Veterinary Microbiology 250 (2020) 108865

Contents lists available at ScienceDirect

Veterinary Microbiology

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

Lack of protection against feline immunodeficiency virus infection among domestic cats in New Zealand vaccinated with the Fel-O-Vax® FIV vaccine

A. Stickney [1], S. Ghosh, N.J. Cave [2], M. Dunowska [*,2]

School of Veterinary Science, Massey University, Private Bag 11 222, Palmerston North 4442, New Zealand

ARTICLE INFO

Keywords:
New Zealand
FIV infection
FIV
Fel-O-Vax® FIV vaccine
Feline immunodeficiency virus
Vaccination

ABSTRACT

Infections with feline immunodeficiency virus (FIV) are common in New Zealand, although the impact of those infections on the health status of the cats remains unclear. Although many cats are vaccinated yearly with a commercial FIV vaccine containing FIV subtypes A and D, the effectiveness of this vaccine in protection against infection with field FIVs is unclear, as a high proportion of New Zealand viruses belong to subtype C. The objective of the study was to compare the frequency of FIV infection among adult FIV-vaccinated and FIV-unvaccinated domestic cats with access to outdoors. Buccal swabs were collected by the participating veterinarians and tested for the presence of FIV provirus by quantitative PCR. Overall, 26/185 (14.0 %) samples were positive for FIV, including 7/82 (8.5 %) samples from FIV-unvaccinated and 19/103 (18.4 %) from FIV-vaccinated cats. There was no protective effect of vaccination on FIV infection among sampled cats ($p = 0.05$). Partial sequences of the FIV envelope gene from five New Zealand viruses were analysed by the maximum likelihood method. All clustered with other New Zealand FIV sequences from subtypes A ($n = 2$), C ($n = 2$) or putative recombinant viruses ($n = 1$). While the FIV vaccination did not prevent FIV infection among sampled cats, it may have had an impact on transmissibility of the virus or on disease progression. As neither was addressed in the current study, further research is needed to fully assess the potential benefits of FIV vaccination. Considering the frequency of FIV infection in FIV-vaccinated cats, FIV infection status should be monitored not only before the first vaccination, but before each yearly booster.

1. Introduction

Feline immunodeficiency virus (FIV) is classified in the genus *Lentivirus* within the family *Retroviridae*. The virus has a diploid positive sense RNA genome which, typical for RNA viruses, is prone to mutations (Beczkowski et al., 2015). Variants of FIV viruses have been classified into different subtypes (designated A through F) based on the envelope (env) gene (Weaver, 2010). Recombinant viruses or viruses of unknown subtypes have also been described (Hayward and Rodrigo, 2010). Under experimental conditions, primary FIV infection may be subclinical or associated with mild transient clinical signs that include fever, lethargy, respiratory tract disease, conjunctivitis, lymphadenopathy, gastrointestinal disturbances, stomatitis, or dermatitis (Hartmann, 2011). Most cats recover and enter an asymptomatic phase of infection which may last for years. Some, but not all, eventually succumb to feline acquired immunodeficiency disease syndrome similarly to people infected with a closely related human immunodeficiency virus. The presenting clinical signs vary and reflect a variety of secondary viral, bacterial or fungal infections with common presentations of stomatitis, neoplasia, ocular lesions due to uveitis or chorioretinitis, anemia and leukopenia, renal insufficiency, lower urinary tract disease, or endocrinopathies (Hartmann, 2011). However, older FIV-negative cats can also present with similar clinical signs due to other causes, and hence the true impact of FIV infection on cats' health remains somewhat undetermined (White et al., 2011).

In contrast, others reported rapid disease progression and high mortality rates following either natural or experimental FIV infection (Diehl et al., 1995). The discrepancies between results of various studies may be related to the characteristics of the FIV used or age and immune status of the infected cats, but such varied disease presentation is an obvious impediment in the assessment of the efficacy of an FIV vaccine. This highlights the need for availability of local efficacy data in order to

* Correspondence author.
E-mail address: M.Dunowska@massey.ac.nz (M. Dunowska).
[1] Current affiliation: Peninsula Vet Emergency and Referral Hospital, Mornington, Victoria, 3931, Australia.
[2] Authors contributed equally.

https://doi.org/10.1016/j.vetmic.2020.108865
Received 8 June 2020; Accepted 17 September 2020
Available online 28 September 2020

provide evidence-based recommendations for small animal veterinarians.

The prevalence of FIV infection in New Zealand cats appears to be higher than in many other countries. In one study, 14.4 % of pet cats of variable health status were positive for FIV antibody (Swinney et al., 1989). The frequency of detection of FIV seropositive feral cats ranged from 11 % to 36 % between different New Zealand regions (Hayward et al., 2007). By comparison, the seroprevalence of FIV among pet cats North America was 2.5 % (Levy et al., 2006). The seroprevalence of FIV infection among Australian cats appears to be similar to that observed in New Zealand, with values ranging from 6% to 15 % between different populations of cats (Westman et al., 2016c).

The Fel-O-Vax® FIV vaccine has been available to veterinarians in New Zealand for the past 12 years. A recent survey showed that the majority of New Zealand veterinarians recommend the use of Fel-O-Vax® FIV vaccine to their clients (Cave et al., 2015), despite that fact that its efficacy in the field remains uncertain. The reported protection rates against infection with various FIV subtypes following vaccination vary between 0% and 100 % (Coleman et al., 2014; Yamamoto et al., 2010). However, in most of the studies vaccinated cats were challenged with subtype A or B of FIV, while the most common subtype among New Zealand cats appears to be subtype C (Hayward et al., 2007; Kann et al., 2007b).

As the Fel-O-Vax® FIV vaccine contains inactivated virus, replication and integration of the viral genome into the host cell does not occur. As such, detection of FIV provirus by PCR has been considered to be a valid method to detect FIV-infection among vaccinated cats (Ammersbach et al., 2013). Infection of cats with FIV following vaccination has been documented, with one Australian study showing that 4.2 % (5/119) of vaccinated cats versus 8.79 % (21/239) unvaccinated cats were infected with FIV (as confirmed by virus isolation) (Westman et al., 2015). These data indicate a preventable fraction of only 52.2 % for the Fel-O-Vax® FIV vaccine in Australia, a value much lower than that suggested by most of experimental challenge studies using viruses of a subtype expected within Australia (A or B)(Kann et al., 2006).

We hypothesised that the frequency of detection of FIV infection among FIV-vaccinated pet cats is lower than that among FIV-unvaccinated pet cats in New Zealand.

2. Materials and methods

2.1. Enrolment of cats

Cross-sectional retrospective study design was used to investigate the frequency of FIV infection among cats vaccinated with the Fel-O-Vax FIV® vaccine and FIV-unvaccinated cats. A total of 493 veterinary clinics were contacted via email and asked to participate in this study. Of these, 42 responded and were sent supplies to collect samples. Each clinic was asked to supply an equal number of samples from FIV-vaccinated and FIV-unvaccinated cats. To be eligible for enrolment into the study, both FIV-vaccinated and FIV-unvaccinated cats had to be $>$ 2 years of age and had access to outdoors. Only cats that have received a complete course of vaccination according to the manufacturer's directions, with the most recent vaccine administered within the past 12 months were included. All cats older than 6 months of age at the time of first vaccination must had tested negative for FIV antibody to ensure that they were not FIV infected. Unvaccinated cats must have been never vaccinated against FIV. All samples were accompanied by submission forms, which provided information about the cat, including details of prior FIV testing, and vaccination history. The study protocol was approved by the Massey University Animal Ethics Committee (Protocol # 14/129).

