The enteric virome of cats with feline panleukopenia differs in abundance and diversity from healthy cats

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Abstract

Feline panleukopenia (FPL) is a severe, often fatal disease caused by feline parvovirus (FPV). How infection with FPV might impact the composition of the entire eukaryotic enteric virome in cats has not been characterized. We used metatranscriptomic and viral particle enrichment metagenomic approaches to characterize the enteric viromes of 23 cats naturally infected with FPV (FPV-cases) and 36 age-matched healthy shelter cats (healthy controls). Sequencing reads were detected from 11 mammalian infecting viral families mostly belonging to *Coronaviridae, Parvoviridae* and *Astroviridae*. Among the healthy control cats the most abundant viruses were Feline coronavirus, *Mamastrovirus 2* and *Carnivore bocaparvovirus 3* (Feline bocavirus 1) with frequent co-infections of all three. Feline chaphamaparvovirus was only detected in healthy controls (6/36, 16.7%). Among the FPV-cases, in addition to FPV, the most abundant viruses were *Mamastrovirus 2*, Feline coronavirus and *Carnivore bocaparvovirus 4* (Feline bocaparvovirus 2). The latter and Feline bocaparvovirus 3 were detected significantly more frequently in FPV-cases than in healthy controls. *Feline calicivirus* was present in a high proportion of FPV-cases (11/23, 47.8%) compared to healthy controls (5/36, 13.9%, p=0.0067). Feline kobuvirus infections were also common among FPV-cases (9/23, 39.1%) and were not detected in any healthy control cats (p<0.0001). While abundant in both groups, astroviruses were more frequently present in FPV-cases (19/23, 82.6%) than in healthy controls (18/36, p=0.0142). The differences in eukaryotic virome composition found in this study indicate that further investigations to determine associations between enteric viral co-infections on clinical disease severity in cats with FPL are warranted.

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Abstract

Feline panleukopenia (FPL) is a severe, often fatal disease caused by feline parvovirus (FPV). How infection with FPV might impact the composition of the entire eukaryotic enteric virome in cats has not been characterized. We used metatranscriptomic and viral particle enrichment metagenomic approaches to characterize the enteric viromes of 23 cats naturally infected with FPV (FPV-cases) and 36 age-matched healthy shelter cats (healthy controls). Sequencing reads were detected from 11 mammalian infecting viral families mostly belonging to *Coronaviridae, Parvoviridae*and *Astroviridae* . Among the healthy control cats the most abundant viruses were Feline coronavirus, *Mamastrovirus 2* and*Carnivore bocaparvovirus 3* (Feline bocavirus 1) with frequent co-infections of all three. Feline chaphamaparvovirus was only detected in healthy controls (6/36, 16.7%). Among the FPV-cases, in addition to FPV, the most abundant viruses were *Mamastrovirus 2* , Feline coronavirus and *Carnivore bocaparvovirus 4* (Feline bocaparvovirus 2). The latter and Feline bocaparvovirus 3 were detected significantly more frequently in FPV-cases than in healthy controls.*Feline calicivirus* was present in a high proportion of FPV-cases (11/23, 47.8%) compared to healthy controls (5/36, 13.9%, p=0.0067). Feline kobuvirus infections were also common among FPV-cases (9/23, 39.1%) and were not detected in any healthy control cats (p<0.0001). While abundant in both groups, astroviruses were more frequently present in FPV-cases (19/23, 82.6%) than in healthy controls (18/36, p=0.0142). The differences in eukaryotic virome composition found in this study indicate that further investigations to determine associations between enteric viral co-infections on clinical disease severity in cats with FPL are warranted.

1. Introduction

Feline parvovirus (FPV), a small non-enveloped linear ssDNA virus with a 5.1 kb genome is a variant of the species *Carnivore Protoparvovirus 1 (* family *Parvoviridae).* Feline parvovirus causes an enteric and systemic disease known as feline panleukopenia (FPL) and can also infect cats subclinically (Barrs 2019). The virus is shed in high copy numbers in all secretions of infected cats and is transmitted faeco-orally, especially via fomites. Infection by this remarkably environmentally resilient virus occurs most commonly in unvaccinated kittens that first encounter the virus, especially in animal shelters, after maternal antibodies wane. Disease in susceptible cats is characterised by vomiting, diarrhoea, dehydration, sepsis and in severe cases, death (Barrs 2019).

Co-infections of FPV with other enteric viruses have been detected in sick and/or healthy cats including viruses in the families*Parvoviridae* (Genus *Bocaparvovirus* and*Chaphamaparvovirus*), *Astroviridae* (Genus*Mamastrovirus*), *Coronaviridae* (Genus Alphacoronavirus),*Caliciviridae* (Genus *Vesivirus, Norovirus*) and*Picornaviridae* (Genus Kobuvirus) (Brussel et al., 2020; Castro et al., 2015; Di Profio et al., 2021; Ji et al., 2020; Piewbang et al., 2019).

