Felis catus papillomavirus type 2 virus-like particle vaccine is safe and immunogenic but does not reduce FcaPV-2 viral loads in adult cats.

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Abstract

Felis catus papillomavirus type 2 (FcaPV-2) commonly infects the skin of domestic cats and has been associated with the development of skin cancer. In the present study, a FcaPV-2 virus-like-particle (VLP) vaccine was produced and assessed for vaccine safety, immunogenicity, and impact on FcaPV-2 viral load. This is the first report of the use of a papillomavirus VLP vaccine in domestic cats. The FcaPV-2 VLP vaccine was given to ten adult cats that were naturally infected with FcaPV-2, and a further ten naturally infected cats were sham vaccinated as a control group. The rationale for vaccinating cats already infected with the virus was to induce neutralizing antibody titers that could prevent reinfection of new areas of skin and reduce the overall viral load, as has been demonstrated in other species. Reducing the overall FcaPV-2 viral load could reduce the risk for subsequent PV-associated cancer. The vaccine in this study was well-tolerated, as none of the cats developed any signs of local reaction or systemic illness. In the treatment group, the geometric mean anti-papillomavirus endpoint antibody titers increased significantly following vaccination from 606 (95% CI 192–1913) to 4223 (2023 –8814), a 7.0-fold increase, although the individual antibody response varied depending on the level of pre-existing antibodies. Despite the immunogenicity of the vaccine, there was no significant change in FcaPV-2 viral load in the treatment group compared to the control group, over the 24 week follow-up period. A possible reason is that FcaPV-2 was already widespread in the basal skin layer of these adult cats and so preventing further cells from becoming infected had no impact on the overall viral load. Therefore,

these results do not support the use of a FcaPV-2 VLP vaccine to reduce the risk for PV-associated cancer in cats in which FcaPV-2 infection is already well established. However, these results justify future studies in which the vaccine is administered to younger cats prior to FcaPV-2 infection becoming fully established.

Keywords: Cat, FcaPV-2, papillomavirus, virus-like particle, vaccine, viral load

1. Introduction

Felis catus papillomavirus type 2 (FcaPV-2) is a non-enveloped DNA virus that infects the skin of domestic cats. Infection appears to be common as FcaPV-2 DNA has been detected in skin swabs from 39–98% of clinically normal cats (Geisseler et al., 2016; Munday & Witham, 2010; Thomson et al., 2015). In some cats, however, FcaPV-2 is thought to cause pre-neoplastic skin lesions such as viral plaques and Bowenoid *in-situ* carcinomas, and the virus has been associated with cutaneous squamous cell carcinoma (SCC); the evidence for these associations has been recently reviewed by Munday et al., 2017. Cutaneous SCCs are the most common malignant skin cancer of cats (Miller et al. 1991). Recent studies have suggested that FcaPV-2 may be involved in the development of 33–45% of cutaneous SCCs, particularly those that occur in densely haired (UV-protected) areas of skin and around one third of those on the nasal planum. (Munday et al., 2011; Munday et al., 2013; Thomson et al., 2016). Briefly, the evidence for a role of FcaPV-2 in these cancers includes the demonstration of FcaPV-2 DNA and gene-expression associated with alterations in host-cell protein expression within the cancers (Munday et al., 2011; Munday et al., 2012; Thomson et al., 2016). PV-associated SCCs also have a different biological behavior to non-PV-associated SCCs (Munday et al., 2003). Furthermore, the transforming properties of FcaPV-2 viral proteins have been demonstrated in cell culture (Altamura et al., 2016). In contrast, the majority of cutaneous SCCs that occur in UV-exposed areas, such as the pinna, do not exhibit evidence of PV involvement and are thought to be caused by the accumulation of UV-induced DNA damage (Munday et al., 2011; Thomson et al., 2016). SCCs are relatively slow growing and do not readily metastasize, yet they invade deep into surrounding tissues (Gross et al., 2005). Large SCCs on the nasal planum of cats may require nasal planum resection- an advanced surgery that is not always available in general practice (Jarrett et al. 2013). Given that FcaPV-2 is likely involved in a proportion of these cancers, it may be possible to prevent some of these cancers by vaccinating with a FcaPV-2 virus-like

particle vaccine, similar to those used to prevent human papillomaviral (HPV) induced cervical cancer in women (Paavonen et al. 2009).

Papillomavirus virus-like particle (VLP) vaccines consist of recombinantly expressed papillomavirus (PV) capsid (L1) proteins that self-assemble into empty PV capsids (Kirnbauer et al. 1992). Vaccination with these PV VLPs induces a strong antibody response in the recipient (Breitburd et al. 1995). These antibodies prevent infection of basal epithelial cells in the skin or mucosal surfaces by blocking the association of the native PV with the basement membrane, or at lower concentrations the antibodies prevent formation of a stable association with the cell surface (Day et al., 2010). This prevents the PV from entering the basal cells and establishing infection at that site. However, if the PV is already present in some basal cells at the time of vaccination, then the antibodies will have no effect on the intracellular virus (Kreider, 1963). The PV will persist in the basal cells and replicate in the overlying skin cells for some time until eventually being repressed by the cell-mediated immune response or, in very rare instances, inducing cancer development (Kreider, 1963). Thus, vaccination has no effect on existing infection and so should ideally be given prior to first exposure to the virus. In women, the HPV types that cause cervical cancer are sexually transmitted, so HPV vaccination is given to adolescent girls to prevent infection and any risk of subsequent cancer development (Paavonen et al. 2009).

Unfortunately, prophylactic vaccination against FcaPV-2 is not feasible in cats as the virus is widespread and kittens are exposed at birth, or soon thereafter, leaving no practical opportunity to vaccinate prior to first exposure to the virus (Thomson et al. 2015; Thomson et al., 2018). However, a recent study of vaccination against a cutaneous papillomavirus in mice has shown that it is possible to reduce the incidence of PV-induced skin tumors by vaccination regardless of previous exposure to the PV (Vinzón et al., 2014). In the study by Vinzón and colleagues (2014), vaccination of African multimammate mice (*Mastomys coucha*) already infected with *Mastomys natalensis* papillomavirus (MnPV) resulted in a 10- to 20-fold lower viral load and completely prevented the development of skin tumors, compared to non-

vaccinated mice of which 28% developed skin tumors. The authors suggested that this effect was most likely due to neutralizing antibodies blocking reinfection of neighboring cells in traumatized epithelium (Vinzón et al., 2014). At present, little is known about the immune response to FcaPV-2 infection in cats. Only one study has investigated the immune response to the virus, finding a seroprevalence of 22% in dermatologically healthy cats (Geisseler et al., 2016). However, several studies have shown high levels of FcaPV-2 shedding by adult cats (Geisseler et al., 2016; Thomson et al., 2015; Thomson et al., 2018). This may suggest that the natural antibody response against FcaPV-2 is inefficient at preventing reinfection of new areas of skin. Therefore, vaccination against FcaPV-2 might reduce the overall FcaPV-2 viral load in cats in the same way that vaccination against MnPV-1 reduced viral loads in mice (Vinzón et al., 2014). In this scenario, the overall FcaPV-2 viral load may slowly decline as existing foci of infection resolve and the establishment of new sites of infection are blocked by high levels of vaccine-induced antibodies. The overall reduction in viral load would be expected to reduce the risk of subsequent cancer development.