2.2. Collection and processing of samples

Non-invasive sampling method was chosen to facilitate recruitment of cats. A rayon tipped sterile swab (Copan) was inserted into the cat's mouth and rubbed against the inner cheek mucosa for approximately 30 s. The tip of the swab was then cut off and inserted into a sterile cryovial containing 250 µL of RNAlater®. The samples were stored at -20 °C for a period of up to one month or at 4 °C for a period of up to two weeks before being shipped at room temperature to the Virology Laboratory at Massey University (Palmerston North, New Zealand). Once thawed, the cryovial containing the swab tip was vortexed for 30 s and the swab was then transferred to a 0.6 mL microtube with a hole in the base. The swab containing microtube was then inserted into a larger 1.5 mL microctube and the assembly was centrifuged at high speed for 1 min. The swab tip was discarded and the flow-through combined with the remaining volume of RNA later in which the swab was initially submerged. Nucleic acids were extracted from the sample using the High Pure Viral Nucleic Acid Extraction Kit (Roche) according to the manufacturer's instructions.

In order to determine the FIV status of the sampled cats, all samples were initially screened using FIV quantitative PCR (qPCR) with 2 µL of template DNA (Table 1). Samples that produced the correct melting peak on the screening qPCR were considered suspect positives irrespective of whether or not the amplification plots crossed the threashold, and used as a template in conventional FIV-specific nested PCR targeting the env gene. The identity of bands of the correct size was confirmed by sequencing. The remaining suspect positive samples were re-tested using the FIV qPCR and 4 µL of template. Samples positive on the screening qPCR and either conventional PCR or the confirmatory qPCR were regarded as positive for FIV provirus (Fig. 1). All samples that were negative for FIV RNA in the screeningn qPCR were tested in qPCR with 28S rDNA housekeeping gene primers. Samples negative in 28S rDNA qPCR were excluded from the study.

2.3. FIV-specific qPCR

Quantitative PCR was used to amplify proviral DNA using previously published primers targeting a 164 bp product of the *gag* gene (Wang et al., 2010)(Table 1). Each reaction consisted of 0.1 µM of FIV.F primer, 1 µM of FIV.R primer and 2 µL (in screening qPCR) or 4 µL (in confirmatory qPCR) of template DNA in 1 x reaction buffer with SYTO 9 dye (Accumelt HRM, Quanta Biosciences) in a total volume of 10 µL. The cycling conditions included an initial denaturation step (95 °C for 5 min), followed by 40 cycles of denaturation (95 °C for 5 s), annealing (58 °C for 16 s) and elongation (72 °C for 15 s), with the melting step from 65 °C to 95 °C at 12 °C/min. The performance of the assay was initially assessed based on a standard curve produced using serial dilutions of the cDNA from the Fel-O-Vax FIV® vaccine, and the sensitivity of the assay was later determined by amplification of serial dilutions of an 874 bp PCR product containing a pre-determined copy numbers of the target sequence ("long PCR" in Table 1). At least one standard (10^7 copies/µL of template), a positive control (cDNA obtained from a blood of a known FIV-positive cat), and a non-template control (water) were included in each run. All samples were tested in duplicate. A standard curve (10^7 to 10° copies/µL of template) with automatically set cut-off was imported into the analysis of each run. A sample was considered positive in the confirmatory qPCR if the amplification plot crossed the baseline and the melting temperature of the product was between 79.5 and 83.5 °C (which was the temperature range obtained in the amplification of FIV-positive samples that were confirmed by sequencing).

2.4. Conventional FIV-specific PCR targeting env gene

The PCR reactions were performed in a total volume of 10 µL, consisting of 2.0 µL HOT FIREPol® Blend Master mix (Solis BioDyne), 0.1 µM of each primer (Table 1), and 1.0 µL of template DNA. For the nested reaction, 1.0 µL of the primary PCR product was used as template DNA. Amplification conditions for both primary and nested reactions consisted of the initial denaturation at 95 °C for 10 min, followed by 35

Table 1
Primers used in the study.

PCR	Primer	Location[1]	Product size (bp)	Sequence (5' to 3')	Reference
Env (Primary)	VE1.F	7128 – 7148	1234	GAGTAGATACWTGGTTRCAAG	(Nishimura et al., 1996)
	VE1.R	8342 – 8361		CATCCTAATTCTTGCATAGC	
Env (Nested)	VE2.F	7320 – 7339	862	CAAAATGTGGATGGTGGAAY	
	VE2.R	8162 – 8181		ACCATTCCWATAGCAGTRGC	
House-keeping gene	28SrRNA.F	N/A	97	CGCTAATAGGGAATGTGAGCTAGG	(Helps et al., 2003)
	28SrRNA.R	N/A		TGTCTGAACCTCCAGTTTCTCTGG	
Gag qPCR	FIV.F	628 – 650	164	ATGGGGAAYGGACAGGGGCGAGA	(Wang et al., 2010)
	FIV.R	762 – 791		TCTGGTATRTCACCAGGTTCTCGTCCTGTA	
Long	FIV.long.F	353 – 372	874	GCAGTTGGCGCCCGAACAGG	
	FIV.long.R	1206 – 1226		TTATCTGCAGCGCACCCTGGT	

[1] Nucleotide positions in the reference FIV_{PET} (GenBank accession number M25381).

