Virology 460-461 (2014) 100–107

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Felis catus gammaherpesvirus 1; a widely endemic potential pathogen of domestic cats

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ARTICLE INFO

Article history:
Received 22 March 2014
Returned to author for revisions
7 April 2014
Accepted 7 May 2014
Available online 29 May 2014

Keywords:
Gammaherpesvirus
Feline
Molecular epidemiology
Copathogen
Transmission

ABSTRACT

Felis catus gammaherpesvirus 1 (FcaGHV1), recently discovered in the USA, was detected in domestic cats in Australia (11.4%, 95% confidence interval 5.9–19.1, $n=110$) and Singapore (9.6%, 95% confidence interval 5.9–14.6, $n=176$) using qPCR. FcaGHV1 qPCR positive cats were 2.8 times more likely to be sick than healthy. Risk factors for FcaGHV1 detection included being male, increasing age and coinfection with pathogenic retroviruses, feline immunodeficiency virus (FIV) or feline leukaemia virus. FcaGHV1 DNA was detected in multiple tissues from infected cats with consistently high virus loads in the small intestine. FcaGHV1 viral load was significantly higher in FIV-infected cats compared with matched controls, mimicking increased Epstein–Barr virus loads in human immunodeficiency virus-infected humans. FcaGHV1 is endemic in distant geographic regions and is associated with being sick and with coinfections. Horizontal transmission of FcaGHV1 is supported, with biting being a plausible route. A pathogenic role for FcaGHV1 in domestic cats is supported.

Introduction

Gammaherpesviruses (GHVs) are one of three subfamilies in the family *Herpesviridae*, order *Herpesvirales* (King et al., 2012). GHVs are classified within four genera; Lymphocryptovirus, Rhandinovirus, Macavirus and Percavirus. They infect a diverse range of vertebrates including humans and other primates, several ruminants, squirrels, badgers and sea lions (King et al., 2012). Persistent infection is characteristic of GHVs but their pathogenicity is variable. In the virus's natural host, infection is often inapparent as long as antiviral immunity is maintained. In the absence of an effective immune response, either because of immune dysfunction or infection of a susceptible but non-adapted host, GHVs cause devastating diseases including lymphoma and malignant catarrhal fever.

We recently reported the molecular identification of the first GHV of the domestic cat, *Felis catus* gammaherpesvirus 1 (FcaGHV1), bobcat (LruGHV1), both clustering in the Percavirus genus, and a puma GHV (PcoGHV1) most closely related the rhadinoviruses (Troyer et al., 2014). The global domestic cat population is estimated at 600 million (Peterson et al., 2012). The position of cats as the world's principal companion animal drives an expanding market for specialist veterinary care (Batson, 2008). With an owned cat population of 74 million in North America alone, demand for vaccines and therapeutics targeting a novel feline pathogen is likely to be high (AVMA, 2012). Increasing levels of cat-human contact demands vigilance regarding the zoonotic potential of novel feline infections; zoonoses are the predominant source of emerging infectious diseases (Jones et al., 2008). Feline pathogens may also impact wildlife biodiversity. Pathogen spill-over from free-roaming cats accompanying expanding human habitats presents a potential risk to sympatric wildlife populations (Bevins et al., 2012). Thus, determination of the pathogenic potential of FcaGHV1 is a priority for feline, human and wildlife health.

The development of a vaccine for the GHV Epstein–Barr virus (EBV), a lymphocryptovirus, has been identified as a significant goal for human medicine (Cohen et al., 2011), though the absence of a suitable natural host-virus model is a hurdle to progress in this area (Yajima et al., 2008). EBV is responsible for 200,000 new

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http://dx.doi.org/10.1016/j.virol.2014.05.007
0042-6822/

malignancies/year, or 2% of the world's cancers (Thun et al., 2010; Fukayama and Ushiku, 2011), as well as significant morbidity from infectious mononucleosis in young adults (Macsween et al., 2010). It was the role of human GHVs as copathogens in HIV infection (Cesarman, 2011) that raised suspicion for the existence of a GHV in the domestic cat (Beatty et al., 2012). In HIV-infected patients, EBV is causal in 80% of non-Hodgkin's lymphomas (Gloghini et al., 2013). Cats infected with feline immunodeficiency virus (FIV) are susceptible to immune dysfunction and aggressive B cell lymphomas that mirror the consequences of HIV infection (Vahlenkamp et al., 2006; Shelton et al., 1990). Therefore, if FcaGHV is revealed to be a feline pathogen, its suitability as natural model for human GHV infections would be relevant.

We reported widespread FcaGHV1 infection of free-ranging feral cats in the USA with a mean prevalence of 16% (95% confidence interval 10% to 22%), with increasing age, geographic location, and being male as risk factors for GHV1 qPCR detection (Troyer et al., 2014). In this study, we investigated whole blood from client-owned or shelter cats from Australia and Singapore and found that FcaGHV1 DNA was also present in these populations at similar prevalence rates. Sequence analysis revealed identical or nearly identical GHV sequences in cats from all three continents over a 315 bp region of the genome. The association between FcaGHV1 infection and health status was also investigated. Risk factor analyses were conducted for populations from Australia, Singapore and the previously reported USA population. We also evaluated the effect of FIV infection on FcaGHV1 virus load, and FcaGHV1 DNA was quantitated in tissues from infected cats to investigate tissue tropism.