A host can be exposed to multiple viruses with similar routes of transmission and high prevalence at the same or within a short space of time. In young children, co-infection and simultaneous detection of enteropathogens is common (Makimaa et al., 2020). Interactions among co-infecting viruses may be synergistically pathogenic and enteric virus co-infections are commonly identified for all viruses linked to acute gastroenteritis in humans (Simsek et al., 2021). Similarly, enteric co-pathogen infections may play a role in determining clinical outcomes in cats infected with FPV. Since FPV damages gastrointestinal epithelium and bone-marrow to cause local and systemic immunocompromise, the abundance of viral co-infections might be higher in cats with FPL than among healthy cats.

We incorporated both metatranscriptomics, the analysis of non-ribosomal RNA (rRNA) transcripts, and viral particle enrichment metagenomics in this study to characterise the enteric virome of sick FPV-infected and clinically healthy cats. Our specific aim was to reveal any differences in the gut virome of FPV-infected cats, particularly enteric viruses that are absent from the enteric virome of healthy control cats.

2. Methods and materials

2.1 Ethics

The collection of faecal samples from cats in this study was approved by the University of Sydney Animal Ethics Committee (AEC approval number N00/7-2013/3/6029).

2.2 Faecal sample collection

From December 2016 to October 2017, faecal samples were collected from 24 domestic cats (FPV-cases) presenting to veterinary clinics in Sydney, Australia with signs associated with FPL including diarrhoea, vomiting, fever and/or unexpected death (Supplementary Data S1) (Van Brussel et al., 2019). FPV infection was confirmed using PCR and Sanger sequencing of the VP2 protein (Van Brussel et al., 2019). Faecal samples were also collected from 36 heathy, age-matched cats (healthy controls) from two shelters in Sydney from April to August 2017 (Shelter 1-AWL and Shelter 2-CPS). Age, sex, breed, admission date and date of last vaccination were recorded for healthy cats (Supplementary Data S2), all of which had been vaccinated at least once with a Feligen RCP live modified vaccine (Virbac, France) that contains attenuated FPV, *Feline calicivirus*and *Felid alphaherpesvirus* . Faecal samples were stored at -80°C after collection.

2.3 Total RNA extraction and NGS sequencing – metatranscriptome RNA

The isolation of total RNA from faecal samples, rRNA depletion, library preparation and sequencing were performed as previously described (Brussel et al., 2020; Chong et al., 2019). Briefly, the RNeasy plus mini kit (Qiagen, Germany) and Zymo-Seq RiboFree Total RNA Library Preparation Kit (Zymo Research, USA) were used for total RNA isolation, rRNA depletion and RNA sequencing library construction (Brussel et al., 2020). RNA libraries were sequenced on the Novaseq6000 platform (Illumina, USA, 150bp paired end) at the Australian Genome Research Facility (AGRF, Melbourne, Australia) (Brussel et al., 2020). Faecal samples from all 24 FPV-cases and 36 healthy controls were processed and RNA libraries sequenced.

2.4 Virion enrichment, viral nucleic acid extraction, random amplification and NGS sequencing – metagenome cDNA and DNA

Virion enrichment from faecal samples was performed using a previously published protocol (Chong et al., 2019; Conceicao-Neto et al., 2015). Modifications were introduced to prevent DNA sequencing bias as follows: after the enrichment of virions and isolation of nucleic acids (Chong et al., 2019; Conceicao-Neto et al., 2015), nucleic acid extracts were divided into two aliquots of equal volume. One aliquot was subjected to DNase treatment (Invitrogen, Thermo Fisher Scientific, USA) to remove viral DNA, leaving viral RNA (Brussel et al., 2020), while the second, untreated aliquot represented the viral DNA. The viral RNA underwent random amplification using the Whole Transcriptome Amplification Kit (WTA2, Sigma-Aldrich, Merck, USA) and the maximum PCR cycle number (22 cycles) to produce cDNA (Brussel et al., 2020; Chong et al., 2019; Conceicao-Neto et al., 2015). The viral DNA also underwent random amplification using the Whole Genome Amplification Kit (WGA2, Sigma-Aldrich, Merck, USA) following the manufacturer's instructions. The products from both viral RNA (cDNA) and DNA random amplification were purified using the GenElute PCR Clean-Up Kit (Sigma-Aldrich, Merck, USA). Libraries for sequencing were created and sequenced as previously described (Brussel et al., 2020) Briefly, cDNA and DNA libraries were produced using the Nextera XT library preparation kit (Illumina, USA) and were sequenced on the NovaSeq6000 platform (Illumina, USA, 150bp paired end) at the AGRF (Melbourne, Australia). Four of the 24 FPV-cases faecal samples had cDNA or DNA extracts that failed quality control measurements after library preparation and were excluded from sequencing (Supplementary Data S1). Additionally, five of the 24 FPV-cases cDNA libraries were of low quality and therefore sequenced on the NextSeq500 platform (Illumina, USA, 150bp paired end) at the AGRF (Melbourne, Australia). In total 21 FPV-cases cDNA, 22 FPV-cases DNA, 36 healthy control cDNA and 36 healthy control DNA libraries were processed and sequenced.