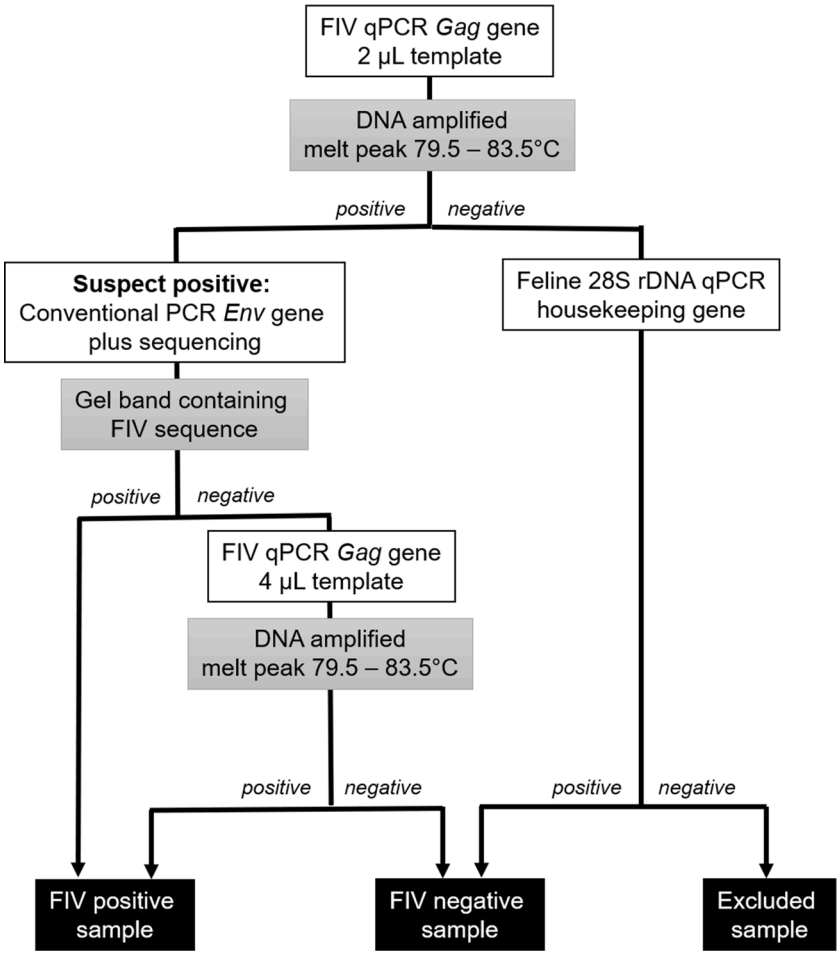

Fig. 1. Flow chart describing processing of the buccal swab samples collected from feline immunodeficiency (FIV) vaccinated and unvaccinated cats from New Zealand. All samples were initially screened using a quantitative PCR (qPCR) to amplify a region of the FIV *gag* gene using 2 µL of template DNA. To determine the presence of amplifiable DNA, all FIV-negative samples were tested in qPCR with 18S rDNA housekeeping gene primers. Samples negative in 18S rDNA qPCR were excluded from the study. Samples that produced the correct melting peak on the screening qPCR were considered suspect positives and used as a template in conventional FIV-specific PCR with subsequent sequencing of the product to confirm the identity of the product. Negative samples were re-tested using the FIV qPCR and 4 µL template DNA. Samples positive on the screening qPCR and either conventional PCR or the confirmatory qPCR were regarded as positive for FIV provirus.

cycles of denaturation (15 s at 95 °C), annealing (15 s at 50 °C), and elongation (1 min at 72 °C), with the final elongation step at 72 °C for 5 min. PCR products were subjected to gel electrophoresis (100 V for 40 min) through a 1% ethidium bromide stained agarose gel. DNA was extracted from bands of the expected size (862 bp) and submitted for sequencing to the Massey Genome Service (Massey University, New Zealand). Sequence reads were analysed using Geneious Pro 9.1.8 software (Biomatters Ltd, 2009, Auckland, New Zealand) and identified using BLAST algorithms.

2.5. House-keeping gene qPCR

Quantitative PCR targeting a 97 bp region of feline 28S ribosomal DNA using previously published primers (Helps et al., 2003) was used to confirm the presence of amplifiable DNA in buccal swab samples. The reaction was performed in a total volume of 10 µL, consisting of 0.5 µM of the forward primer, 0.4 µM of the reverse primer, and 2.0 µL of template in Accumelt HRM mastermix. Amplification conditions consisted of initial denaturation at 95 °C for 5 min, then 45 cycles of template denaturation (95 °C for 5 s), primer annealing (60 °C for 20 s), and elongation (72 °C for 15 s). The cycling was followed by a melting step from 55 to 95 °C.

2.6. Phylogeny

Maximum likelihood (ML) trees were constructed in Mega X (Kumar et al., 2018) based on the Clustal W alignment of the five New Zealand FIV sequences and representative sequences from subtypes A, B, C, D

and E obtained from GenBank. The alignment was done within Geneious Pro 9.1.8 software with default settings.

2.7. The effect of RNAlater® on detection of FIV provirus

Whole blood (2 mL in EDTA) was collected from a known FIV positive cat recruited from the Massey University Veterinary Teaching Hospital (VTH). The cat had been previously diagnosed with FIV by serology and had no history of FIV vaccination. Nucleic acid was extracted from 200 μL of the whole blood as well as from blood diluted (2:3 and 1:9) in RNAlater® (Sigma-Aldrich) or in RNase-free water. All extractions were performed using the High Pure Viral Nucleic Acid Extraction Kit (Roche) according to manufacturer's instructions. Nucleic acids were eluted in 50 μL of prewarmed elution buffer. FIV specific qPCR was used to amplify FIV provirus from 2 μL of template DNA in a total volume of 10 μL, as described above. In addition, a buccal swab was collected from the same cat into RNAlater and tested for the presence of FIV provirus in the same way.

2.8. Statistical analysis

Submission forms were reviewed and any samples with an incomplete history were excluded. A logistic regression model was created, with FIV status as the outcome variable. Explanatory variables included sex, age and FIV vaccination status of the cat. The analysis was then repeated following exclusion of all cats that were first vaccinated against FIV as kittens and hence had not been tested for FIV antibody prior to vaccination. Statistical analyses were performed using R statistical software (R: A Language and Environment for Statistical Computing, R foundation for Statistical Computing, Vienna) or an on-line regression analysis tool (https://easystat.com/). A p-value of < 0.05 was considered significant.

2.9. GenBank accession numbers

The FIV sequences obtained in this study have been submitted to GenBank under accession numbers MW012627 to MW012631.

3. Results

3.1. FIV qPCR performance

The FIV qPCR showed an average efficiency of 90.5 %, with an R^2 value consistently exceeding 0.99. The assay was sensitive enough to detect 1 copy equivalent of FIV genome per μL of template.

3.2. The effect of RNAlater® on detection of FIV provirus

The swabs were collected in RNAlater® in order to minimize the possible detrimental effect of variable storage conditions at the participating veterinary clinics on the quality of nucleic acids in the sample, and to allow transport to the laboratory at room temperature (Gray et al., 2013). FIV proviral DNA was amplified from the nucleic acid extracted from undiluted whole blood from the FIV positive cat, as well as from all samples diluted at various proportions in either RNAlater® or water. The mean Cq values for the FIV-positive blood sample diluted 2:3 in RNAlater® or water were 25.9 and 25.8, with the melting peak at 81.7 °C and 81.4 °C, respectively. The amplification plot produced from the undiluted blood sample in the same run showed a mean Cq of 22.5 with the melting peak at 81.5 °C. The mean Cq values for the FIV-positive blood sample diluted 1:9 in RNAlater® or water were 28.9 and 27.5, respectively, with the melting peak at 82.0 °C for both sample types. The undiluted blood in the same qPCR run produced a curve with a mean Cq value of 25.3 and a melting peak at 81.4 °C. We concluded from these results that RNAlater® did not interfere with viral nucleic acid extraction or subsequent FIV qPCR. Proviral DNA was detected in nucleic acid extracted from the buccal swab of the same FIV seropositive cat with a Cq value of 31.2 and Tm of 80.9 °C. While this result confirmed our ability to detect FIV provirus in a known FIV positive cat, the high Cq value suggested that the concentration of proviral DNA in this sample was low. The slight Tm variations between different qPCR runs may reflect the presence of several genotypes of FIV in the clinical samples used.