Results

Prevalence and geographic distribution of FcaGHV1

FcaGHV1 DNA was detected in whole blood from domestic cats from Australia and Singapore (Fig. 1A). The prevalence in Australia, 11.4% (95% confidence interval [CI] 5.9% to 19.1%), was similar to that in Singapore, 9.6% (95% CI 5.9% to 14.6%) (Fig. 1B). In Australia, the probability of FcaGHV1 infection did not differ between states; Queensland, New South Wales and Victoria ($D_{2,87}=2.299$, $P=0.317$). In the USA, the prevalence of FcaGHV1 was 19.1% (95% CI 12.5% to 27.1%), which is slightly higher than previously reported for a larger population from the USA (see Troyer et al., 2014 for prevalence differences among states and counties) and higher than prevalence in Australia or Singapore ($D_{2,373}=5.310$, $P=0.070$). The average FcaGHV1 prevalence across all three countries was 12.8% (95% CI 9.7% to 16.5%).

FcaGHV sequence variation

Partial gB sequences (315 nucleotides) from Australian cats (GenBank KJ561572) were identical to those from USA. A single, identical, synonymous nucleotide polymorphism was detected in all Singapore samples sequenced (GenBank KJ561573). At nucleotide 126 of the published partial FcaGHV1 gB sequence (GenBank KF840715) (Troyer et al., 2014). cytosine (USA and Australia) is replaced with thymidine (Singapore).

Effect of FcaGHV1 on health status

Data on health status were available for cats from Australia and Singapore. FcaGHV1 DNA positive cats were more likely to be classified as sick (Table 1). On average, FcaGHV1 DNA positive domestic cats were 2.82 (95% CI 1.24% to 6.58%) times more likely to be sick than FcaGHV1 negative cats. An effect of age was detected on health status too, with older cats being more likely to be sick (Table 1). There was no association between sex and health status (Table 1).

Sex, age and copathogens as risk factors for FcaGHV1 detection

Overall (all countries combined), FcaGHV1 detection was positively associated with individual sex (male > female) and age (being adult) (Table 2). These findings were most distinct in Australia and USA, which is consistent with our recent findings (Troyer et al., 2014). A notable difference was found in Singapore where FcaGHV1 status was unrelated to sex or age (Table 2). Remarkably, in Australia and USA, the FcaGHV1 positive populations were 90% (9 of 10) and 100% (21 of 21) male respectively, where the total population from each country comprised 65.9% and 56.9% male cats. In contrast, the Singapore FcaGHV1 positive population was 50% male (7 of 14) in a population that was 47.6% male.

The most striking positive copathogen association was noted between FIV infection and FcaGHV1 detection in Singapore (Table 2). In the USA, there were fewer FIV-infected cats tested for FcaGHV1 DNA, and this relationship was not statistically significant (Table 2). Nevertheless, a greater proportion of the FcaGHV1 positive population in the USA was FIV-infected compared with FcaGHV1 negative cats

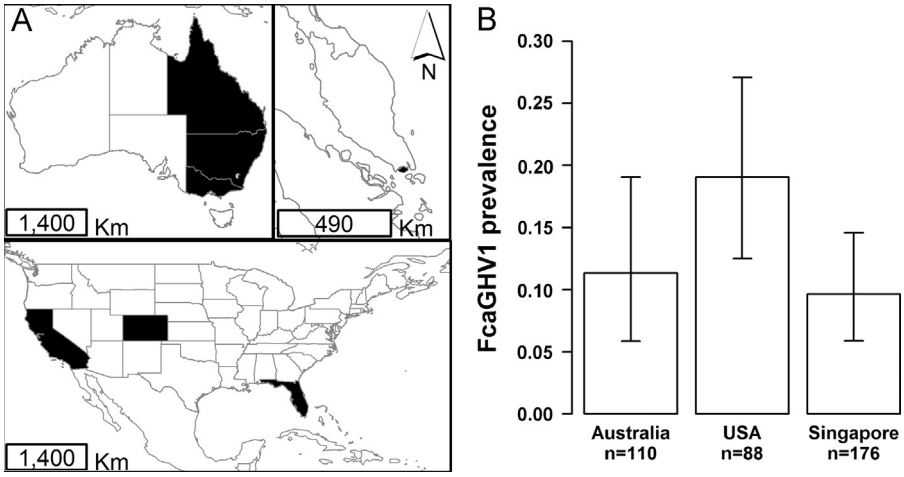

Fig. 1. Location of samples (A) and maximum likelihood estimates (± 95% CIs) (B) of FcaGHV1 prevalence among countries. States within Australia (Queensland, New South Wales and Victoria from north to south) and the USA (California, Colorado and Florida, from west to east) from which samples were sourced shaded black. Similarly, Singapore, off the southern tip of the Malaysian peninsular, is shaded black.