2.5 Pre-processing of reads, de novo assembly and abundance mapping

Raw sequence reads were processed to remove adapters and primers sequences, PCR duplicates, ribosomal

RNA (rRNA), host (*Felis catus*) reads and poor-quality terminal regions as previously described (Brussel et al., 2020). Briefly, rRNA reads were removed using SortMeRNA and host reads were identified and removed by mapping to the *Felis catus* genome (Brussel et al., 2020). The filtered metatranscriptomic reads (RNA) were *de novo* assembled using Trinity version 2.8.5 and the filtered metagenomic reads (cDNA and DNA) were *de novo*assembled using IDBA-UD version 1.1.2 (Brussel et al., 2020). The contigs were compared to the non-redundant protein database using Diamond version 2.0.4. The taxonomic classification for the filtered reads was calculated using KMA version 1.3.9a (Clausen et al., 2018) and CCMetagen version 1.2.4 (Marcelino et al., 2020) by comparing the filtered paired-end reads to the NCBI nucleotide database that contains all NCBI sequences except those of environmental eukaryotic and prokaryotic, unclassified and artificial origin. In CCMetagen read depth, specified as reads per million (RPM), was calculated and the threshold function was disabled to allow all taxonomy levels to be reported (Marcelino et al., 2020). Read abundance was further calculated by mapping filtered reads to the *de novo* assembled contigs observed in this dataset using Bowtie2 version 2.3.4.3. Geneious version 2020.2.5 was used to predict ORFs and annotate genomes. The extent of index-hopping between libraries sequenced on the same lane was minimized by comparing contigs and identifying any identical sequences. The library with the highest read abundance for that sequence was then used to exclude any library that had a read abundance below 0.01% of that number.

2.6 Vaccine PCR and sequencing

The non-structural (NS) and VP2 genes from the FPV Feligen RCP modified live vaccine (Virbac, France) were amplified and sequenced as previously described (Pérez et al., 2014; Van Brussel et al., 2019). Amplicons were sequenced at the AGRF (Melbourne, Australia) and Geneious version 2020.2.5 was used to determine the consensus contig of the NS and VP2 based on the forward and reverse sequence.

2.7 Phylogenetic, recombination and statistical analysis

Nucleotide and amino acid sequences downloaded from NCBI GenBank were used for phylogenetic analysis and aligned employing the E-INS-I algorithm in MAFFT version 7 (Katoh et al., 2013). IQ-TREE version 1.6.7 (Nguyen et al., 2015) was used to determine the best-fit nucleotide and amino acid substitution models for each alignment using the ModelFinder program and the Akaike information criterion (AIC) (Kalyaanamoorthy et al., 2017). Phylogenetic trees were inferred using the maximum likelihood method in IQ-TREE employing the SH-like approximate likelihood ratio test (SH-aLRT) and ultrafast bootstrap with 1000 replicates to assess nodal support (Hoang et al., 2018). Branch support of >70 SH-aLRT and >70% UFBoot is shown as a grey circle on the branch node of all phylogenetic trees. Nucleotide alignments using MAFFT version 7 were screened for recombination events using RDP4 and possible recombination events were then confirmed using Simplot. To compare the virus composition in FPV-cases and healthy controls, a two-tailed Fisher's Exact Test was used and a p-value of <0.05 was considered significant.

2.8 Excluded sequences – Contaminants, endogenous viruses and bacteriophages

Circoviridae and *Genomoviridae* contigs and a complete pneumovirus genome detected in our sequencing libraries were considered likely reagent-associated contaminants (Porter et al., 2021) and not analysed in this study. Additionally, any retrovirus and bacteriophage sequences detected in this study were not analysed. The picobirnaviruses detected here were also disregarded, since although commonly detected in mammalian faeces they contain a bacterial motif that is only detected in the viral genome of bacterial RNA viruses, consistent with their recent classification as bacteriophages (Krishnamurthy et al., 2018).

3. Results

3.1 Overview

For FPV-cases, a total of 23 metatranscriptomic and 42 metagenomic (22 DNA and 20 cDNA) sequencing libraries were generated, while for the healthy control cats, we obtained 36 metatranscriptomic and 72 metagenomic (36 DNA and cDNA) sequencing libraries. Results from one FPV-case sample were excluded due to poor sequencing quality in both metatranscriptomic and metagenomic libraries. Sequencing produced 2,316,187,631 paired end reads after filtering, including 1,174,728,799 from metatranscriptomic and

1,141,458,832 from metagenomic sequencing.*De novo* assembly generated 9,492,829 contigs from the metatranscriptomic sequencing libraries and 538,922 contigs from the metagenomic sequencing libraries. Viral read abundance calculated by CCMetagen and grouped by taxonomy classification is depicted in Figure 1. Enteric virus co-infections detected in FPV-cases and healthy control cats are summarised in Figure 2.

3.2 Parvoviridae

3.2.1 Protoparvovirus

FPV contigs were detected in 2/8 healthy control cats from shelter 1 and 25/28 cats from shelter 2. The FPV sequence in the two cats from shelter 1 had 100% nucleotide sequence identity to a sequence isolated from sick FPV cats in this study, which is circulating throughout the greater Sydney region and has only been identified in cats from New South Wales (NSW) (Van Brussel et al., 2019). At the time of collection both shelter 1 cats were clinically healthy, consistent with a subclinical infection of FPV. For cats from shelter 2 the nucleotide sequence similarity of FPV contigs compared to the FPV vaccine strain was 99.9-100% for NS1 and 99.8-99.9 for VP2, and 23/25 had been vaccinated within the previous 30 days. Read abundance for the healthy control cats was calculated by mapping to a complete FPV genome isolated from a healthy control cat (Supplementary Data S3). Thus, the FPV detected in shelter 2 cats likely represents the vaccine strain or a variant of the vaccine strain. In addition, one healthy control cat from shelter 2 had an FPV VP2 sequence (1448bp contig) with 100% identity to an FPV sequence isolated from an Australian outbreak of FPL in Mildura, Victoria in 2015 (Van Brussel et al., 2019).