3.3. Enrolled cats

A total of 191 samples were received from 15 different veterinary clinics from the North Island (116/191, 60.7 % of samples) and the South Island (75/191, 39.3 % of samples) of New Zealand between March and July 2015. Six samples were excluded either due to non-compliance with the sampling requirements ($n = 3$) or lack of amplifiable DNA based on 18SrDNA qPCR ($n = 3$). Of the remaining 185 samples, 103 (55.7 %) were from cats vaccinated against FIV, and 82 (44.3 %) were from cats not vaccinated against FIV (Fig. 2). Of the 103 FIV-vaccinated cats, 50 (48.5 %) were males and 53 (51.5 %) were females. Of the 82 FIV-unvaccinated cats, 51 (62.2 %) were males and 31 (37.8 %) were females. The age of the cats ranged from 2 to 18 years, with a median age of 5 years for both FIV-vaccinated (95 % CI 4.8–6.0) and FIV-unvaccinated (95 % CI 5.9–7.8) cats.

3.4. The frequency of FIV infection among FIV-vaccinated versus FIV-unvaccinated cats

Overall, 26 of 185 (14.0 %) samples were considered positive for FIV, including 7/82 (8.5 %) samples from FIV-unvaccinated cats (5 males and 2 females) and 19/103 (18.4 %) from FIV-vaccinated cats (11 males and 8 females). The calculated concentration of FIV DNA ranged from <1 to 140,125 copies/μL of template, with <100 copies/μL of template detected in 15/26 (57.7 %) FIV-positive samples. Only five qPCR-positive samples produced a band of the expected size in conventional PCR targeting the env gene, all of which were confirmed to be derived from FIV by sequencing (Table 2). An example of amplification plots and melting curves from confirmatory qPCR is shown in Fig. 3.

Logistic regression showed that vaccination status did not significantly affect the FIV infection status of the sampled cats ($p = 0.05$). Age and sex were included in the model to assess for sampling bias between FIV-vaccinated and FIV-unvaccinated cats, and there was no effect of these factors on the FIV status ($p = 1.0$ and 0.3, respectively).

Review of the submission form data revealed that 10 FIV positive cats received their first dose of vaccine as kittens and were therefore not tested for FIV prior to vaccination, and the age of first vaccination was unknown for one additional cat. The analysis was repeated following exclusion of these 11 cats. Results again showed that there was no effect of FIV vaccination on FIV infection status, with the frequency of FIV-positive samples of 8.7 % (8/92) among FIV-vaccinated cats versus 8.5 % (7/82) among FIV-unvaccinated cats ($p = 1.00$).

3.5. Phylogeny

The five New Zealand FIV sequences all clustered with older New Zealand FIV sequences: three sequences from FIV-vaccinated cats clustered with either subtype C or putative A/C recombinant viruses, while two sequences from FIV-unvaccinated cats clustered with subtype A viruses (Fig. 4).

4. Discussion

No protective effect of Fel-O-Vax® FIV vaccination on FIV infection status was detected among household cats from New Zealand. The most likely explanation for these results is lack of cross-protection between immune responses raised to the vaccine strains of FIV and viruses circulating in the field. Other possibilities include selection bias,

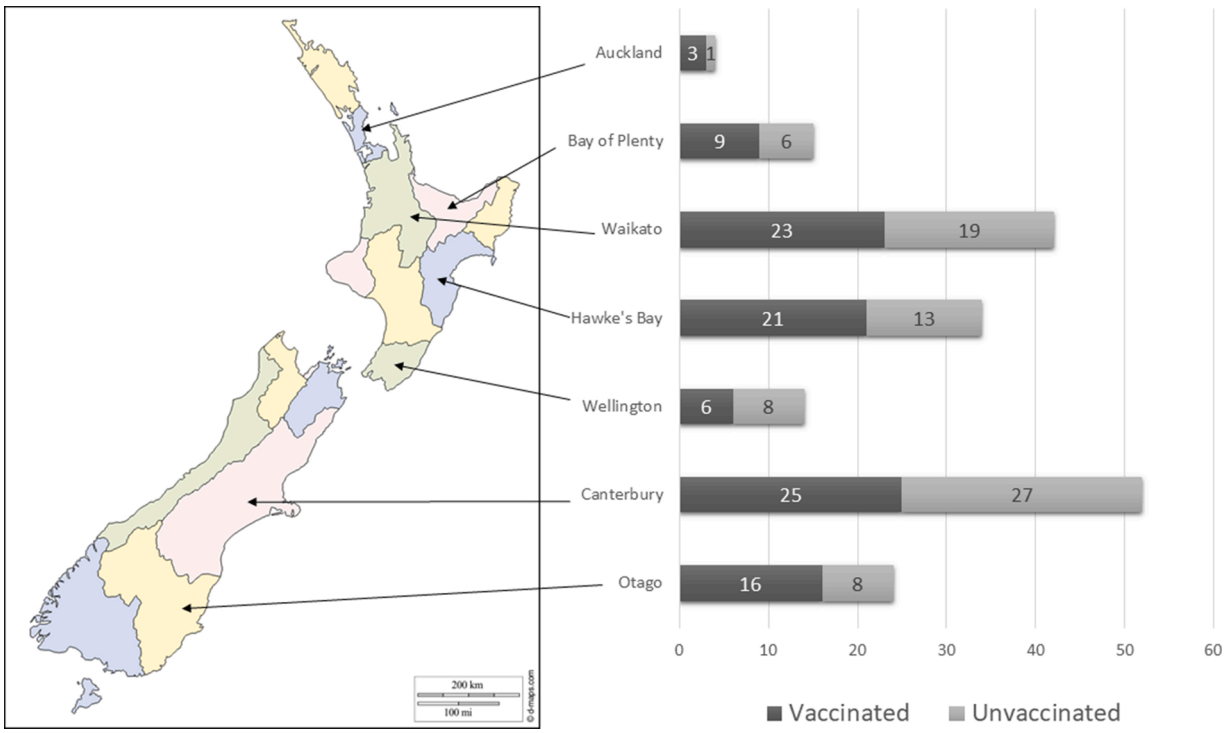

Fig. 2. Number of feline immunodeficiency (FIV) vaccinated ($n = 103$, black bars) and unvaccinated ($n = 82$, grey bars) cats that were retained in the final dataset, stratified by the geographical location of origin. The map has been sourced from https://d-maps.com/m/oceania/nzelande/nzelande34.gif.

Table 2
Signalment data for cats that tested positive for FIV in conventional nested FIV PCR, with the identity of amplicons confirmed by sequencing.

FIV sequence ID	Cat ID	Date of sampling	Location	Cat's sex	Cat's age (years)	FIV vaccination
FIV.NZ.20015/1	U01U	May 2015	unknown	M	11	No
FIV.NZ.20015/2	VAH02V	July 2015	Hawke's Bay	M	3	Yes
FIV.NZ.20015/3	VAH03V	July 2015	Hawke's Bay	F	4	Yes
FIV.NZ.20015/4	TV03V	April 2015	Bay of Plenty	F	3	Yes
FIV.NZ.20015/5	VAH12U	July 2015	Hawke's Bay	M	15	No

vaccine-induced enhancement of infection, poor specificity of the test employed, or timing of FIV infection (prior to primary vaccination for kittens or before development of full protective immunity for adult cats).