Table 1
FcaGHV1 detection and demographic factors associated with health status.[a]

	Deviance	df	*P*	Deviance	df	*P*	Deviance	df	*P*
	Combined			Australia			Singapore		
FcaGHV1	6.138	1	**0.013**	2.873	1	0.090*	2.948	1	0.086*
Age	26.12	1	< **0.001**	19.272	1	< **0.001**	9.762	1	**0.002**
Sex	0.007	1	0.935	0.257	1	0.612	0.098	1	0.754
Residuals		255			81			173	

[a] Statistics represent outcomes of binomial regression with all predictor variables included in the model. Predictor variables are also binomial (0 or 1), with 1 equal to male, adult, or a positive FcaGHV1 test result. Bold = significant results ($P < 0.05$).
* trending results ($P = 0.05$–0.1). Significant or trending results represent positive risk factor relationships.

Table 2
Demographic factors and pathogens associated with FcaGHV1 detection.[a]

	Deviance	df	*P*	Deviance	df	*P*
	Combined			Australia		
Sex	18.031	1	< **0.001**	3.895	1	**0.048**
Age	10.310	1	**0.001**	4.991	1	**0.025**
Bartonella IgG	3.191	1	0.074*	2.188	1	0.139
B. henselae	0.039	1	0.843	2.685	1	0.101
B. clarridgeiae	0.172	1	0.678	0.367	1	0.544
Mhm	20.120	1	< **0.001**	3.649	1	0.056*
Mhf	6.602	1	**0.010**	4.032	1	**0.045**
Residuals		83			343	
	USA			Singapore		
Sex	27.183	1	< **0.001**	0.084	1	0.771
Age	8.750	1	**0.003**	0.080	1	0.777
FIV Ab	1.108	1	0.292	16.538	1	< **0.001**
FeLV Ag				4.605	1	**0.032**
Toxoplama IgG	0.001	1	0.999	0.013	1	0.908
Bartonella IgG	1.780	1	0.182	0.004	1	0.948
B. henselae	1.289	1	0.256	0.166	1	0.684
B. clarridgeiae	0.395	1	0.530	0.255	1	0.614
Mhm	2.984	1	0.084*	9.717	1	**0.002**
Mhf	0.012	1	0.913	7.899	1	**0.005**
Residuals		92			166	

[a] Statistics represent outcomes of binomial regression with all variables included in the model. Variables were also binomial (0 or 1), with 1 equal to male, adult, or a positive serological or PCR test result. Bold = significant results ($P < 0.05$).
* trending results ($P = 0.05$–0.1). Significant or trending results represent positive risk factor relationships. Correlated copathogen status is shown in Table S2. Ab = antibody, Ag = antigen, *B. henselae, B. clarridgeiae, Candidatus Mycoplasma haemominutum* (Mhm) and *Mycoplasma haemofelis* (Mhf) were detected using PCR.

(Table 3). FIV-infected cats from Australia, investigated in a separate group with a well-defined age and sex-matched control group, had a significantly greater likelihood of having detectable FcaGHV1 relative to matched controls ($D_{1,52} = 8.22$, $P = 0.004$; odds ratio 5.500 [1.69–20.51]). Of the 27 individuals that were FIV-infected, 15 were also FcaGHV1 positive whereas, of the 27 FIV-uninfected individuals only 5 were FcaGHV1 positive. Data on another pathogenic feline retrovirus, FeLV, were available for cats from Singapore. FeLV infection was significantly associated with FcaGHV1 detection in this group.

There were also positive associations between detection of FcaGHV1 DNA and Mhm or Mhf infection (Table 2). This relationship was significant for both hemoplasmas in Singapore and for Mhf in Australia, but was non-significant in the USA. We found no significant relationship between *Bartonella spp* or *T. gondii* exposure and detection and FcaGHV1 at any site.

The relationships identified in Singapore, where FIV status, but not sex or age, represented a risk factor for FcaGHV1 were unexpected. This is because it is well established that adult, male domestic cats are at increased risk from FIV infection in most international epidemiological studies, including those from Australia and USA (Liem et al., 2013; Levy et al., 2006). Reports of risk factors for FIV infection in Singapore specifically are not available. Therefore, we investigated the relationship between sex, age and FIV status in Singapore to reveal any regional differences. Interestingly, sex was not a determinant of FIV status in Singapore, while age was (Sex $D_1 = 1.85$, $P = 0.174$; Age $D_1 = 7.22$, $P = 0.007$, residual df_{209}). These data demonstrate that being male is a risk factor for FcaGHV1 infection in geographic regions where being male is widely reported as a risk factor for FIV infection. This suggests that being male per se does not necessarily relate to increased inherent GHV exposure, as would be concluded by looking only at USA and Australia populations.