3.2.2 Bocaparvovirus

The genus *Bocaparvovirus* contains 25 recognised species that infect many mammalian species (Jager et al., 2021). Three species of feline bocaparvovirus have been described –*Carnivore bocaparvovirus 3*(feline bocavirus 1), *Carnivore bocaparvovirus 4* (feline bocaparvovirus 2) and *Carnivore bocaparvovirus 5* (feline bocaparvovirus 3) (Lau et al., 2012; Ng et al., 2014; Zhang et al., 2014). We identified feline bocaparvovirus contigs in 11/23 (47.8%) FPV-case libraries and 19/36 (55.5%) healthy control libraries (Figure 2). Feline bocavirus 1 contigs were detected in 18/36 healthy controls (50%) and in 9/23 (39%) of FPV-cases (39% 9/23) (p=0.4317). Among healthy controls, feline bocavirus 1 was the bocaparvovirus with the most abundant reads. By contrast, feline bocaparvovirus 2 was the most abundant bocaparvovirus detected in FPV-infected cats and was significantly more frequent in these (21.7%, 5/23) than in healthy control cats (2.8%, 1/36; p =0.0291). Finally, 6/23 (26%) FPV-cases and 1/36 (2.8%) healthy control cats had feline bocaparvovirus 3 contigs (p = 0.0114) (Table 1). Our phylogenetic analysis revealed that feline bocavirus sequences from the case and control libraries were scattered throughout the phylogeny. Feline bocaparvovirus 2 and 3 sequences were more tightly clustered, but clades lack sufficient sequence numbers to assess diversity (Figure 3).

3.2.3 Chaphamaparvovirus and Dependoparvovirus

Chaphamaparvovirus is a recently proposed genus in the*Parvoviridae* family, subfamily *Hamaparvovirinae* that includes species isolated from pigs, dogs, rodent, duck, owl and bat hosts (Palinski et al., 2016; Palombieri et al., 2020; Roediger et al., 2018; Souza et al., 2017; Vibin et al., 2020; Yang et al., 2016). Feline chaphamaparvovirus was recently discovered in the faeces of shelter-housed cats with diarrhoea (Li et al., 2020). We did not detect feline chaphamaparvovirus contigs in any of the FPV-case libraries. However, read abundance data showed the presence of feline chaphamaparvovirus DNA in FPV-case samples at relatively low levels (maximum 25 reads in the metagenomic library). By contrast, in the healthy controls feline chaphamaparvovirus contigs were detected in 6/36 libraries (p = 0.0724) (Figure 2). In the six sequencing libraries, abundance values ranged from 61 to 6,749 RPM for the metagenomic libraries and 87 and 4,648 RPM for the metatranscriptomic libraries.

Dependoparvovirus, another genus in the *Parvoviridae*family, includes the species *Feline dependoparvovirus,* previously detected in a single cat in the same shelter cat population in which feline chaphamaparvovirus was discovered (Li et al., 2020). Here*Feline dependoparvovirus* DNA was detected in 1/23 FPV-case libraries and 5/36 healthy control libraries (p=0.3886).

3.3 Astroviridae

The family *Astroviridae* contains two genera *Avastrovirus*and *Mamastrovirus* that infect birds and mammals, respectively. Astroviruses are commonly associated with acute gastroenteritis in humans (Vu et al., 2017; Walter et al., 2001). The recognised feline astrovirus species is *Mamastrovirus 2,* although several novel feline astroviruses have been identified (Brussel et al., 2020; Zhang et al., 2014). Recently, we proposed two novel feline astroviruses,*Feline astrovirus 3* and *Feline astrovirus 4* , identified in a pilot study of four cats included here, one with FPV and diarrhoea and three healthy control cats (Brussel et al., 2020). Further analysis on this larger data set showed the presence of astrovirus contigs at a higher frequency in FPV-cases (19/23, 82.6%) compared to healthy controls (18/36, 50%) (p = 0.0142) (Figure 2). Two FPV-case libraries had contigs with 96-99% nucleotide similarity to *Feline astrovirus 4* and one contig, identified in an FPV-case library (cat #FPV166), contained the full ORF1a with 99% nucleotide similarity. The*Mamastrovirus 2* sequence infecting two healthy controls is identical to the *Mamastrovirus 2* sequence AUS/AWL isolated in our pilot study, with read abundance counts of 932,723 and 312,958 RPM for the two metatranscriptomic libraries and 896,065 and 862,160 RPM for the two metagenomic libraries. The capsid protein phylogeny revealed two groupings of *Mamastrovirus 2* sequences (Figure 4). Interestingly, two *Mamastrovirus 2* sequences - FPV-2 and FPV-8 - formed a basal group to the human astroviruses in the capsid phylogeny although not in the ORF1b phylogeny (Figure 4). RDP4 and Simplot analysis detected a possible recombination event in the*Mamastrovirus 2* sequence FPV-1 and sequence FPV-10 in the ORF1b and capsid overlap (Supplementary Data S4). However, no recombination events were detected in *Mamastrovirus 2* sequence FPV-2 and FPV-8, contrary to the positioning of both sequence in the ORF1b and capsid phylogenies. It is likely that the currently limited number of complete*Mamastrovirus 2* genomes available on GenBank precludes identification of recombination events.