Therefore, the purpose of this study was to investigate the response to a FcaPV-2 VLP vaccine in adult cats. There were three specific study objectives. Firstly, to determine if the vaccine is safe; particularly in regards to local reactions at the vaccine site. This is important because persistent reactions at injection sites are a risk factor for injection-site sarcomas in cats (Hartmann et al., 2015). A FcaPV-2 VLP vaccine that reduces the risk for one type of cancer but increases the risk for another type would have no overall benefit. The second objective was to assess the immunogenicity of the vaccine. In particular, whether FcaPV-2 VLP vaccination could boost anti-PV antibody levels above the level induced by natural infection. The third and final objective was to assess whether vaccination against FcaPV-2 reduces the FcaPV-2 viral load in adult cats that have already been exposed to the virus. This study is the first published report of a PV VLP vaccine being used in domestic cats and the results will help to determine whether such a vaccine could be a feasible way to reduce the incidence of cutaneous SCCs in cats.

2. Material and Methods

2.1. Vaccine production

To create the FcaPV-2 virus-like particles (VLPs), a custom synthetic gene (Genscript, Piscataway, NJ, U.S.A.) encoding the full-length FcaPV-2 L1 capsid protein (Genbank EU796884) was cloned into a pAcUW51-derived vector containing the baculovirus p10 promoter. The recombinant baculovirus was generated using linearized baculovirus DNA (FlashBac ULTRA, Oxford Expression Technologies, Oxford, U.K.), and transfected into *Spodotera frugiperda* (Sf21) insect cells, then further amplified through two rounds of infection until an adequate viral titer was reached. To express and purify the FcaPV-2 VLPs, a 400 mL Sf21 suspension culture was infected at a multiplicity of infection of 1.0 and incubated for 3 days at 27°C with shaking. Following infection, the cells were lysed in 1% Triton-X100, and the VLP pelleted by ultracentrifugation at 100,000g after an initial 10,000g centrifugation step to remove cellular debris. The VLP-containing pellet was resuspended in phosphate buffered saline (PBS), loaded onto a CsCl gradient containing 1.2 and 1.4 g/cm^3 densities, and centrifuged at 100,000g for 18 hours at 4°C. The resulting VLP band was collected, desalted and concentrated by dialysis into 50% glycerol/PBS. The VLP was run on a Coomassie-stained SDS-PAGE gel to confirm the purity and expected size of the L1 protein. The protein band was extracted and submitted to mass spectrometry analysis (Center for Protein Research, University of Otago, New Zealand) to confirm identity. The structural integrity of the VLPs was confirmed by electron microscopy (Figure 1). VLP concentration was measured by spectrophotometry and a total of 6 mg, sufficient for the entire study, was produced and pooled at the start of the study to avoid any batch variation. A preservative (thimerosal 0.01%) was added, and the VLPs in glycerol solution was stored at −20°C prior to use. VLP production was timed so that the vaccination course was completed within 3 months of VLP production, preventing the need for prolonged storage of the VLPs. Each 500 µL dose of the final FcaPV-2 VLP vaccine contained 50 µg of FcaPV-2 VLPs adsorbed onto Alum adjuvant

(Imject Alum, Thermo Scientific, Waltham, MA, U.S.A.) in PBS with 1% glycerol and 0.0002% thimerosal. The sham vaccine contained all components except the FcaPV-2 VLPs.

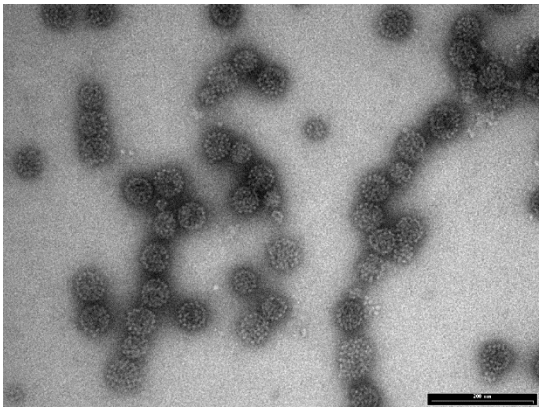


Figure 1. Electron micrograph showing FcaPV-2 virus-like particles (VLPs). The scale bar bottom right shows 200 nm and the VLPs are around 55 nm in diameter.

2.2. Animals

The study was conducted at a feline research colony where FcaPV-2 infection is ubiquitous and the cats are exposed to the virus early in life (Thomson et al., 2018). Twenty adult domestic short hair cats were allocated into two groups of ten cats with similar FcaPV-2 viral loads, based on a preliminary skin swab sample. It was not possible to randomly allocate the cats as some combinations of cats had to be avoided because they were known to fight. Fighting would compromise the welfare of the cats and also induce skin wounds which could promote FcaPV-2 infection- a potential confounding factor (Nafz et al., 2007). Once the two groups were established, one group of cats was randomly selected to be the treatment group, identified here as cats A–J. The other group became the control group, identified as cats K–T. The two groups were housed in separate runs, with no direct contact between the two groups or with other cats in the colony. Management practises were adjusted to minimize the possibility of

169 virus transfer via fomites between the two groups. This study was carried out in accordance with the
170 New Zealand regulations for animal welfare and the study protocol was approved by the Massey
171 University Animal Ethics Committee.

172

173 2.3. Vaccination and sampling schedule

174 Cats in the treatment group were vaccinated with 50 µg of FcaPV-2 VLPs in alum adjuvant buffer,
175 subcutaneously, three times at two-week intervals. At the same time, the control group cats were sham
176 vaccinated with the alum adjuvant buffer only. In both groups, the skin was shaved over the injection
177 site on the dorsal thorax prior to the first injection. This site was inspected for swelling or redness on a
178 daily basis for the first 8 weeks following vaccination and then fortnightly for the next 16 weeks.
179 Appetite and behavior were also monitored during the 24 week study, and the cats were weighed on a
180 weekly basis. Blood samples were collected from all cats to measure anti-PV antibody titers prior to
181 vaccination, and then again at the mid- and end-points of the study (weeks 12 and 24). Approximately 2
182 ml of blood was collected from the external jugular vein and allowed to clot at room temperature for 1
183 hour. Serum was separated by centrifugation and stored at −70°C prior to ELISA analysis. Skin swabs
184 were collected from all cats to measure viral load prior to vaccination, and then at six weekly intervals
185 for 24 weeks. Skin swab samples were collected from the shaved area of skin on the dorsal thorax as
186 previously described (Thomson et al., 2018). The swab samples were collected into DNA shield solution
187 (Zymo Research, Irvine, CA, U.S.A.) for storage at –20°C. An additional follow-up skin swab was collected
188 from all of the cats 10 months after the end of the study.