Effect of FIV or FeLV infection on FcaGHV1 load

FIV-infected cats from Australia had significantly higher FcaGHV1 loads than matched FIV-uninfected cats ($F_{1,18} = 8.25$, $P = 0.010$, Fig. 2). For cats from Singapore and USA, no significant effect of retrovirus infection on FcaGHV1 load was detected (USA—FIV $F_{1,19} = 0.29$, $P = 0.6$; Singapore—FIV $F_1 = 1.5$, $P = 0.25$, FeLV $F_1 = 2.04$, $P = 0.18$, residual df_{14}). The difference in observed results between regions is most likely due to the greater numbers of FIV-infected cats and the robust control group, matched for age and sex, in Australia although regional influences cannot be fully excluded. Our results suggest that, when cat age and sex are tightly controlled, FIV infection is accompanied by higher FcaGHV1 loads.

Quantitative FcaGHV1 tissue distribution

We quantified FcaGHV1 DNA load in tissues from 3 FIV-infected cases. Most tissue types yielded at least one positive qPCR result for FcaGHV1 DNA. Tissues that were consistently positive in all cases were small intestine, liver and spleen. We detected significant individual variability in FcaGHV1 viral loads among tissues (Fig. 3A) using Chi-square goodness of fit test assuming uniform tissue distribution (Cat 1 $X^2_{14} = 17{,}544$, $P < 0.001$; Cat 2 $X^2_{13} = 192{,}624$, $P < 0.001$; Cat 3 $X^2_{14} = 640{,}129$, $P < 0.001$). Differences were observed between tissues in DNA load, ranging from 88 to 2645 FcaGHV1 DNA copies per million cells. Small intestine was the only tissue found to have higher than expected viral loads, relative to the assumption of even distribution among tissues, in all cats (Fig. 3B). We detected inter-individual variability in whether viral loads were greater or less than expected for spleen, bone marrow and kidney (Fig. 3B). For the remaining tissues examined, FcaGHV1 loads were lower than expected. Notably, these samples included tumour tissue from two cats with neoplasia.

Discussion

We have shown that FcaGHV1 infects domestic cats from Australia and Singapore. Together with our previous findings from the USA, this demonstrates that the virus is present on at least three continents. Sequence comparisons demonstrated a minor strain difference in a conserved region of the genome in Singapore

Table 3
Numbers of FcaGHV1 negative and positive individuals associated with each variable, sex, age and co-pathogen status, for regions combined and individually.[a]

		Combined		Australia		USA		Singapore	
		Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
Sex	Female	152	8	29	1	44	0	79	7
	Male	156	38	49	9	37	21	70	8
Age	Young	105	6	29	1	19	0	57	5
	Adult	214	41	49	9	63	21	102	11
FIV Ab	Negative	–	–	–	–	79	16	139	6
	Positive	–	–	–	–	10	5	19	10
FeLV Ag	Negative	–	–	–	–	–	–	142	10
	Positive	–	–	–	–	–	–	16	6
T. gondii IgG	Negative	–	–	–	–	88	21	145	15
	Positive	–	–	–	–	1	0	8	1
Bartonella IgG	Negative	244	29	44	4	65	10	135	15
	Positive	72	18	30	6	24	11	18	1
B. henselae	Negative	300	45	63	10	80	18	157	17
	Positive	22	3	11	0	9	3	2	0
B. clarridgeiae	Negative	310	45	68	10	85	18	157	17
	Positive	12	3	6	0	4	3	2	0
Mhm	Negative	269	21	63	6	59	6	147	9
	Positive	45	27	11	4	22	15	12	8
Mhf	Negative	307	39	74	9	76	16	157	14
	Positive	7	9	0	1	5	5	2	3

[a] Variability in the number of sample combinations for FcaGHV1 and each variable is controlled for in risk factor analysis through reduction to a complete dataset (see Table 1). Dashed lines indicate variables not evaluated. Ab=antibody, Ag=antigen, Mhm=*Candidatus Mycoplasma haemominutum*, Mhf=*Mycoplasma haemofelis*.

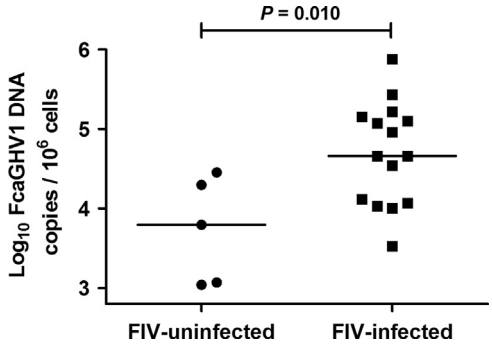

Fig. 2. Effect of FIV infection on FcaGHV1 load for Australian cats. FcaGHV1 loads are significantly higher among FIV-infected cats. Data was obtained from FIV-infected cats ($n=27$) and FIV-uninfected age and sex matched controls ($n=27$). All cats were FeLV-antigen negative.