3.4 Coronaviridae

Alphacoronavirus 1 belongs to the *Coronaviridae* family that includes strains infecting cats, dogs, pigs, the spotted hyena, cheetahs and raccoon dogs (East et al., 2004; Pearks Wilkerson et al., 2004; Vijaykrishna et al., 2007). The prevalence of feline coronavirus RNA in the FPV-case libraries, 60.8% (14/23), was not dissimilar to that in healthy controls (80.5% 26/36) (p = 0.4026, Figure 2) and its read abundance was high in both groups (Table 1). The available spike proteins identified were screened for the amino acid mutations M1058L and S1060A, which are more commonly associated with systemic replication of feline infectious peritonitis virus, which is an endogenous mutant feline coronavirus that causes feline infectious peritonitis, a severe multisystemic viral disease (Chang et al., 2012). None of the spike proteins analysed contained these spike mutations, consistent with their isolation from faecal samples.

3.5 Caliciviridae

3.5.1 Vesivirus

Feline calicivirus, genus *Vesivirus,* family*Caliciviridae,* is a highly contagious pathogen of domestic cats and other *Felidae* (Gaskell et al., 2004). It typically causes self-limiting upper respiratory tract disease (URTD) (Gaskell et al., 2004). Recently enteric-adapted *Feline calicivirus* strains have been isolated from cats with enteritis in single-agent infections or together with other enteric viruses (Di Martino et al., 2020). Here,*Feline calicivirus* contigs were significantly more frequent in FPV-cases (47.8%, 11/23) compared to healthy controls (13.9%, 5/36) (p= 0.0067, Figure 2). With the exception of 2,889 and 6 reads from the FPV-case and healthy control libraries, respectively, no *Feline calicivirus* contigs were identified as *Feline calicivirus*vaccine strain F9. In contrast, 409,597 reads (320 RPM) mapped to the*Feline calicivirus* contigs identified in the FPV-case libraries (Table 1). *Feline calicivirus* contigs identified in the other nine FPV-case libraries ranged from 203 to 7864bp. In the control libraries the longest *Feline calicivirus* contig was 815bp. The phylogenetic analysis of the full genome and capsid protein shows the enteric sequences in this study do not group with the other identified enteric sequences in the phylogeny, instead forming a clade with other sequences from Australia (Figure 5).

3.5.2 Norovirus

The species *Norwalk virus* , genus *Norovirus* , is separated into 10 genogroups that are further classified into genotypes. In humans, norovirus infections by genogroups GI, GII, GIV and GVIII result in gastroenteritis in 50% of cases, whereas nearly all feline norovirus infections have been detected in cats with diarrhoea and belong to genogroups GIV and GVI (Di Martino et al., 2019; Patel et al., 2008). We detected feline norovirus RNA in 1/23 FPV-cases and 1/36 healthy control cats (Table 1) (p=1, Figure 2), both in genogroup GIV (Figure 6). The FPV-case library (cat #FPV202) contained a complete genome. The nine feline norovirus contigs isolated from the healthy control library (cat #CPS73) were 310 to 1927bp in length and contained sections of the polyprotein, capsid protein and VP3 regions.

3.6 Picornaviridae

Feline kobuvirus belongs to the species *Aichivirus A*and was first identified in cats with diarrhoea in South Korea. Other studies have reported an association between feline kobuvirus and diarrhoea in cats (Lu et al., 2018; Niu et al., 2019). Notably, we only detected feline kobuvirus contigs in FPV-cases in 9/23 (39.1%) FPV cat libraries ($p < 0.0001$, Figure 2). Seven of the nine kobuvirus-infected cats were kittens (6 – 9 weeks), two were adults (1-2 years) and four were known to have had diarrhoea. The feline kobuvirus sequences in this study formed a clade in both the full genome and VP1 phylogenies, although feline kobuvirus sequence FPV-9 branches off from the other sequences in this study in both phylogenies (Figure 7).

We also identified *Feline picornavirus* contigs in 0/23 FPV-case libraries and 2/36 healthy controls (p= 0.5161, Figure 2). One healthy control library (cat #AWL8) contained a complete genome with 85.6% nucleotide and 96.1% amino acid similarity to *Feline picornavirus* strain 073F in the complete polyprotein. Comparison of the 3D protein identified in both libraries showed 96.1% amino acid similarity to *Feline picornavirus* strain 127F. The read abundance in the metatranscriptome libraries was 82 and 57 RPM and in the metagenome libraries 42,971 and 6,436 RPM for cats #AWL8 and #AWL9, respectively.