189

190 2.4. ELISA development

191 For the FcaPV-2 VLP ELISA, 96-well plates (Maxisorb immunoplates, Nalge Nunc, Rochester, NY, U.S.A.)
192 were coated overnight at 4°C with 8 µg/mL VLPs in 50 mM carbonate buffer (pH 9.6). This equated to

400 ng VLPs per well, which was the optimal antigen concentration determined by checkboard titration. After coating with antigen, the plates were washed three times in PBS with 0.05% Tween 20, and then blocked with 5% (w/v) skim milk in PBS with 0.05% Tween 20, for 2 hours. Plates were then washed and incubated with a series of twofold dilutions of cat sera, in duplicate, for 1 hour at 21°C. At least six dilutions of positive and negative control sera were included on every plate. *Felis catus* IgG was detected with a HRP-conjugated goat anti-cat IgG Fc fragment antibody (Jackson Immunoresearch laboratories, West Grove, PA, U.S.A.) diluted 1:30,000 in blocking buffer. Colour development was performed by addition of 50 µL of TMB substrate (Thermo Scientific), and stopped with 50 µL of 2M sulphuric acid. Absorbance was measured at 450 nm.

The endpoint titer of each sample was defined as the reciprocal of the highest dilution that gave a positive reaction. A positive reaction was defined as an absorbance reading that was greater than three times the absorbance of the negative control sera on the same plate, at each specific dilution. The same negative control sera was used on every plate in the same dilutions as the test samples. The negative control was sera obtained from a cat in the vaccine group prior to vaccination. This serum had a very low absorbance, and further evidence for a lack of pre-existing anti-FcaPV-2 antibodies was obtained by determining the avidity index after vaccination. Briefly, following vaccination the antibody avidity index increased from 30% at 4 weeks post vaccination, to 84% at 6 weeks post vaccination. This increase from low to high avidity suggests that the humoral immune system had not previously encountered the antigen. Sera that did not read an absorbance over three-times the negative control sera at the starting 200-fold dilution was assigned an endpoint titer of 100, which was the minimum endpoint titer that could be determined by the assay.

2.5. DNA extraction and real-time PCR

DNA was extracted from the swab samples using a High Pure PCR Template Preparation kit (Roche Applied Science, Penzberg, Germany) as previously reported (Thomson et al., 2015). A cow wart sample was included as a negative control for the DNA extraction process. DNA was extracted from this sample along with the cat samples and, as expected, this sample contained bovine PV DNA but no FcaPV-2 DNA. This confirms that FcaPV-2 DNA was not being introduced to the samples during the extraction process. The extracted DNA was used as template for real-time PCR, along with recombinant plasmid standards, to calculate the FcaPV-2 DNA copy number (Thomson et al., 2015). The FcaPV-2 DNA copy number was normalized to copies per swab because it was not possible to normalize to feline genomic DNA as the virus-containing mature skin cells had little to no genomic DNA. This was expected because the normal cornification process of mature skin cells involves endonuclease degradation of genomic DNA (Eckhart et al., 2013). The viral DNA however is protected from nucleases by the papillomavirus capsid (Buck et al., 2005). In this study, viral load was reported as copies of FcaPV-2 DNA per swab. Given that most of the viral DNA detected was probably from intact virions, which have double-stranded DNA, the copies of FcaPV-2 DNA would equal twice the number of intact virions. However, previous studies have reported copies of FcaPV-2 DNA rather than the number of virions because the physical state of the virus was unknown, so the same was reported here for consistency (Thomson et al., 2015; Thomson et al., 2016).

2.6. Statistical analysis

Data were analyzed using SPSS statistics 23 (IBM, Armonk, NY, U.S.A.). Log base 10 transformed data were used to compare viral loads between the two groups at the start of the study (independent t-test), and over time (two-way mixed model ANOVA). The transformed data was approximately normally distributed and fulfilled the necessary assumptions of homogeneity of variance (Levene's test $p > .05$), homogeneity of covariance (Box's test of equality of covariance matrices, $p = .64$), and sphericity

(Mauchly's test of sphericity, p = .55). One outlier was present in the data, with a studentized residual of 3.23. This was a true unusual value, which represented a particularly high viral load in one cat in the control group at the beginning of the study. Subsequent removal of all data from this cat had no effect on the overall outcome of either analysis, so the outlier did not appear to have excessive influence on the analysis and was included in the presented results.

Change in anti-PV antibody titer over time was compared separately in the treatment and control groups using one-way repeated measures ANOVA, followed by planned contrasts between weeks 0 and 12, and weeks 12 and 24. Large differences in variances and covariances between the titers in the two groups precluded direct comparison with a two-way mixed model ANOVA. In both groups, antibody titer data was log transformed. The log transformed data was approximately normally distributed in the vaccine group and had a positive skew at all time-points for the control group. Repeated measures ANOVAs are fairly robust to non-normality so long as the distributions are all skewed in a similar manner, so this was another reason to analyze the treatment and control groups separately. There were no outliers identified on box-plots, however as the assumption of sphericity was violated in the treatment group, a Greenhouse-Geisser correction was used. Log-transformed data were back transformed for reporting.

Non-parametric tests, including Mann-Whitney U and Spearman's rank-order correlations, were used to compare the distributions of age and anti-PV antibody titers in the treatment and control group at the start of the study, and assess how these variables correlated together and with the FcaPV-2 viral load. Probability values were adjusted for multiple comparisons where appropriate.