FcaGHV1 isolates compared with those from Australia and USA. Comparison of longer sequences from multiple genes will be informative to reveal the extent of regional heterogeneity. This resembles a previous observation in GHV-infected bobcats (*Lynx rufus*) where several nucleotide substitutions in gB were detected in LruGHV1 isolates from bobcat populations in Florida compared with those in California (Troyer et al., 2014). While data from more locations will be informative, detection of FcaGHV1 DNA in peripheral blood at a similar prevalence among cats from the first three regions to be investigated supports FcaGHV1 being widely endemic in domestic cats worldwide.

Our qPCR assay, the first diagnostic tool for FcaGHV1, allows rapid quantitation of FcaGHV1 DNA in clinical samples. While this assay is highly specific for FcaGHV1, its sensitivity for detecting infected individuals has not yet been evaluated. Since latent infection is characteristic of herpesviruses, qPCR almost certainly underestimates the true prevalence of FcaGHV1 infection in domestic cats. In comparison, peripheral EBV DNA load in asymptomatic adult humans is low or undetectable, contrasting with a seroprevalence of around 95% (Stevens et al., 2001; Rowe et al., 1997). It is worth noting that serology is therefore unhelpful in diagnosing EBV-associated disease whereas measurement of EBV DNA load by qPCR is an important clinical tool in the management of several EBV-associated disorders (Kimura et al., 2008). For FcaGHV1, an antibody detection assay will be informative to identify cats that have been exposed to FcaGHV1 but do not have DNA detectable in whole blood and may shed further light on natural routes of transmission.

Our findings provide insight into potential mechanisms of natural transmission. The increased infection risk in adult cats supports our previous contention that horizontal mechanisms of transmission predominate. Evidence points to transmission of FcaGHV1 during aggressive territorial behaviour being a credible route. Territorial behaviour of male wood mice has been suggested as a major route of natural transmission of murine gammaherpesviruses (Knowles et al., 2012). It is plausible that FcaGHV1 shares common routes of transmission with copathogens identified as risk factors. Inoculation of saliva during a cat fight is the major natural mode of FIV transmission (Yamamoto et al., 1989). There is now mounting evidence that intercat aggression represents an important mode of transmission for feline hemoplasmas. Hemoplasma DNA is found in saliva (Dean et al., 2008) and risk factors for hemoplasma infection include increasing age, male sex, outdoor access, FIV-infection and evidence of a cat bite (Tasker, 2010). FeLV spreads rapidly through non-aggressive contact between cohabiting cats but can also be effectively transmitted by biting (Goldkamp et al., 2008; Hardy et al., 1976). The observed increased risk of FcaGHV1 detection in FIV or FeLV infection may have an alternative or additional explanation. Immunosuppression resulting from retrovirus coinfection may permit increased replication of FcaGHV1. For some cats, this might elevate a peripheral FcaGHV1 DNA load that is otherwise below the limit of detection of the qPCR into the detectable range. It should also be considered that the increased FcaGHV load identified here in FIV-infection may increase the risk of transmission from FIV-infected cats if a higher dose of virus is inoculated in blood or saliva during fighting.

Unique regional considerations may underlie the difference in risk factors identified for FcaGHV1 infection in Singapore. Most notable is the absence of a sex predisposition in Singapore which contrasts sharply the detection of only a single female cat among FcaGHV1-infected populations in Australia and USA. Singapore is a densely-populated, urban environment with a large street cat population. Street cats live in free-roaming colonies with food

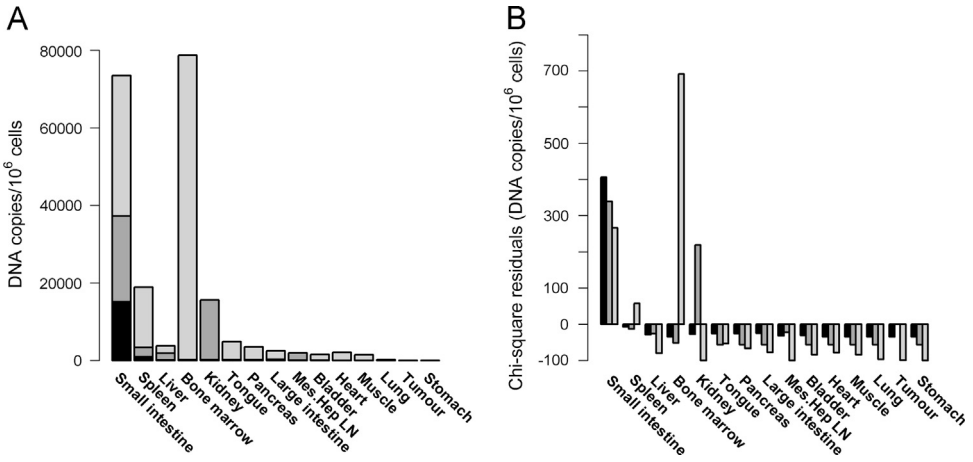

Fig. 3. Tissue tropism of FcaGHV1. (A) Tissue FcaGHV1DNA loads for three cases (denoted by bar shading) and (B) residual viral load scores from the chi-square goodness of fit analysis supporting tropism in some tissues over others and individual variability among cats.