3.7 Anelloviridae

Anelloviruses are small single stranded circular DNA viruses in the*Anelloviridae* family and are the most abundant member of the human eukaryotic virome, comprising around 70% of eukaryotic viruses detected, which include over 700 viruses from 23 families (Gregory et al., 2020; McElvania TeKippe et al., 2012; Ng et al., 2009; Zhang et al., 2016). Until now feline anellovirus reads have been identified in sick animals (Jarošová et al., 2015; Li et al., 2020; Zhang et al., 2014). We observed diverse anellovirus contigs in 65.2% (15/23) FPV-case and 83.3% (32/36) healthy control cats (p=0.1289). Contigs were relatively divergent and shared ~30-90% amino acid sequence similarity. In total >700 anellovirus contigs were detected, of which more than 400 exhibited <50% amino acid similarity to the nearest NCBI virus protein hit. Therefore, read abundance for *Anelloviridae* was not calculated and because these sequences are so divergent we did not perform a phylogenetic analysis.

3.8 Papillomavirdae, Polyomaviridae

Papillomavirus contigs were detected in 3/23 (13%) FPV-cases and in 0/36 the healthy controls (p=0.0545, Figure 2). The papillomavirus contigs covered sections of the E1, E2, E6, E7, L1 and L2 genes of*Felis catus papillomavirus 3* and *Felis catus papillomavirus 4* . In 0/23 FPV-cases and 4/36 (11%) healthy control cat libraries, polyomavirus contigs were detected (p=0.1489). Two healthy control cats had complete polyomavirus genomes with high sequence similarity (93% nucleotide identity) to *Cat associated lyon-IARC polyomavirus* , isolated from a cat with diarrhoea.

3.9 Picobirnaviridae

We observed a large diversity of picobirnaviruses in the faecal samples of 11/23 (47.8%) FPV-cases and 5/36 (13.8%) healthy control libraries (p=0.0067). The complete genome sequence of both segment 1 and segment 2 was detected in one FPV-case library and four healthy control libraries.

4. Discussion

We characterised the enteric eukaryotic virome of 23 FPV-cases and 36 healthy control cats using metatranscriptomic and viral particle enrichment metagenomic approaches. Metagenomic sequencing revealed a large abundance of viruses in families *Astroviridae* ,*Parvoviridae* , *Coronaviridae* , *Caliciviridae* ,*Picornaviridae* , *Anelloviridae* , *Polyomaviridae* and

Papillomavirdae.

Despite the relatively small sample size, several viruses were significantly more likely to be detected in FPV-cases compared with healthy controls including feline kobuvirus, feline astroviruses,*Feline calicivirus* , feline bocaparvovirus 2 and feline bocaparvovirus 3. Indeed, it is notable that feline kobuvirus was only detected in FPV-cases (Figure 2) and the prevalence of detection (39.1%) is the highest reported to date (Chung et al., 2013; Di Martino et al., 2015; Lu et al., 2018). In previous reports, co-infections of FPV, feline coronavirus and/or feline bocavirus 1 were common in cats naturally infected with feline kobuvirus (Chung et al., 2013; Di Martino et al., 2015; Lu et al., 2018; Niu et al., 2019). However, in those investigations targeted conventional PCR was used to screen for a few common enteric viruses only. The use of unbiased sequencing techniques, as performed here, demonstrates that other enteric viruses including feline astroviruses, feline bocaparvovirus 2 and feline bocaparvovirus 3, as well as *Feline calicivirus* are also common co-infections (Figure 2). The feline kobuvirus sequences detected here are the first in Australia, suggesting active circulation among Australian cats and highlighting the importance of screening for this virus in Australian cats with gastroenteritis of unknown cause. Similarly, here we documented the first Australian sequences of *Feline picornavirus* .

Feline astrovirus infections have been identified in both healthy and sick cats (Brussel et al., 2020; Zhang et al., 2019; Zhang et al., 2014), although experimental infection of specific-pathogen kittens with feline astrovirus induces acute enteritis and viral shedding (Harbour et al., 1987). Here, although astrovirus shedding was detected in healthy controls, the prevalence of astrovirus in FPV-cases was significantly higher. Recently feline astrovirus infection was associated with acute gastroenteritis in shelter-housed cats, where 91% of affected cats and 56% of healthy cats were found to be shedding feline astrovirus, using a metagenomics approach (Li et al., 2021).

We detected *Feline calicivirus* at a significantly higher rate in FPV-cases than in healthy control cats. Although primarily a feline respiratory pathogen, evidence is mounting for an aetiological role in naturally occurring viral gastroenteritis (Castro et al., 2015; Di Martino et al., 2020). Experimental infection of cats with *Felinecalicivirus* causes diarrhoea (Povey et al., 1974) and enteric strains of *Feline calicivirus* are resistant to low pH, trypsin and bile salts (Di Martino et al., 2020). In a previous study,*Feline calicivirus* was not detected in healthy cat faeces but was found in 25.9% of diarrhoeic faeces and co-infections with FPV or feline coronavirus were common (Di Martino et al., 2020). In the cats shedding *Feline calicivirus* here, enteric co-infections, especially with feline astroviruses were common in both FPV-cases and healthy control cats (Figure 2).