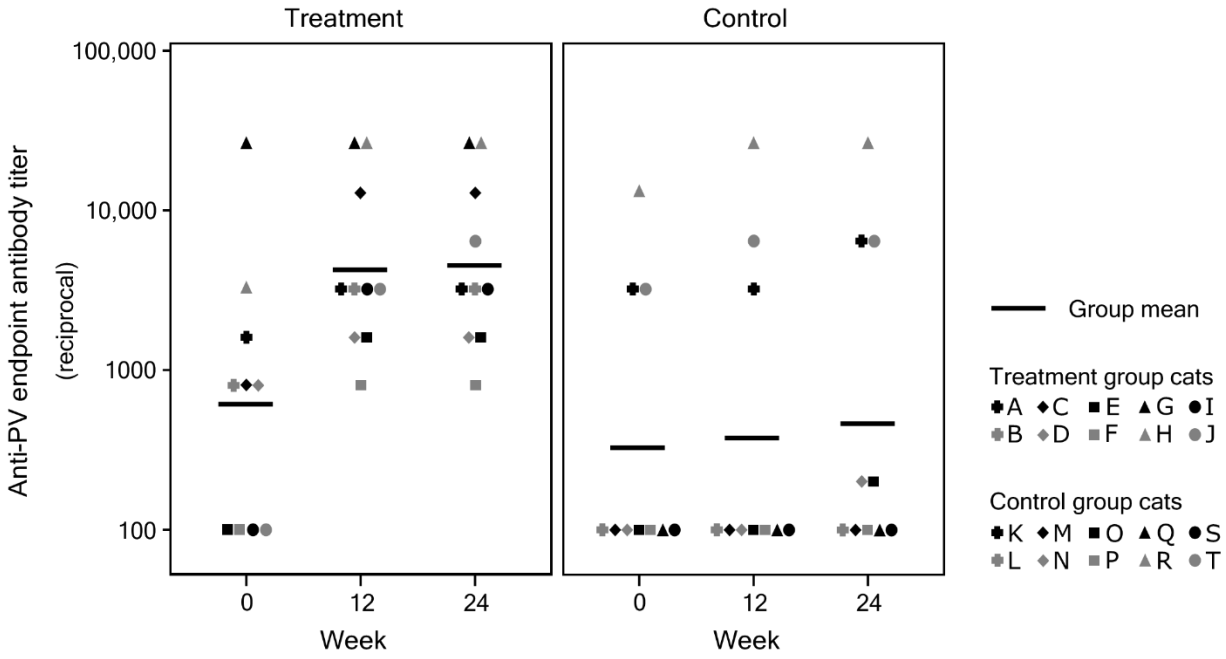
3. Results

3.1. Viral loads and antibody titers at the start of the study

The viral loads on the cats at the start of the study varied considerably, ranging from 109 to 730,165 copies of FcaPV-2 DNA per swab. As expected, there was no significant difference in the mean viral load between the treatment and control groups at the start of the study (p = .30). The geometric mean viral load in the treatment group was 1084 (95% CI 409–2870) copies FcaPV-2 DNA per swab; in the control group it was 2674 (710–10,067) copies of FcaPV-2 DNA per swab.

Endpoint anti-PV antibody titers also varied considerably at the start of the study, ranging from 100 (the lower limit of detection) to 25,600 as shown at the first time-point on Figure 2. The geometric mean endpoint anti-PV antibody titer in the treatment group was 606 (192–1913) while in the control group it was 325 (98–1078). Antibody titers were subsequently compared within each group over time, rather than between groups. There was a moderate positive relationship between anti-PV antibody titer from natural infection and FcaPV-2 viral load (r_s = .45, p = .045; Supplementary Figure 1) at the start of the study, with high viral-load cats tending to also have high anti-PV endpoint antibody titers. All data for individual cats is presented in Supplementary Table 1.

Both the treatment and control groups had two male and eight female cats. The cats were of mixed age, ranging from 1 year and 3 months old to 10 years and 3 months old. There was a significant difference in age between the two groups: the median age for the treatment group was 7 years 6 months, compared to 3 years 10 months for the control group (Mann-Whitney U test, p = .015). There was no correlation between age and FcaPV-2 viral load (r_s = -.016, p = .95). There was a moderate positive correlation between cat age and anti-PV antibody titer (r_s = .57, p = .008), with older cats having higher anti-PV antibody titers.



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284 **Figure 2.** Individual cat anti-PV endpoint antibody titers over time in the treatment and control groups.

285 Geometric mean antibody titers for each group are indicated by a solid line (*n* = 10 in each group).

286 Vaccination was given after sampling at week 0, then repeated at weeks 2 and 4.

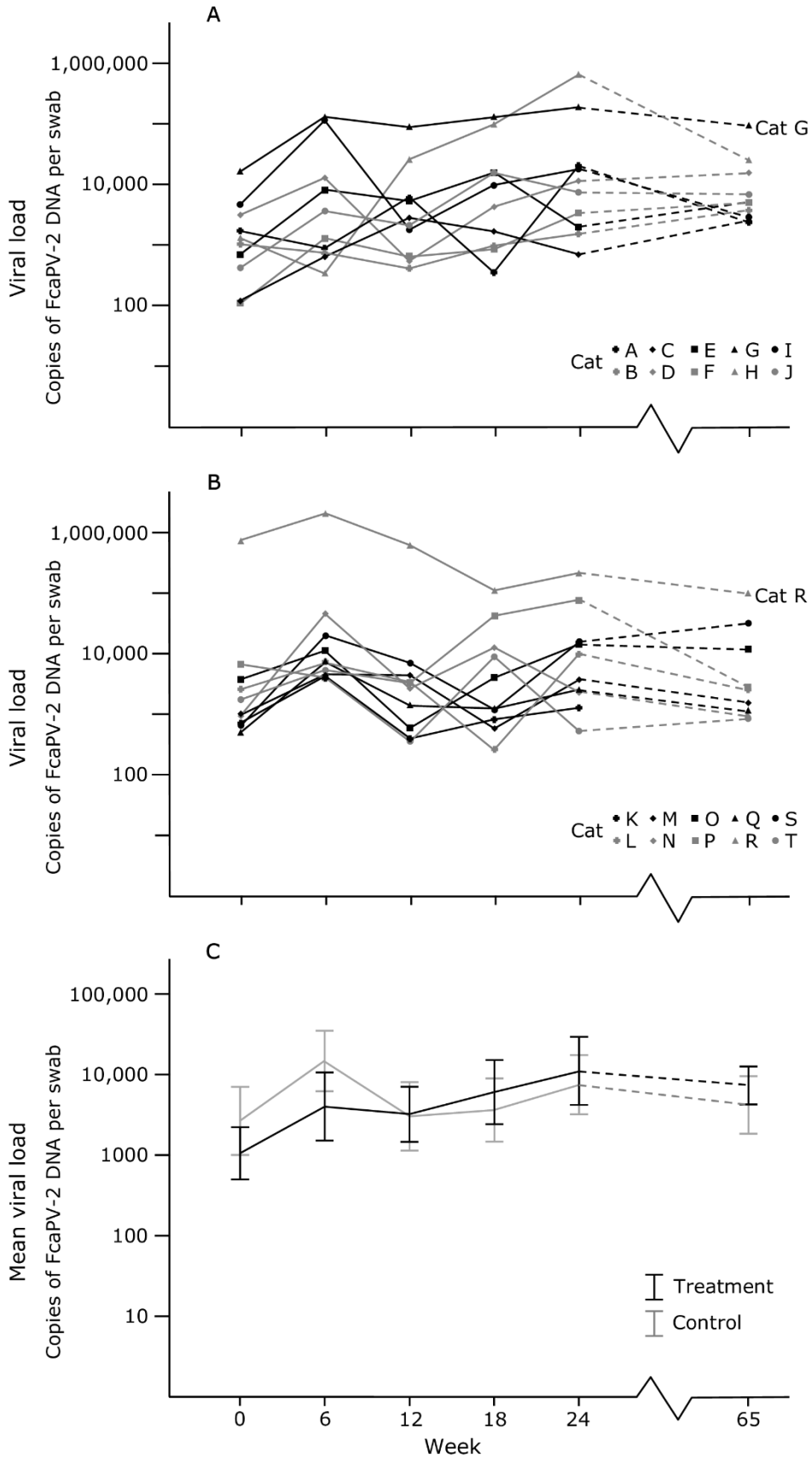
A
1,000,000
10,000
100
Viral load
Copies of FcaPV-2 DNA per swab
Cat G
Cat A C E G I
B D F H J
B
1,000,000
10,000
100
Viral load
Copies of FcaPV-2 DNA per swab
Cat R
Cat K M O Q S
L N P R T
C
100,000
10,000
1000
100
10
Mean viral load
Copies of FcaPV-2 DNA per swab
Treatment
Control
0
6
12
18
24
65
Week

