuri, N. & Hilden, J. Evaluation of nucleic acid amplification tests in the absence of a perfect gold-standard test: a review of the statistical and epidemiologic issues. *Epidemiology* **16**, 604-612 (2005).

- 60 Alonzo, T. A. & Pepe, M. S. Using a combination of reference tests to assess the accuracy of a new diagnostic test. *Statistics in medicine* **18**, 2987-3003 (1999).
- 61 Nystad, T. W. & Myrnes, H. Prevalence of primary versus reactivated Epstein-Barr virus infection in patients with VCA IgG-, VCA IgM- and EBNA-1-antibodies and suspected infectious mononucleosis. *Journal of clinical virology* **38**, 292-297 (2007).
- 62 Keymeulen, B. *et al.* Transient Epstein-Barr virus reactivation in CD3 monoclonal antibody-treated patients. *Blood* **115**, 1145-1155 (2010).
- 63 Quinlivan, E. B. *et al.* Longitudinal sero-reactivity to human herpesvirus 8 (KSHV) in the Swiss HIV Cohort 4.7 years before KS. *J Med Virol* **64**, 157-166 (2001).
- 64 McDowell, M. E., Purushothaman, P., Rossetto, C. C., Pari, G. S. & Verma, S. C. Phosphorylation of Kaposi's Sarcoma-Associated Herpesvirus Processivity Factor ORF59 by a Viral Kinase Modulates Its Ability To Associate with RTA and oriLyt. *Journal of virology* **87**, 8038-8052 (2013).
- 65 Vittone, V. *et al.* Determination of interactions between tegument proteins of herpes simplex virus type 1. *J Virol* **79**, 9566-9571, doi:10.1128/JVI.79.15.9566-9571.2005 (2005).
- 66 Chouljenko, D. V. *et al.* Functional hierarchy of herpes simplex virus 1 viral glycoproteins in cytoplasmic virion envelopment and egress. *J Virol* **86**, 4262-4270, doi:10.1128/JVI.06766-11 (2012).
- 67 Zhou, Z. H., Chen, D. H., Jakana, J., Rixon, F. J. & Chiu, W. Visualization of tegument-capsid interactions and DNA in intact herpes simplex virus type 1 virions. *J Virol* **73**, 3210-3218 (1999).

66 Tasker, Séverine. "Haemotropic mycoplasmas: what's their real significance in cats?." *Journal of feline medicine and surgery* 12.5 (2010): 369-381.

67 de Oliveira Medeiros, Sheila, et al. "Natural transmission of feline immunodeficiency virus from infected queen to kitten." *Virology journal* 9.1 (2012): 1-7.