Results of several studies indicated that immune responses following FIV vaccination are protective against challenge with a homologous virus but may not be protective against challenge with all heterologous viruses. For example, cats immunized with a vaccine containing FIV_{PET} were protected against challenge with the homologous subtype A FIV_{PET} strain, but only one of four cats was protected against challenge with a heterologous subtype D virus, FIV_{SHI} (Hohdatsu et al., 1997). Similarly, none of the cats vaccinated with Fel-O-Vax® FIV was protected against infection with another type A virus, FIV_{GL8} (Dunham et al., 2006). Others, however, demonstrated protection against challenge with both homologous and heterologous FIV strains following Fel-O-Vax® FIV vaccination, although the level of protection differed between studies (Coleman et al., 2014; Yamamoto et al., 2010). The discrepancies between results of various studies most likely reflect the differences in the source and amount of the challenge virus used, the route of infection, as well as the ages and immune status of challenged cats. Only two studies used a New Zealand isolate of FIV as a challenge virus (Coleman et al., 2014; Yamamoto et al., 2010) – results of both showed low preventable fraction of between 40 and 44 %, consistent with results from the current study. It has been suggested, however, that the efficacy of Fel-O-Vax® FIV vaccine following natural exposure may be better than that following experimental infection due to the fact that naturally infected cats may be exposed to lower doses of infectious virus than those used in challenge studies. To support this view, half (4/8) FIV-unvaccinated cats become infected with FIV, while none of the FIV-vaccinated cats showed evidence of infection when both groups lived together with cats that had been experimentally infected with FIV_{AMORI} (Kusuhara et al., 2005). It took, however, over two years of co-mingling between FIV infected and non-infected unvaccinated cats for the latter to become FIV-positive, highlighting difficulties with prospective studies designed to mimic natural exposure. We used a cross-sectional retrospective study design to investigate the frequency of FIV infection in FIV-vaccinated and FIV-unvaccinated client-owned cats. There was no protection observed among Fel-O-Vax® vaccinated cats, which suggests that even under natural exposure Fel-O-Vax® vaccination was not effective against FIVs circulating in New Zealand.

To our knowledge, only one other group investigated the protection offered by Fel-O-Vax® FIV vaccine using client-owned cats (Westman et al., 2016a). Based on the data from that study, 4.2 % of 212 FIV-vaccinated and 8.8 % of 212 age-, sex-, and post-code matched FIV-unvaccinated pet cats in Australia tested positive for FIV provirus, resulting in 56 % (95 % CI 20–84) protective rate for Fel-O-Vax® FIV vaccination against infection with FIV circulating in Australia. Subtype A virus was identified in all 5/212 FIV-vaccinated cats that became FIV positive. While these protection levels were considerably lower than those obtained in several studies with laboratory adapted strains of subtype A viruses (Coleman et al., 2014), they were higher than those observed in our study.

Similar to the Australian study, we used FIV-vaccinated and FIV-

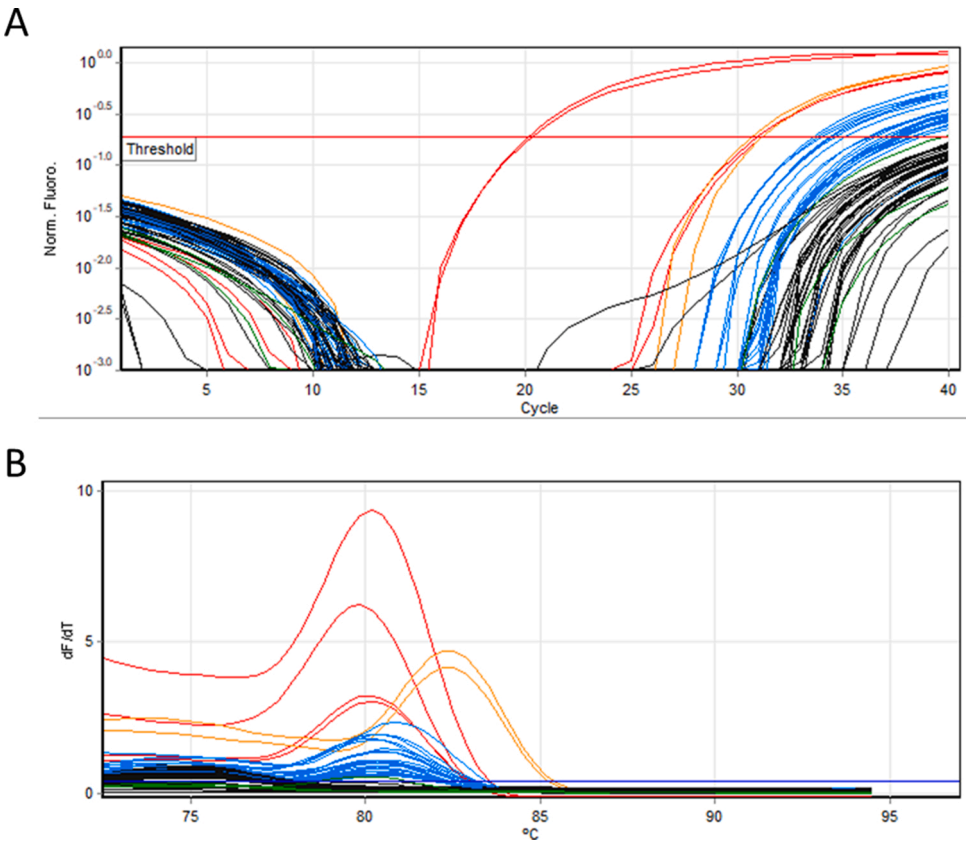

Fig. 3. An example of feline immunodeficiency virus (FIV) qPCR run using 4 μL of template. The amplification plots (A) and corresponding derivative melting curves (B) are shown. Standards (10^7 and 10^4 copies of target DNA/μL of template) are shown in red, positive control (DNA extracted from blood of FIV-infected cat) in orange, positive test samples in blue, negative test samples in black, and non-template control in dark green. Horizonal lines indicate thresholds.

unvaccinated cats from the same veterinary clinic (as a proxy for geographical location). Both New Zealand and Australia have relatively high prevalence of FIV infection among cats, between 0% and 32 % among various cat populations in Australia (Westman et al., 2016c; and references within) and an average of 14 % among cats in New Zealand, with infection rates typically lower among client-owned cats in comparison with stray cats (Gates et al., 2017). However, FIVs circulating in the two countries appear to differ, with most common detection of subtype A viruses in Australia (Kann et al., 2006), and subtype C viruses in New Zealand (Hayward et al., 2007; Kann et al., 2007a). Viruses from different subtypes typically differ between 17 and 27 % at the nucleotide sequence of the *Env* gene (Sodora et al., 1994), and hence the sequence variation between the *Env* gene of New Zealand field viruses and those contained within the Fel-O-Vax® FIV vaccine may contribute to the apparent lack of protection offered by Fel-O-Vax® FIV vaccination in New Zealand (Stickney et al., 2013). The fact that all three viruses from FIV-vaccinated cats that were sequenced in the current study clustered with either subtype C or putative subtype A/C recombinant viruses seems to support this view, although more viruses from both FIV-vaccinated and FIV-unvaccinated cats would need to be sequenced to draw any solid conclusions. An on-going molecular surveillance of FIV among FIV-vaccinated and FIV-unvaccinated cats not only in New Zealand but also in other countries where FIV vaccination is routinely offered to cat owners would help to identify whether or not there are any common characteristic for viruses that escape vaccine-induced protection.