Figure 3. FcaPV-2 viral loads in skin swab samples from the treatment and control cats over time**. Panel A:** FcaPV-2 viral loads for the individual cats in the treatment group (received FcaPV-2 VLP vaccine). **Panel B:** FcaPV-2 viral loads for the individual cats in the control group (received sham vaccine). **Panel C:** Geometric mean FcaPV-2 viral loads, with 95% confidence intervals, for both groups at each time point. Vaccination was given after sampling at week 0, then repeated at weeks 2 and 4. An additional follow-up swab was collected at week 65 under different conditions.

3.2. Vaccine safety

The sham and FcaPV-2 VLP vaccines used in this study appeared to be well-tolerated as none of the cats in either group developed any swelling or redness of the injection site in the 24 weeks following the first vaccine dose. There were no signs of systemic illness during the 24 week follow-up period, with all cats maintaining good appetites and normal behavior. Body weight was stable over the 24 week follow-up period for all cats except control Cat L, whose weight mildly reduced between week 12 (2.73 kg) and week 24 (2.50 kg).

3.3. Immunogenicity

Following vaccination, the endpoint anti-PV antibody titers increased in 9 of 10 cats in the treatment group with a mean 7.0-fold increase (95% CI 2.5–19.5) between weeks 0 and 12. As shown in Figure 2, the mean antibody titer for the treatment group increased from 606 (192–1913) to 4223 (2023–8814). The individual response to vaccination varied depending on the existing anti-PV antibody titer. For instance, the four cats (E, F, I, J) with titers at the lower limit of detection at the start of the study had the greatest increase following vaccination from 100 to 800–3200, a 22-fold mean increase. A further

five cats (A–D, H) had moderate starting titers of 800–3200 that increased 6.4-fold following vaccination (to 1600–25600). At the other extreme, Cat G had a very high starting anti-PV antibody titer of 25,600 that remaining the same following vaccination. In the later part of the follow-up period, between weeks 12 and 24, there was no significant change in mean anti-PV antibody titer (fold change 1.1, 95% CI 0.89–1.3) with a final mean titer of 4526 (2165–9459). The results of the one-way repeated measures ANOVA confirmed that the anti-PV antibody titers were significantly different between different time points over the course of the study (p = .021).

As expected, there was no significant change in endpoint anti-PV antibody titers in the control group following sham vaccination, between weeks 0 and 12 (mean fold change 1.1, 95% CI 0.90–1.5). The mean antibody titer for the group was 325 (98–1078) at week 0, and 373 (97–1440) at week 12. The individual titers, shown in Figure 2, either stayed high (at around 3200–12800 in cats K, R, and T) or stayed at the lower limit of detection (100) in the remaining cats. There was no significant change between week 12 and week 24 (mean fold change 1.2, 95% CI 0.93–1.6), with a final mean antibody titer of 460 (118–1782). Despite the lack of significant change between successive time points in the control group, the results of the one-way repeated measures ANOVA showed that the anti-PV antibody titers were significantly different between different time points over the course of the study (p = .014), due to a slight upwards trend over the three time points.

3.4. Effect of vaccination on viral load

FcaPV-2 viral loads did not reduce following vaccination in the treatment group of cats. Rather, the viral loads on the individual cats ranged from showing no trend to an increasing trend (with r^2 values of 0.0006–0.85) over the 24 week follow-up period. This can be seen in the top panel of Figure 3. The geometric mean viral loads for the group, shown in the bottom panel of Figure 3, increased slightly from

1084 (95% CI 409–2870) at week 0, to 3228 (1115–9351) at week 12, to 11,110 (2951–41,820) at week 24.

A similar pattern was also seen in the control group of cats, with individuals having either no trend in viral load over time, or mild to moderate positive or negative trends (with r^2 values of 0.0003–0.65). The geometric mean viral loads for the control group were similar to those of the treatment group: 2674 (710–10,067) at week 0, 3044 (809–11455) at week 12 and 7485 (2376–23,584) at week 24. Consistent with this, the results of the two-way mixed model ANOVA showed no statistically significant interaction between vaccination and time (p = .094). This means that the rate at which the viral load changed over time was no different between the treatment and control cats, confirming that the FcaPV-2 VLP vaccine was not effective at reducing FcaPV-2 viral loads.

After completion of the study at 24 weeks post vaccination, the cats returned to their usual housing with other cats. One extra follow-up swab was collected 10 months after completion of the study (week 65), with the purpose of investigating the particularly high viral loads found on two cats (discussed later). Data from this follow-up swab was not included in the main analysis as it was collected under different conditions. However, paired samples t-tests of the viral load at week 24 compared to week 65 showed no significant change in viral load in the treatment group (p = .41), nor control group (p = .079).