provided by persons in the community. Anecdotally, client-owned cats are commonly derived from the street cat population. Equal participation of both sexes in territorial aggression, perhaps related to increased competition for resources, may explain the absence of a sex predisposition for FcaGHV1 infection in Singapore. In support of this notion, we found male cats in Singapore were as likely as female cats to be infected with FIV, a virus spread through biting and fighting. We reported a similar absence of sex as a risk factor in wild cat GHVs (Troyer et al., 2014), which might similarly reflect more opportunities for spread among intact female felids with opportunities for territorial disputes. In Singapore, age did not predict FcaGHV1 detection. Fighting at an earlier age in this region was not supported by FIV risk which was higher in adults. The association between FcaGHV1 and age should thus be studied in cat populations from other regions to associate behaviours relating to disease risk.

Recent data supporting arthropod-borne transmission for a GHV comes from the finding that immature ticks, *Ixodes ricinus,* can harbour the murine gammaherpesvirus, MuHV4 (MHV68, genus Rhadinovirus) (Ficova et al., 2011). No significant role for flea-borne transmission of FcaGHV1 is suggested by our data. In contrast to the copathogens discussed above, the major route of natural inter-cat transmission of *Bartonella spp* is indirect, via the flea vector, *Ctenocephalides felis*, or via flea feces. *Bartonella spp* exposure or DNA detection were consistently unrelated to FcaGHV1 infection. Further, the prevalence of FcaGHV1 DNA detection in a population selected for having a live flea burden (Australia) was not significantly different from Singapore cats, none of which had evidence of flea infestation at the time of sampling. It will be of interest to determine whether ectoparasites can harbour FcaGHV1 DNA to elucidate their potential as vectors or the existence of reservoir hosts.

We conducted the first investigation of FcaGHV1 DNA tissue loads. FcaGHV1 exhibits the ability to infect a broad range of tissues, but displays putative tropism for the small intestine. While amplification of virus from peripheral blood within tissues is expected, this source would not explain the consistent differences in FcaGHV1 load detected between regions of the gastrointestinal tract that are similarly vascularized. Similarly, viral load did not parallel the expected tissue blood supply in a previous study of ovine gammaherpevirus 2 (OvHV2, genus Macavirus) tissue distribution in sheep where, for example, cornea which is avascular, had a higher OvHV2 DNA load than skeletal muscle (Hussy et al., 2002). In agreement with our observations, compartmentalization of virus in the small intestine has been reported in sheep naturally infected with the OvHV2 (Hussy et al., 2002). It should be noted that one cat had a very high FcaGHV1 DNA load in the bone marrow. Prior FIV infection may alter FcaGHV1 tissue loads or even tropism. Future studies including a broad range of tissues in more cats, including FIV-uninfected cats, will determine the significance of these findings. Of particular interest will be the high FcaGHV1 load in the small intestine in relation to shedding or pathogenesis.

An interesting association between FcaGHV1detection and health status was identified, with FcaGHV1 positive cats having higher odds of being sick. This broad categorization of health status, even when performed by a veterinarian, will inevitably result in misclassification of some individuals. While this method is not ideal, it is accepted for epidemiological studies, since full investigation of individual patients is not practical (Levy et al., 2006; O'Connor et al., 1991). It was important to also examine the effect of age and sex on health status since both were strongly associated with FcaGHV1 status in Australia and the USA. Sex was not related to health status. That cats older than 2 years were more likely to be sick than cats aged 6 months to 2 years is perhaps not surprising. The older group encompasses a greater range of potential age-associated problems and, in the younger group, paediatric problems have been excluded. Our findings do not prove that FcaGHV1 infection is a cause of poor health in cats. As described previously with FIV, concurrent illness might result in escape of chronic FcaGHV1 from latency, providing a greater likelihood of detecting infection using qPCR methodology. Further studies of a role for FcaGHV1 in feline disease are warranted.

Our observation that cats coinfected with FIV have higher FcaGHV1 loads than retrovirus-negative cats is intriguingly similar to elevated EBV loads in HIV-infected people. HIV infection of EBV-infected individuals is followed by an early rise in EBV load that occurs even before CD4 counts fall (Piriou et al., 2004). The source of the increased EBV load is postulated to be proliferation of B cells (memory B cells being the reservoir of EBV infection), a feature of the HIV-mediated immune dysregulation (Pietersma et al., 2008). In FIV-infected cats, polyclonal B-cell stimulation is well documented (Flynn et al., 1994), indicating the potential for similar mechanisms underlying the increased FcaGHV1 load observed in FIV- infection. Future studies will determine whether FcaGHV1 infection in the domestic cat might represent a natural model for GHV pathogenesis.