There are conflicting reports on the prevalence of detection of bocaparvoviruses in cats with enteritis. These viruses have been detected in the faeces of cats with and without diarrhoea and in the oropharynx of healthy cats (Abayli et al., 2021; Li et al., 2020; Yi et al., 2018). Here, feline bocaparvovirus 2 and feline bocaparvovirus 3 were both detected at a significantly higher prevalence in the faeces of FPV-cases compared to healthy controls. Although, evidence for an association between feline bocavirus 1 infection and gastroenteritis in cats is accumulating, we found no significant difference in the prevalence of feline bocavirus 1 in FPV-cases and healthy control cats. One study reported a prevalence of 24.8% in 105 cats with diarrhoea compared to 9.8% in 92 healthy cats (Yi et al., 2018). Two other reports concluded there was an association between feline bocavirus 1 infection and severe gastroenteritis in cats, although sample numbers were small and co-infections with other pathogenic enteric viruses including FPV were common (Liu et al., 2018; Piewbang et al., 2019).

4.2 The virome of healthy cats

Excluding anelloviruses, read counts for feline coronavirus*, Mamastrovirus 2 and Carnivore bocaparvovirus 3* were high in the healthy control cats (Table 1). The finding of high feline coronavirus read counts in this

cohort is not surprising since feline coronavirus is endemic in shelters where the housing of multiple cats in close proximity favours virus transmission and one or more chronically infected "super-shedders" maintain cycles of infection and re-infection, since immunity is short-lived (Addie et al., 2000; Cave et al., 2004). After feline coronavirus, *Mamastrovirus 2* was the most abundant virus detected in shelter-housed cats in this study, corroborating the findings of others that infection rates of astroviruses are high among clinically healthy shelter cats (Zhang et al., 2014).

We observed FPV contigs in core-vaccinated healthy control cats. All FPV vaccine contigs that contained the VP2 region of the genome displayed the amino acid leucine at position 562. This leucine amino acid is present in the FPV attenuated vaccine virus in the Feligen (Virbac, France) vaccine and in other vaccine strains Felocell (Zoetis, USA) and Purevax (Boehringer Ingelheim, Germany). Notably the 562-leucine amino acid was missing from all field strains in the 23 FPV cats in this study. The FPV read counts in the cases were markedly higher than FPV vaccine virus read counts in the healthy controls, consistent with active infection. On average FPV vaccine read counts in healthy controls were 3-6 log lower than the average read count for FPV-cases. Furthermore, our read abundance data suggests that FPV vaccine virus can be shed in faeces up to four weeks after vaccination. Previous studies have demonstrated vaccine virus shedding up to 28 days after vaccination with a live modified FPV vaccine (Bergmann et al., 2019; Jacobson et al., 2022). FPV vaccine virus was detected in one control cat (#CPS35) several months after vaccination with reads detected in the metagenomic library but not in the metatranscriptomic library (Supplementary Data S3). It is also possible that the read count for this healthy control cat is a result of index-hopping during sequencing and not active shedding since a preliminary faecal PCR test was negative for FPV DNA.

Here, feline chaphamaparvovirus sequences were only detected in healthy control cats from both shelters sampled. Similar to other studies, co-infections with other enteric viruses were common (Di Profio et al., 2021; Li et al., 2020). While feline chaphamaparvovirus has been detected in faecal or oropharyngeal samples from healthy cats elsewhere (Abayli et al., 2021; Di Profio et al., 2021), there is some evidence to suggest that feline chaphamaparvovirus may have pathogenic potential as a co-pathogen rather than as a single agent. One study detected feline chaphamaparvovirus in 14/38 (36.8%) sick cats with acute gastroenteritis and 1/51 (2%) controls and all but one of the positive sick cats were co-infected with FPV, feline kobuvirus and/or feline norovirus (Di Profio et al., 2021).

4.3 Methodological observations

When comparing the metatranscriptomic and virion enrichment library read counts it is evident that the metagenomic libraries produced higher read depth than the metatranscriptomic libraries (Supplementary data S6). Therefore, conducting both a metatranscriptomic and virion enrichment sequencing approach produces a broader perspective on virome diversity.

4.4 Conclusions

In conclusion, we revealed differences in viral abundance and diversity between FPV-cases and healthy controls, with infections with feline kobuvirus, feline astroviruses, *Feline calicivirus* , feline bocaparvovirus 2 and feline bocaparvovirus 3 more common in FPV-cases. Despite these differences in virome composition, whether the clinical outcome of FPV-infection is influenced by the presence of particular co-pathogen infections, or by the combined contribution of multiple co-infecting viruses remains to be determined.

Conflict of interest : The authors declare no conflict of interest

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Table 1. Enteric viral species abundance in FPV-cases and healthy control cats calculated using bowtie2.

	FPV-Cases (n=23)	**FPV-Cases (n=23)**	**Healthy controls (n=36)**	**Healthy controls (n=36)**
Virus	**Total reads (Meta-genome and -transcriptome)**	**RPM**	**Total reads (Meta-genome and -transcriptome)**	**RPM**
Parvoviridae				
Feline parvovirus (field or vaccine strains)	470,532,619	12,291,539	7,323,094	197,256
Carnivore bocaparvovirus 3	123,065	3,116	11,456,635	278,814
Carnivore bocaparvovirus 4	12,039,049	524,515	814	26
Carnivore bocaparvovirus 5	143,947	5,292	223,074	5,960
Feline chaphamaparvovirus	10	2	402,172	579
Feline dependoparvovirus	110	0	16,574	15,154
Papillomaviridae				
Felis catus papillomavirus 3	60	2	42	1
Felis catus papillomavirus 4	199	7	377	9
Polyomaviridae				
Cat associated lyon-IARC polyomavirus	12	0	2,228	42
Astroviridae				
Mamastrovirus 2	151,373,185	3,569,170	112,953,736	3,149,338
Coronaviridae				
Feline coronavirus	29,591,557	1,689,200	31,290,366	9,894,288
Caliciviridae				
Feline calicivirus	409,597	44,710	50	20
Feline calicivirus F9 vaccine	2,889	338	6	1
Norovirus GIV	767,133	21,562	1,690	1,435
Picornaviridae				
Feline kobuvirus	3,830,887	372,095	12	6
Feline picornavirus	6	0	60,474	49,605