3.5. Comparison of viral load between individual cats

An interesting observation from this study was the consistently high viral loads on two individual cats. In the control group Cat R had very high viral loads which continued for the duration of the study. The geometric mean viral load on this cat was 468,038 (95% CI 172,859–1,267,276) copies of FcaPV-2 DNA per swab. The treatment group also had one cat with high viral loads, Cat G, with a geometric mean viral load of 86,174 (37,235–199,436) copies of FcaPV-2 DNA per swab. Data from these cats are annotated in

Figure 3. The remaining cats in both groups had much lower mean viral loads, ranging from 765 to 14,886 copies of FcaPV-2 DNA per swab. Interestingly, both cats G and R also had very high anti-PV antibody titers with endpoint dilutions ranging between 12800–25600.

To further investigate the differences in viral load in the individual cats, a follow-up swab was collected 10 months after the completion of the study. By this time the cats had been re-integrated with the rest of the colony cats and were housed in a number of different runs. Interestingly, cats R and G still had high viral loads at 100,940 and 94,535 copies of FcaPV-2 DNA per swab respectively. Viral loads on the other cats at follow-up were similar to what they had been during the study, ranging from 847 to 31,666 copies of FcaPV-2 DNA per swab.

4. Discussion

This study is the first report of the use of a papillomavirus VLP vaccine in cats, and the vaccine was found to be well-tolerated. The possible occurrence of local post-vaccine reactions was of particular concern in this study as such reactions occur occasionally in cats in response to several commonly used feline vaccines (Hartmann et al., 2015). These reactions are visible as a 1–6 cm diameter subcutaneous swelling at the site of previous injection (Gobar & Kass, 2002). A study based on the observation of injection site swellings considered to be post-vaccine reactions by primary care veterinarians, reported that most reactions resolved within 2 months but a small proportion persisted for longer than 4 months and progressed to injection-site sarcoma within 1–3 years (Gobar & Kass, 2002). While it was reassuring that there was no evidence of local reaction to the vaccine in this study, the sample size was too small to conclude that FcaPV-2 VLP vaccines are safe for use in the general cat population. However, these results could justify future studies with larger numbers of cats.

The results of the present study showed that the cats mounted a strong humoral immune response to the FcaPV-2 VLP vaccine with a 7.0-fold increase in anti-PV antibody endpoint titers observed from the start of vaccination to 8 weeks after the final vaccine had been administered. These high titers were maintained for the duration of the study. Therefore, the FcaPV-2 VLP vaccine was immunogenic and boosted anti-PV antibody titers above the level typically induced by natural infection. However, the mean increase in anti-PV antibody titers in this study was modest compared to titers induced by HPV VLP vaccines. For example, peak antibody titers in young women vaccinated with a commonly used HPV VLP vaccine were at least 100-fold higher than after natural infection, although these levels then declined approximately 10-fold to a long-term protective level over the next 2 years (Schiller et al., 2012). The lower antibody response seen in the cats in this study was partly due the presence of some cats with high pre-existing anti-PV antibody titers, as the antibody response was inversely related to the pre-existing antibody titer from natural infection. For example, the two youngest cats in the treatment group both had low starting antibody titers that increased 32-fold following vaccination, which is more comparable to vaccinating young women with little previous exposure to the high-risk HPV types. At the other extreme, a ten-year-old cat with a very high starting anti-PV antibody titer had no further antibody response following vaccination. Thus, an overall 7.0-fold increase in anti-PV antibody titer was still considered a reasonable response to the vaccine.

In the control group of cats there was a small upwards trend in anti-PV antibody titer, cumulating in a 1.4-fold increase over the 24 week follow-up period, although there was no significant difference between successive time-points. Given the strong correlation between age and anti-PV antibody titer, this mild increase may reflect the normal long-term response to FcaPV-2 infection. While a similar response may have occurred in both groups, the anti-PV antibody response in the treatment group was much more consistent with a response to the vaccine as it was of greater magnitude, statistically

401 significant, occurred immediately following vaccination, and occurred in all of the cats except Cat G
402 which had a very high pre-existing antibody titer.

403 The finding of a statistically significant correlation between age and anti-PV antibody titer from natural
404 infection in this study is consistent with a previous study which found a similar correlation (Geisseler et
405 al., 2016). However, there were some notable exceptions in the present study. Cat E, for instance, was a
406 10-year-old cat with a low starting anti-PV antibody titer which increased 16-fold following vaccination.
407 Therefore, in the wider cat population, younger cats would probably have a better response to FcaPV-2
408 VLP vaccination than older cats but there may be a considerable amount of variation and it is possible
409 that some older cats may also respond to the vaccine.

410 Despite the FcaPV-2 VLP vaccinated cats consistently developing high antibody titers, vaccination did not
411 significantly reduce the FcaPV-2 viral loads on these cats. There are two possible reasons for this. The
412 first possibility is that vaccination failed to induce sufficiently high titers of neutralizing antibodies to
413 prevent reinfection of new areas of skin. The second possibility is that further infection was prevented
414 but this had little to no impact on overall viral load.

415 In regards to the first possibility, the increase in anti-PV antibody concentrations following vaccination
416 varied markedly in this study, ranging from no change to 32-fold increases in antibody titers. However,
417 even the cats with the greatest increases showed no change in FcaPV-2 viral load. The type of vaccine
418 (VLPs composed of the PV L1 protein), dose and adjuvant used in this study were similar to that used
419 previously in mice, dogs and rabbits, which induced neutralizing antibodies that protected these animals
420 from experimental challenge with the native PV (Breitburd et al., 1995; Suzich et al., 1995; Vinzón et al,
421 2014). Furthermore, the antibody titers relative to the level induced by natural infection in this study
422 was similar to the long-term protective levels in young women vaccinated against high-risk HPVs
423 (Schiller et al., 2012). Nevertheless, the magnitude of increase in antibody titers following vaccination

may not relate to the concentration of neutralizing antibodies required to prevent FcaPV-2 from entering new basal cells, so it is still possible that the vaccination in this study did not increase neutralizing antibody titers to a level sufficient to protect against reinfection.

The second possible reason for the lack of reduction in FcaPV-2 viral loads in this study is that FcaPV-2 was already widespread in basal skin cells of the cats and the virus continued to replicate and shed from these foci of infection throughout the study. While PV vaccines are usually given prior to first infection, a recent study of MnPV infection in multimammate mice showed that vaccination of naturally infected animals reduced MnPV viral loads and subsequently prevented skin tumors (Vinzón et al., 2014). The protective effect of vaccination was suggested to be due to a reduction in viral spread on the host, however such a protective effect was not observed in the cats in this study. A major difference between this study and the study of MnPV infected mice was the age of the host animals. The mice in the later study were initially vaccinated at 1.8 months old whereas the cats in our study ranged from 1 to 10 years old. It is possible that the vaccine worked in the young mice because they had recently been infected by MnPV and the infection had not yet spread widely over the body. In contrast, cats have been shown to be infected with FcaPV-2 in the first weeks of life (Thomson et al., 2015; Thomson et al., 2018), so in all of the cats the virus would have had many months to disseminate widely over the skin surface. It is possible that, in the mice, the vaccine stopped an early infection from becoming fully established. In contrast, the viral infections in the cats were most likely fully established prior to vaccination. A further possibility that cannot be excluded is that FcaPV-2 can spread from one skin site to another regardless of the presence of neutralizing antibodies. Further research is needed regarding the normal spread of this virus in cats.