Material and methods

Ethics statement

Samples used this study were collected according to the University of Sydney Animal Ethics Committee approvals (N00/6-2009/1/4985; N00/7-2013/3/6029), Section 53 of the Animals and Birds Act (Cap 5)

Agri-Food and Veterinary Authority of Singapore and the Colorado State University Animal Care and Use Committee or appropriate institutional, local, and state agencies (Bevins et al., 2012).

Samples for molecular epidemiological studies

Australia: Whole blood DNA from client-owned, domestic cats from Eastern Australia was available for FcaGHV qPCR ($n=110$) (Barrs et al., 2010). Data available for analyses were: sex, age (6 months—2 years or >2 years), neuter status, environment (indoor only or outdoor access), state of domicile (New South Wales, Queensland or Victoria), serology for *Bartonella* spp IgG (Lappin et al., 2009) and results of PCR assays for *Mycoplasma haemofelis* (Mhf), *Candidatus Mycoplasma haemominutum* (Mhm) (Jensen et al., 2001), *Bartonella henselae* and *Bartonella clarridgeiae* (Jensen et al., 2000). Cats were classified as either healthy or sick by the attending veterinarian based on the findings of a physical examination and, where available, medical history. The healthy classification included cats that were systemically well but did not exclude cats with minor problems. Cats classified as sick were systemically unwell. All cats had an active flea burden at the time of sampling.

Whole blood DNA was available from a second group of client-owned cats from Australia to evaluate FIV as a risk factor for FcaGHV1 in cats from Australia. These cats were either FIV-infected cats ($n=27$) or age-matched and sex-matched, FIV uninfected controls ($n=27$) from Australia. This population was distinct from the Australian cat population described above. "FIV-infected" and "uninfected" were defined as described previously (Liem et al., 2013). All cats were seronegative for FeLV antigen.

Singapore: Whole blood DNA from client-owned ($n=106$) and shelter-housed ($n=70$) domestic cats from Singapore was available for FcaGHV1 qPCR (Chan et al., 2013). Data available for analyses were as for the Australian sample above, with the addition of serology for FIV antibody, FeLV antigen, (Snap Combo FIV/FeLV, IDEXX Laboratories, Biomed Diagnostics, Singapore) and *Toxoplasma gondii* (*T. gondii*) IgG (Lappin et al., 1989). FeLV status was confirmed using PCR (Pinches et al., 2007). All cats were combed at the time of sampling and found to be free from fleas and flea feces.

USA: Data not previously analyzed were available for a subset ($n=88$) of free-ranging trap-neuter-release cats of known FcaGHV1 qPCR status described previously (Troyer et al., 2014; Bevins et al., 2012). Data available for analyses were: serology for FIV antibody, *T. gondii* IgG and *Bartonella spp*. IgG and results of PCR assays for *B. henselae, B. clarridgeiae*, Mhf and Mhm.

Tissues for FcaGHV1 quantitation

Tissues from three adult, male domestic shorthair cats from Australia were available for FcaGHV qPCR. These client-owned animals were presented for euthanasia and post-mortem examination with major problems of high-grade small intestinal lymphoma (Case 1), acute gastrointestinal hemorrhage (Case 2) and pulmonary carcinoma (Case 3). All cats were infected with FIV and were seronegative for FeLV antigen. Samples from tongue, heart, lung, skeletal muscle, stomach, small intestine, large intestine, spleen, liver, pancreas, abdominal lymph node, kidney, bladder wall and bone marrow, as well as tumor tissue in cases 1 and 3 only, were collected immediately after euthanasia, snap-frozen and stored at −80 °C.

GAPDH PCR

DNA was extracted from snap frozen tissues using proteinase K treatment and phenol-chloroform extraction. To confirm the presence of amplifiable template DNA, conventional PCR for feline glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed. Primers designed to amplify an 80 bp product were (GAPfwd) 5′-AAGGCTGAGAACGGGAAAC-3′ and (GAPrev) 5′-CATTTGATGTTGGCGGGATC-3′. PCR reactions contained 500 nM of each primer, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 1.5 mM $MgCl_2$, 1 unit of Platinum Taq DNA Polymerase (Life Technologies) and 1 μl of template containing up to 1 μg of DNA in a total volume of 50 μl. Cycling conditions were; initial denaturation at 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 45 s, primer annealing at 55 °C for 30 s, extension at 72 °C for 30 s and a final extension step at 72 °C for 7 min. Following electrophoresis on 2.5% agarose gels in Tris-acetate buffer containing 0.5 μg ethidium bromide/ml, the expected 80 bp product was visualized in all samples following ultraviolet illumination.

FcaGHV1 qPCR

FcaGHV1 DNA presence and load in DNA from whole blood or tissues, was detected by qPCR targeting the glycoprotein B (gB) gene of FcaGHV1, as described previously (Troyer et al., 2014).