The approach used in the current study has its limitations. While the use of client-owned cats assured natural challenge in terms of the dose, route and exposure to a range of different viruses circulating in a given geographical area, the frequency of exposure could not be predicted, as it relied on individual cat's behaviour and the level of contact with FIV-positive cats. Ideally, the FIV-vaccinated and FIV-unvaccinated cats would have been age- and sex-matched, but this was considered unrealistic due to differences between participating clinics in the number of cats seen and frequency of FIV vaccination. The latter is most likely the reason for higher numbers of samples from FIV-vaccinated than FIV-unvaccinated cats supplied by some clinics.

We attempted to minimize differences between FIV-vaccinated and FIV-unvaccinated cats by enrolment of only cats with access to outdoors, but we cannot exclude the possibility that cats with more risk-prone behaviours (such as roaming or fighting), and hence higher likelihood of exposure to the virus, were more likely to be FIV-vaccinated compared with cats that lived within a well-established social structure in close proximity to their homes. Such risk-prone behaviour is most common among entire male cats. Although sex was not associated with FIV status based on logistic regression analysis, we did not separate entire males from castrated males due to lack of desexing data. As such, we cannot exclude bias introduced by the composition of the groups with respect to proportions of entire males, which may be one explanation for the higher rates of FIV infection among FIV-vaccinated versus FIV-unvaccinated cats in the current study.

An alternative explanation for the apparent poor protection of the Fel-O-Vax® FIV vaccination observed in this study is infection of cats during the course of primary vaccination, before development of protective immunity. Neither can we fully exclude the possibility that some of the FIV-vaccinated kittens became infected with FIV prior to vaccination. Kittens may acquire FIV antibodies from the colostrum of an infected or vaccinated queen (MacDonald et al., 2004) and hence testing of kittens <6 months of age for the presence of FIV antibody is not recommended. Kittens are also thought to be at low risk of FIV infection, as fighting is the major route of transmission and this is not a common behaviour for kittens. However, in one study, FIV was transmitted to

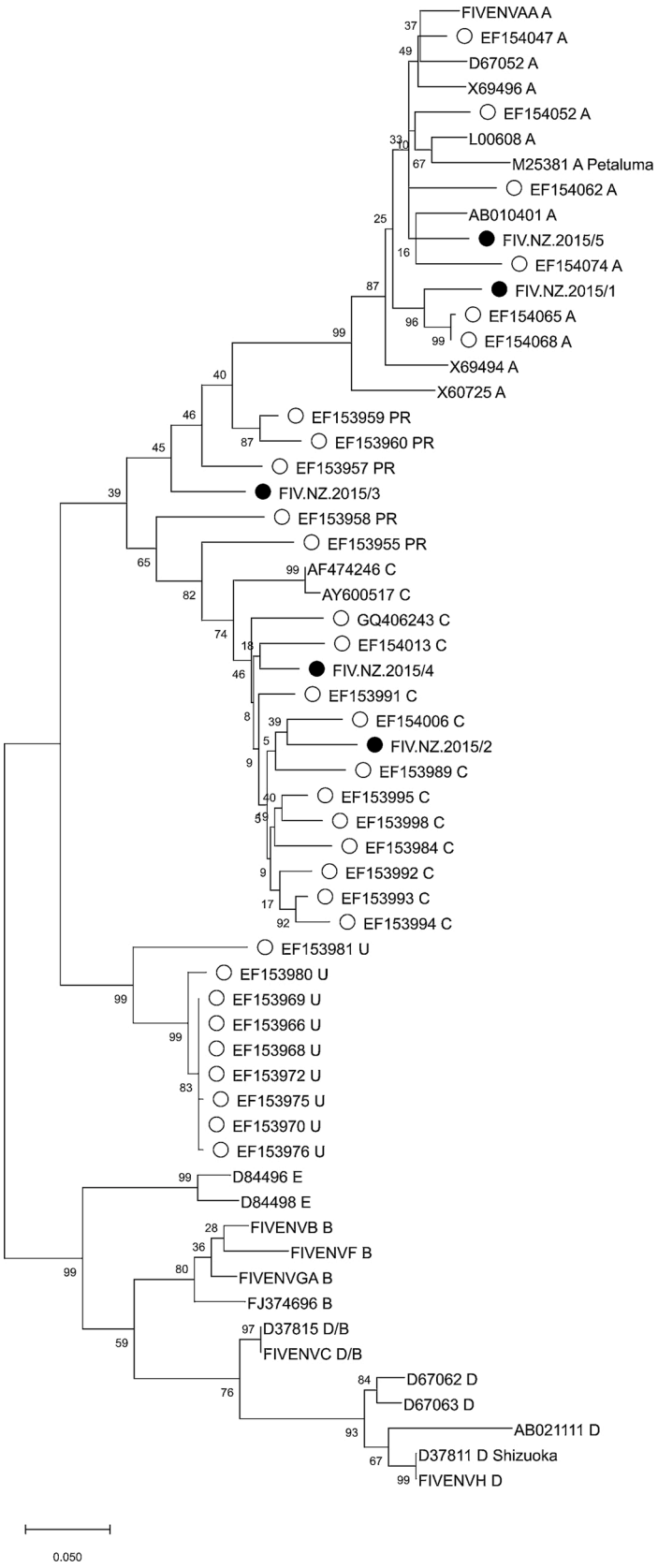

Fig. 4. Evolutionary analysis of feline immunodeficiency virus (FIV) sequences. The evolutionary history was inferred by using the Maximum Likelihood method and Hasegawa-Kishino-Yano model in MEGA X (Kumar et al., 2018). The tree with the highest log likelihood (-9417.13) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 59 nucleotide sequences. All positions with less than 90 % site coverage were eliminated, i.e., fewer than 10 % alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). There were a total of 735 positions in the final dataset. The New Zealand sequences are labelled with open circles (sequences from previous studies available in GenBank) or closed circles close (sequences obtained in the current study). The GenBank accession number is shown next to each node, followed by the type designation of the virus (A, B, C, D, E or recombinant). The additional type designations for New Zealand sequences (PR: possible recombinant, U: unknown) are as defined by Hayward et al. (2007).

kittens from an experimentally infected queen (O'Neil et al., 1995). A total of 11/19 infected FIV-vaccinated cats in the current study received their first FIV vaccination without prior FIV testing. Analysis was therefore repeated to exclude these cats on the basis that we could not be certain that these cats represented true vaccine failures. Results showed that there was still no effect of FIV vaccination on FIV status of the remaining cats.