Therefore, the results of this study do not support the use of a FcaPV-2 VLP vaccine in adult cats with already well-established FcaPV-2 infections. In people, vaccination against cutaneous HPVs has been suggested as a way to reduce the incidence of PV-associated skin cancer following immunosuppression

448 in organ transplant recipients (Vinzón & Rösl, 2015). The results of this study suggest that such an
449 approach may not be effective if the cutaneous HPV is already well-established in the host.

450 Adult cats were used in this study because it was the first time a PV VLP particle vaccine has been used
451 in cats, and any adverse reactions to the vaccine could be more easily be managed in adult cats
452 compared to young kittens. However, given the FcaPV-2 VLP vaccine used in this study was well-
453 tolerated, it would be interesting to repeat this study in young kittens in which FcaPV-2 infection is not
454 yet fully established.

455 In addition to the main findings of this study, an interesting observation was the presence of high viral
456 loads in two of the cats which persisted throughout the 15 month sampling period. Both cats also had
457 very high anti-PV antibody titers. In other species, PV infection results in a period of viral replication and
458 shedding that lasts for several months before the infection is controlled by the development of a cell-
459 mediated immune response and the virus is eliminated, or persists in the basal cells with minimal viral
460 replication (Doorbar et al. 2012; Maglennon et al., 2011). Subsequent PV detection has been attributed
461 to new infection or reactivation of latent infection due to mechanical trauma or immune suppression
462 (Insinga et al., 2010; Maglennon et al., 2011). The presence of persistently high viral loads and high
463 antibody titers in two adult cats in this study may suggest that, in these cats, the immune system failed
464 to suppress existing FcaPV-2 infection. In people, it is well documented that a proportion of women do
465 not effectively suppress or eliminate high-risk HPV infection (Rodriguez et al., 2008; Xi et al., 1995).
466 These women become persistently infected with high-risk HPV and have a greater risk of developing
467 HPV-induced cancer (Remmink et al., 1995; Rodriguez et al., 2008). Therefore, a similar situation may
468 occur in cats and those with persistently high viral loads may be at a greater risk of developing FcaPV-2-
469 associated cancer compared to cats with lower FcaPV-2 viral loads. However, this would need to be
470 confirmed with further studies using greater numbers of cats and longer follow-up periods.

Two major limitations of this study were the lack of FcaPV-2-naïve cats and the difficulties associated with sampling for FcaPV-2. Immunologically naïve cats with no past exposure to FcaPV-2 could have been vaccinated and then challenged with FcaPV-2 to determine the concentration of anti-L1 antibodies required to prevent FcaPV-2 infection *in vivo*. Unfortunately, no FcaPV-2-naïve cats were available in this study. At present, there are no published reports investigating the possibility of prenatal transmission of FcaPV-2. If infection does not occur *in utero*, it may be possible to create FcaPV-2 free cats by delivering kittens by caesarian section, although the implications for the welfare of these kittens would need to be carefully considered.

Sampling methods for detecting FcaPV-2 infection include skin swabs, hair-plucks and skin biopsies (Thomson et al., 2015; Thomson et al., 2018). Hair-plucks and skin biopsies sample only a very small area of skin but contain cells from deeper layers of the epidermis. This makes it easier to account for variations in sample size. However, both of these methods traumatize the epidermis, potentially exposing the epidermal basement membrane. In natural infection, PVs gain access to basal cells by first binding to basement membrane molecules exposed through microwounds in the epidermis (Doorbar et al. 2012). Thus, these sampling methods may promote subsequent PV infection. In contrast, swabbing the surface of the skin does not expose the epidermal basement membrane, making skin swabs a more suitable sampling method for longitudinal studies. However, the lack of nucleated host cells in these samples makes it difficult to normalize to sample size. In this study, one investigator collected all of the swab samples in a standardized manner to minimize variation in sample size. Another disadvantage of skin swabs is the potential to detect FcaPV-2 on the skin surface that was shed from in-contact cats, rather than the cat being sampled (Thomson et al., 2018). Therefore, in the present study it would have been ideal to keep each cat in an individual pen for the duration of the follow-up period. However, given the length of the study, this was not acceptable for welfare reasons. Rather, the two groups were housed in separate runs, so each cat only had contact with the nine other cats in their group. Thus,

although the viral load on an individual cat at any one particular time-point was interpreted with caution, the change in FcaPV-2 viral load over time could be reliably compared between the treatment and control groups.

In summary, he FcaPV-2 VLP vaccine used in this study was well-tolerated and resulted in a significant rise in anti-PV antibody titers. However, the vaccination had no impact on the viral load of the cats. Although this could be due to insufficient concentration of neutralizing antibodies, it is more likely that FcaPV-2 was already widespread in the basal skin layer of these cats and so preventing further cells from becoming infected had no impact on the overall viral load. Therefore, vaccinating adult cats against FcaPV-2 is not likely to reduce the incidence of skin cancer in this species. Future studies should be aimed at vaccination of younger cats. Two cats with persistently high viral loads and antibody titers were identified in this study. It could be speculated that these cats were unable to reduce FcaPV-2 viral loads on their skin and may be at greater risk for FcaPV-2-induced cancer development.