Sequencing of FcaGHV gB

To confirm amplification of FcaGHV1 and to investigate sequence variation between geographic regions, 5 FcaGHV1 qPCR positive DNA samples each from Australian and Singapore were amplified by FcaGHV1-specific PCR. Primers FGHV-F1 (ACCTGCAC-CAGAGCATGAGA) and FGHV-R1 (TGTCCAGTACGTTAGCCAATCTTT) amplified a 360 bp region of the glycoprotein B gene (gB). Reactions contained GoTaq Hot Start Green Master Mix (Promega, Madison, WI), 400 nM primers, 50–500 ng of template DNA in a total volume of 50 μl. Cycling conditions were; initial denaturation at 94 °C for 2 min; followed by 35 cycles of 94 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s; followed by a 5 min extension at 72 °C. The product was visualized following electrophoresis on a 1.5% agarose gel with ethidium bromide. PCR products were purified using a commercial kit (QIAquick PCR purification kit; Qiagen, Valencia, CA) and sequenced in both directions by the Colorado State University Proteomics Facility. After removal of primer sequence, the resulting 315 bp gB sequences were aligned and compared with the FcaGHV1 sequence obtained from cats in USA (Genbank KF840715) using BioEdit (Hall, 1999).

Prevalence and geographic distribution of FcaGHV1

Prevalence of FcaGHV1 infection was estimated using maximum likelihood, with 95% confidence intervals profiled. We evaluated whether the prevalence of FcaGHV1 differed among countries using a binomial regression, using a likelihood ratio test, based on a chi-square, to calculate model deviance (D) and P-values (significance set at <0.05). This approach is more robust than a traditional logistic regression based on a z-statistic. Owing to the spatial distribution of domestic cat samples within Australia, we also evaluated how the probability of FcaGHV1 differed among states, again using a binomial regression. Samples from the USA were also derived across multiple states and we have previously reported their differences (Troyer et al., 2014).

Health status

To evaluate possible effects of FcaGHV1 infection on the health of domestic cats, we analyzed data available from Australia and Singapore on a qualitative measure of health status, as assessed by the attending veterinarian (healthy or sick). We used a binomial

regression to evaluate the effect of FcaGHV1 or age or sex on health status for Australia and Singapore and for both regions combined.

Risk factors for FcaGHV1 infection

A variety of predictors of individual FcaGHV1 infection status were available for analyses, with some variability of available predictors among countries (Table S1). Prior to undertaking analyses, and to avoid type-I statistical error associated with exceeding 1:10 predictor to data ratios (Zar, 2010; Hellard et al., 2012), we conducted a judicious pre-screening of the available suite of predictors among countries (Table S1). Following pre-screening, 'environment' and 'neuter status' were excluded from further analysis.

For all countries, we evaluated the effects of individual sex, age and infection status with *Bartonella* spp. (PCR amplification of *B. henselae*, *B. clarridgeiae* and serology for *Bartonella* IgG) and haemoplasma (PCR amplification of Mhf and Mhm) on FcaGHV1 status. Owing to the availability of additional copathogen information from the USA and Singapore, we also included FIV and *T. gondii* status, and FeLV status (Singapore only), while also maintaining conservative predictor to data ratios. These risk factor analyses were undertaken using binomial regression for all countries combined and individually. For cats from Australia, FIV as a risk factor for FcaGHV1 detection was conducted in a separate group using binomial regression.

Effect of FIV or FeLV infection on FcaGHV1 load

Peripheral virus load increases in EBV-associated diseases (Kimura et al., 2008). Increased GHV load is also seen in humans and other primates following coinfection with immunosuppressive retroviruses (van Baarle et al., 2002; Rivailler et al., 2004). We investigated whether a similar association exists between FcaGHV1 and retroviruses FIV (all regions) and FeLV (Singapore only). For cats from Australia, FcaGHV1 DNA load in FIV-infected cats was compared to load in a robust age and sex matched FIV-uninfected control group. For cats from Singapore and USA, the control population was not matched. In both cases, analyses were undertaken by analysis of variance. Prior to analyses, the distribution of FcaGHV1 load values was evaluated and normalized by log transformation.

FcaGHV1 tissue DNA load analysis

We used a chi-square goodness of fit test, assuming a null hypothesis of even distribution of FcaGHV1 load (DNA copies/10^6 cells) among tissues for each individual cat. Deviations from this null hypothesis were scrutinized by evaluating the chi-square residuals, where positive and negative values indicate higher and lower than expected viral loads respectively. All analyses described were undertaken in the program R (www.r-project.org) using the stats, stats4, and ltm packages.

Acknowledgments

This work was supported by a University of Sydney Thompson Fellowship awarded to JAB, a grant to RMT (PI) and JAB (CI) from the Morris Animal Foundation (D14FE-301), the Sarah & Anne Payten and the Dorothy Minchin Bequests of the Faculty of Veterinary Science, University of Sydney to JB, DVC-Research compact funds, University of Sydney to JB and a grant to SV from the NSF-NIH Ecology of Infectious Disease program (EF 0723676).

We thank our colleagues Mrs J. Bonhs and Drs T. Madsen, S. English, C. Helps, N. Evans, R. Gowan, R. Baral and A. Lingard for assistance with sample testing, collection and preparation.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.05.007.

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