A possibility of enhancement of infection with locally circulating field FIVs in FIV-vaccinated cats should also be considered as yet another explanation for the results presented. While vaccine-induced enhancement of infection has not been observed in experimental challenge with Fel-O-Vax® FIV vaccine, such phenomenon has been described for several other experimental FIV vaccines (Hosie et al., 1992; Huisman et al., 1998; Richardson et al., 1997). It has been suggested that both antibody-mediated enhancement of virus entry and selective expansion of CD4+ T-cells that are the main target for FIV infection may contribute to this process (Huisman et al., 2009).

It is easier and less invasive to collect saliva from cats than blood, and hence buccal swabs were chosen as a test sample in order to facilitate recruitment of cats. While we demonstrated that we could detect FIV DNA in the buccal swab sample collected in RNA later in preliminary experiments, we could not establish diagnostic sensitivity of our sampling procedure in comparison with traditional testing of blood, as blood samples were not available from the enrolled cats. Others, however, have shown that FIV provirus can be reliably detected in saliva (Chang-Fung-Martel et al., 2013; Matteucci et al., 1993; Westman et al., 2016b). In the largest of these three studies, detection of FIV provirus by commercial qPCR assay in saliva was 72 % sensitive and 100 % specific, as compared with 92 % and 99 % sensitivity and specificity, respectively, using blood samples (Westman et al., 2016b). It is hence possible that the use of saliva instead of blood samples resulted in the underestimation of the number of FIV infected cats in the current study. This, however, is unlikely to have affected conclusions reached, as the same sample type was used for detection of FIV in FIV-vaccinated and FIV-unvaccinated cats.

Only 5/26 FIV-positve samples produced the expected band in the conventional gel-based PCR. This is likely a reflection of a low level of target DNA in the samples combined with the high analytical sensitivity of the qPCR used in the current study (1 copy-equivalent of the FIV provirus). This is supported by the fact that the calculated levels of FIV DNA in the majority of the tested samples were low. Alternatively, a low detection rate of FIV in conventional PCR may have also been caused by sequence variation within the primer-binding regions of New Zealand viruses. Finally, non-specific amplicons with melting peaks at the correct temperature may have been generated during qPCR by chance, particularly for samples with low levels of target DNA. Others (Ammersbach et al., 2013; Crawford et al., 2005) reported higher frequency of false-positive qPCR results among FIV-vaccinated than unvaccinated cats, although the reasons for such a discrepancy are unclear. The qPCR testing was performed in-series in the current study to minimize such errors, but we cannot fully exclude the possibility that some of the qPCR results represented false-positives.

The apparent lack of vaccine-induced protection against field FIVs does necessarily justify recommendation of cessation of its use. The current study was not designed to investigate the effect of vaccination on the pathogenicity of the virus following infection, the onset of disease or the level of virus shedding. As it has been suggested by others (Westman et al., 2016a), it may be that sterilizing immunity is an unrealistic expectation and that the focus should shift towards lowering the levels of the virus circulating in the body and shed in saliva, with the goal to protect from disease (as opposed to infection) and to lower the risk that an infected cat will transmit the virus to other susceptible cats.

5. Conclusion

Results from this study suggest that the Fel-O-Vax® FIV vaccine is not effective at preventing FIV infection among New Zealand client-owned cats. As Fel-O-Vax® FIV vaccinated cats can become infected with field viruses, the FIV infection status should be monitored not only before the commencement of vaccination, but before each yearly booster vaccination.

Acknowledgements

Funding for this study was provided by Healthy Pets New Zealand (formerly the Companion Animal Health Foundation). We also wish to thank all the veterinarians and cat owners who contributed clinical samples to the study.

References

Ammersbach, M., Little, S., Bienzle, D., 2013. Preliminary evaluation of a quantitative polymerase chain reaction assay for diagnosis of feline immunodeficiency virus infection. J. Feline Med. Surg. 15, 725–729.
Beczkowski, P.M., Hughes, J., Biek, R., Litster, A., Willett, B.J., Hosie, M.J., 2015. Rapid evolution of the env gene leader sequence in cats naturally infected with feline immunodeficiency virus. J. Gen. Virol. 96, 893–903.
Cave, N.J., Jackson, R., Bridges, J.P., 2015. Policies for the vaccination of cats and dogs in New Zealand veterinary practices. N. Z. Vet. J. 1–9.
Chang-Fung-Martel, J., Gummow, B., Burgess, G., Fenton, E., Squires, R., 2013. A door-to-door prevalence study of feline immunodeficiency virus in an Australian suburb. J. Feline Med. Surg. 15, 1070–1078.
Coleman, J.K., Pu, R., Martin, M.M., Noon-Song, E.N., Zwijnenberg, R., Yamamoto, J.K., 2014. Feline immunodeficiency virus (FIV) vaccine efficacy and FIV neutralizing antibodies. Vaccine 32, 746–754.
Crawford, P.C., Slater, M.R., Levy, J.K., 2005. Accuracy of polymerase chain reaction assays for diagnosis of feline immunodeficiency virus infection in cats. J. Am. Vet. Med. Assoc. 226, 1503–1507.
Diehl, L.J., Mathiasondubard, C.K., Oneil, L.L., Obert, L.A., Hoover, E.A., 1995. Induction of accelerated feline immunodeficiency virus-disease by acute-phase virus passage. J. Virol. 69, 6149–6157.
Dunham, S.P., Bruce, J., MacKay, S., Golder, M., Jarrett, O., Neil, J.C., 2006. Limited efficacy of an inactivated feline immunodeficiency virus vaccine. Vet. Rec. 158, 561–562.
Gates, M.C., Vigeant, S., Dale, A., 2017. Prevalence and risk factors for cats testing positive for feline immunodeficiency virus and feline leukaemia virus infection in cats entering an animal shelter in New Zealand. N. Z. Vet. J. 65, 285–291.
Gray, M.A., Pratte, Z.A., Kellogg, C.A., 2013. Comparison of DNA preservation methods for environmental bacterial community samples. FEMS Microbiol. Ecol. 83, 468–477.
Hartmann, K., 2011. Clinical aspects of feline immunodeficiency and feline leukemia virus infection. Vet. Immunol. Immunopathol. 143, 190–201.
Hayward, J.J., Rodrigo, A.G., 2010. Molecular epidemiology of feline immunodeficiency virus in the domestic cat (Felis catus). Vet. Immunol. Immunopathol. 134, 68–74.
Hayward, J.J., Taylor, J., Rodrigo, A.G., 2007. Phylogenetic analysis of feline immunodeficiency virus in feral and companion domestic cats of New Zealand. J. Virol. 81, 2999–3004.
Helps, C., Reeves, N., Egan, K., Howard, P., Harbour, D., 2003. Detection of Chlamydophila felis and feline herpesvirus by multiplex real-time PCR analysis. J. Clin. Microbiol. 41, 2734–2736.
Hohdatsu, T., Okada, S., Motokawa, K., Aizawa, C., Yamamoto, J.K., Koyama, H., 1997. Effect of dual-subtype vaccine against feline immunodeficiency virus infection. Vet. Microbiol. 58, 155–165.
Hosie, M.J., Osborne, R., Reid, G., Neil, J.C., Jarrett, O., 1992. Enhancement after feline immunodeficiency virus vaccination. Vet. Immunol. Immunopathol. 35, 191–197.
Huisman, W., Karlas, J.A., Siebelink, K.H., Huisman, R.C., de Ronde, A., Francis, M.J., Rimmelzwaan, G.F., Osterhaus, A.D., 1998. Feline immunodeficiency virus subunit vaccines that induce virus neutralising antibodies but no protection against challenge infection. Vaccine 16, 181–187.
Huisman, W., Martina, B.E., Rimmelzwaan, G.F., Gruters, R.A., Osterhaus, A.D., 2009. Vaccine-induced enhancement of viral infections. Vaccine 27, 505–512.
Kann, R.K., Kyaw-Tanner, M.T., Seddon, J.M., Lehrbach, P.R., Zwijnenberg, R.J., Meers, J., 2006. Molecular subtyping of feline immunodeficiency virus from domestic cats in Australia. Aust. Vet. J. 84, 112–116.
Kann, R., Seddon, J., Kyaw-Tanner, M., Meers, J., 2007a. Co-infection with different subtypes of feline immunodeficiency virus can complicate subtype assignment by phylogenetic analysis. Arch. Virol. 152, 1187–1193.
Kann, R.K.C., Seddon, J.M., Meers, J., Zwijnenberg, R.J.G., 2007b. Feline immunodeficiency virus subtypes in domestic cats in New Zealand. N. Z. Vet. J. 55, 358–360.
Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol. Biol. Evol. 35, 1547–1549.
Kusuhara, H., Hohdatsu, T., Okumura, M., Sato, K., Suzuki, Y., Motokawa, K., Gemma, T., Watanabe, R., Huang, C., Arai, S., Koyama, H., 2005. Dual-subtype vaccine (Fel-O-Vax FIV) protects cats against contact challenge with heterologous subtype B FIV infected cats. Vet. Microbiol. 108, 155–165.