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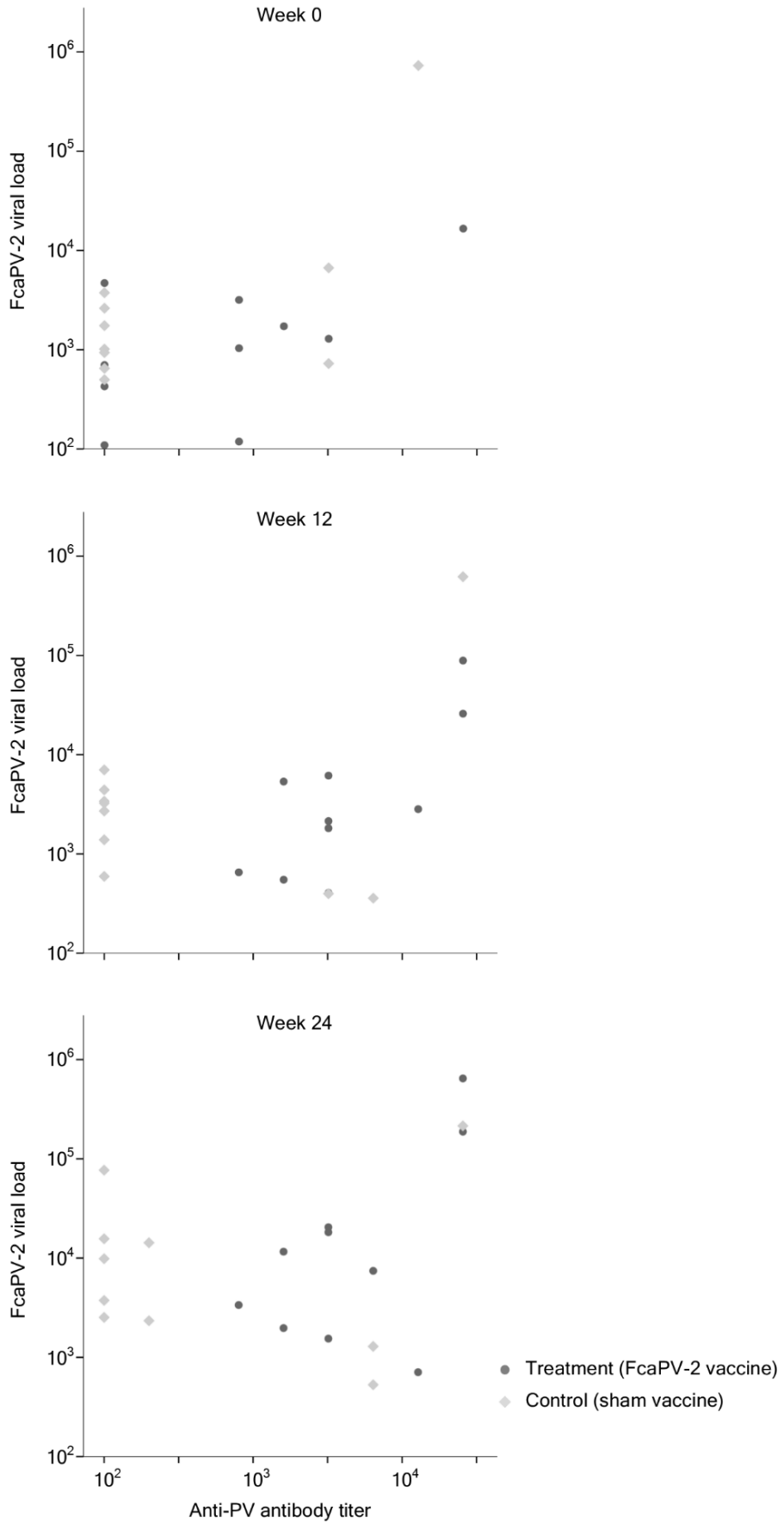
			Anti-PV antibody titers			Viral Load					
Cat	Sex	Age	Week 0	Week 12	Week 24	Week 0	Week 6	Week 12	Week 18	Week 24	Week 65*
Treatment group – received FcaPV-2 VLP vaccine at weeks 0, 2 and 4											
Cat A	F	9y 10m	1600	3200	3200	1725	902	6160	356	20484	2381
Cat B	F	7y 6m	800	3200	3200	1037	739	406	981	1546	3716
Cat C	M	7y 2m	800	12800	12800	119	649	2830	1691	709	2588
Cat D	F	7y 6m	800	1600	1600	3178	12936	551	4300	11620	15757
Cat E	F	10y 3m	100	1600	1600	702	8183	5367	15804	1974	5035
Cat F	F	10y 3m	100	800	800	109	1302	653	872	3370	5159
Cat G	F	10y 3m	25600	25600	25600	16623	131635	88900	129903	188055	94535
Cat H	F	5y 4m	3200	25600	25600	1291	344	25925	98280	646450	25218
Cat I	M	5y 4m	100	3200	3200	4702	114748	1819	9756	18228	2926
Cat J	F	2y 1m	100	3200	6400	428	3665	2145	15668	7446	6927
Control group – received sham vaccine at weeks 0, 2 and 4											
Cat K	F	10y 1m	3200	3200	6400	726	4359	398	829	1289	
Cat L	F	1y 3m	100	100	100	2619	6969	3400	263	9865	2567
Cat M	F	3y 1m	100	100	100	1015	4489	4422	587	3749	1537
Cat N	M	1y 4m	100	100	200	936	45763	2708	12730	2340	917
Cat O	F	1y 3m	100	100	200	3750	11221	594	4034	14287	11912
Cat P	M	1y 4m	100	100	100	1748	5434	3252	42175	76948	2832
Cat Q	F	2y 1m	100	100	100	499	7438	1389	1246	2527	1158
Cat R	F	9y 1m	12800	25600	25600	730275	2072700	621250	111143	214900	100940
Cat S	F	2y 7m	100	100	100	649	20022	7035	1182	15663	31666
Cat T	F	6y 3m	3200	6400	6400	6678	4104	359	8860	530	847

Supplementary Table 1: Individual cat data. Anti-PV antibody titers are reported as endpoint titers- the reciprocal of the highest dilution that gave a positive reaction. Viral load is reported as copies of FcaPV-2 per swab. *An extra swab was collected 10 months after completion of the study, when the cats had been re-integrated with the rest of the colony.


Week 0
Week 12
Week 24
FcaPV-2 viral load
10^2
10^3
10^4
10^5
10^6
Anti-PV antibody titer
Treatment (FcaPV-2 vaccine)
Control (sham vaccine)

Supplementary Figure 1. Scatter plots relating FcaPV-2 viral load to anti-PV antibody titer. FcaPV-2 viral load measured in copies of FcaPV-2 DNA per swab. Anti-PV antibody titer is the reciprocal of the endpoint dilution on ELISA. Each point represents samples from one cat. At Week 0 the cats were in separate groups but the samples were collected prior to vaccination, so this represents the baseline FcaPV-2 viral load and anti-PV antibody titer from natural infection. There was a moderate correlation between FcaPV-2 viral load and anti-PV antibody titer on Spearman's rank correlation (non-parametric): r_s = .45, p = .045. The treatment group was vaccinated with a FcaPV-2 VLP vaccine and the control group was given a sham vaccine at weeks 0, 2 and 4. This increased the anti-PV antibody titer in the treatment group but did not have a significant impact on FcaPV-2 viral load (see manuscript). Therefore, at weeks 12 and 24 there was no longer a significant correlation between FcaPV-2 viral load and anti-PV antibody titer. Week 12: r_s = .17, p = .45. Week 24: r_s = .10, p = .70.