Levy, J.K., Scott, H.M., Lachtara, J.L., Crawford, P.C., 2006. Seroprevalence of feline leukemia virus and feline immunodeficiency virus infection among cats in North America and risk factors for seropositivity. J. Am. Vet. Med. Assoc. 228, 371–376.
MacDonald, K., Levy, J.K., Tucker, S.J., Crawford, P.C., 2004. Effects of passive transfer of immunity on results of diagnostic tests for antibodies against feline immunodeficiency virus in kittens born to vaccinated queens. J. Am. Vet. Med. Assoc. 225, 1554–1557.
Matteucci, D., Baldinotti, F., Mazzetti, P., Pistello, M., Bandecchi, P., Ghilarducci, R., Poli, A., Tozzini, F., Bendinelli, M., 1993. Detection of feline immunodeficiency virus in saliva and plasma by cultivation and polymerase chain reaction. J. Clin. Microbiol. 31, 494–501.
Nishimura, Y., Nakamura, S., Goto, N., Hasegawa, T., Pang, H., Goto, Y., Kato, H., Youn, H.Y., Endo, Y., Mizuno, T., Momoi, Y., Ohno, K., Watari, T., Tsujimoto, H., Hasegawa, A., 1996. Molecular characterization of feline immunodeficiency virus genome obtained directly from organs of a naturally infected cat with marked neurological symptoms and encephalitis. Arch. Virol. 141, 1933–1948.
O'Neil, L.L., Burkhard, M.J., Diehl, L.J., Hoover, E.A., 1995. Vertical transmission of feline immunodeficiency virus. Semin. Vet. Med. Surg. Small Anim. 10, 266–278.
Richardson, J., Moraillon, A., Baud, S., Cuisinier, A.M., Sonigo, P., Pancino, G., 1997. Enhancement of feline immunodeficiency virus (FIV) infection after DNA vaccination with the FIV envelope. J. Virol. 71, 9640–9649.
Sodora, D.L., Shpaer, E.G., Kitchell, B.E., Dow, S.W., Hoover, E.A., Mullins, J.I., 1994. Identification of three feline immunodeficiency virus (FIV) env gene subtypes and comparison of the FIV and human immunodeficiency virus type 1 evolutionary patterns. J. Virol. 68, 2230–2238.
Stickney, A.L., Dunowska, M., Cave, N.J., 2013. Sequence variation of the feline immunodeficiency virus genome and its clinical relevance. Vet. Rec. 172, 607–614.
Swinney, G.R., Pauli, J.V., Jones, B.R., Wilks, C.R., 1989. Feline t-lymphotropic virus (FTLV) (feline immunodeficiency virus infection) in cats in New Zealand. N. Z. Vet. J. 37, 41–43.
Wang, C., Johnson, C.M., Ahluwalia, S.K., Chowdhury, E., Li, Y., Gao, D., Poudel, A., Rahman, K.S., Kaltenboeck, B., 2010. Dual-emission fluorescence resonance energy transfer (FRET) real-time PCR differentiates feline immunodeficiency virus subtypes and discriminates infected from vaccinated cats. J. Clin. Microbiol. 48, 1667–1672.
Weaver, E.A., 2010. A detailed phylogenetic analysis of FIV in the United States. PLoS One 5, e12004.
Westman, M.E., Malik, R., Hall, E., Sheehy, P.A., Norris, J.M., 2015. Determining the feline immunodeficiency virus (FIV) status of FIV-vaccinated cats using point-of-care antibody kits. Comp. Immunol. Microbiol. Infect. Dis. 42, 43–52.
Westman, M.E., Malik, R., Hall, E., Harris, M., Norris, J.M., 2016a. The protective rate of the feline immunodeficiency virus vaccine: an Australian field study. Vaccine 34, 4752–4758.
Westman, M.E., Malik, R., Hall, E., Norris, J.M., 2016b. Diagnosing feline immunodeficiency virus (FIV) infection in FIV-vaccinated and FIV-unvaccinated cats using saliva. Comp. Immunol. Microbiol. Infect. Dis. 46, 66–72.
Westman, M.E., Paul, A., Malik, R., McDonagh, P., Ward, M.P., Hall, E., Norris, J.M., 2016c. Seroprevalence of feline immunodeficiency virus and feline leukaemia virus in Australia: risk factors for infection and geographical influences (2011-2013). JFMS Open Rep. 2, 2055116916646388.
White, J., Stickney, A., Norris, J.M., 2011. Feline immunodeficiency virus: disease association versus causation in domestic and nondomestic felids. Vet. Clin. North Am. Small Anim. Pract. 41, 1197–1208.
Yamamoto, J.K., Sanou, M.P., Abbott, J.R., Coleman, J.K., 2010. Feline immunodeficiency virus model for designing HIV/AIDS vaccines. Curr. HIV Res. 8.