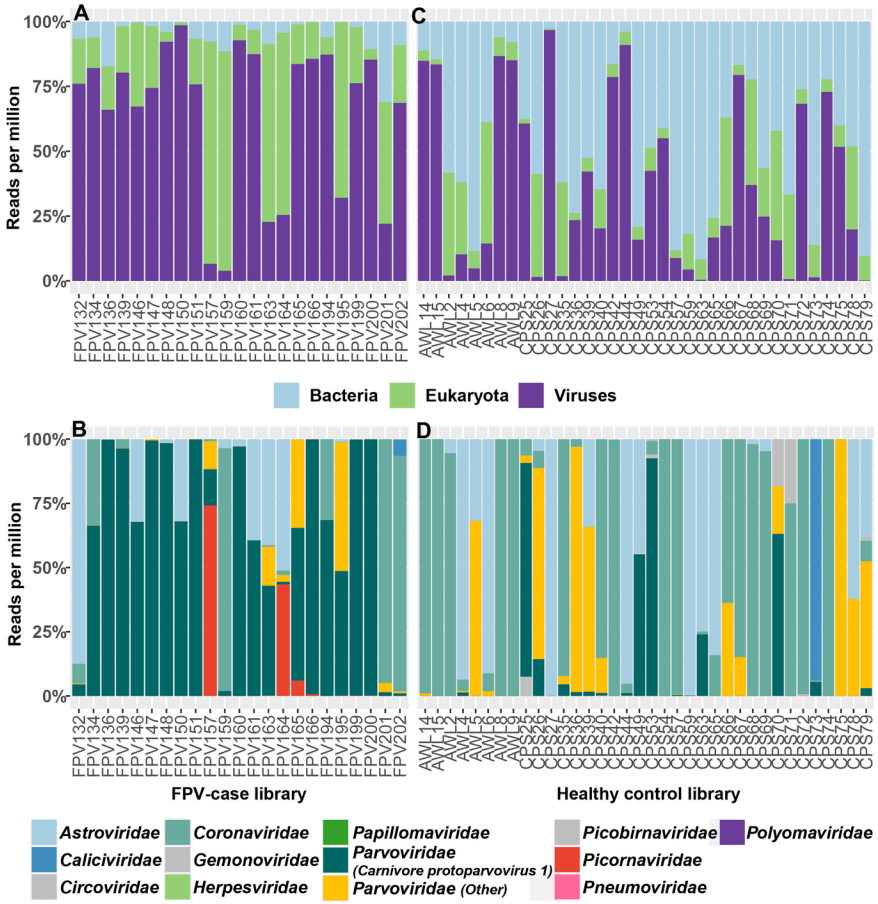


Figure 1. Viral read abundance calculated by CCMetagen and grouped by taxonomy classification. Abundance read count for FPV-case cat libraries by (a) super kingdom and (b) family, and healthy control cat libraries by (c) super kingdom and (d) family. *Anelloviridae*read abundance is not represented in this analysis and*Picobirnaviridae* , *Circoviridae* and *Genomoviridae*are coloured grey as these virus families were disregarded in this study.

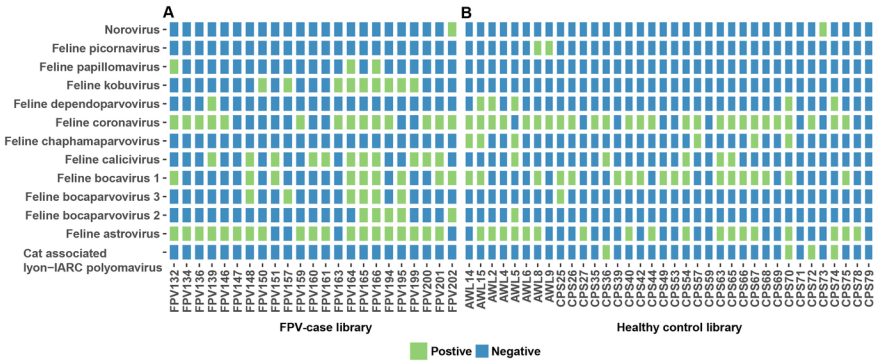


Figure 2. Enteric virus co-infections detected in (A) FPV-cases, and (B) healthy control cats from shelter 1 (AWL) and shelter 2 (CPS). Positive indicates detection of virus contigs in the data set, whereas viral contigs were not identified in negative samples. FPV, picobirnaviruses, circoviruses and genomoviruses have

been excluded from this analysis.

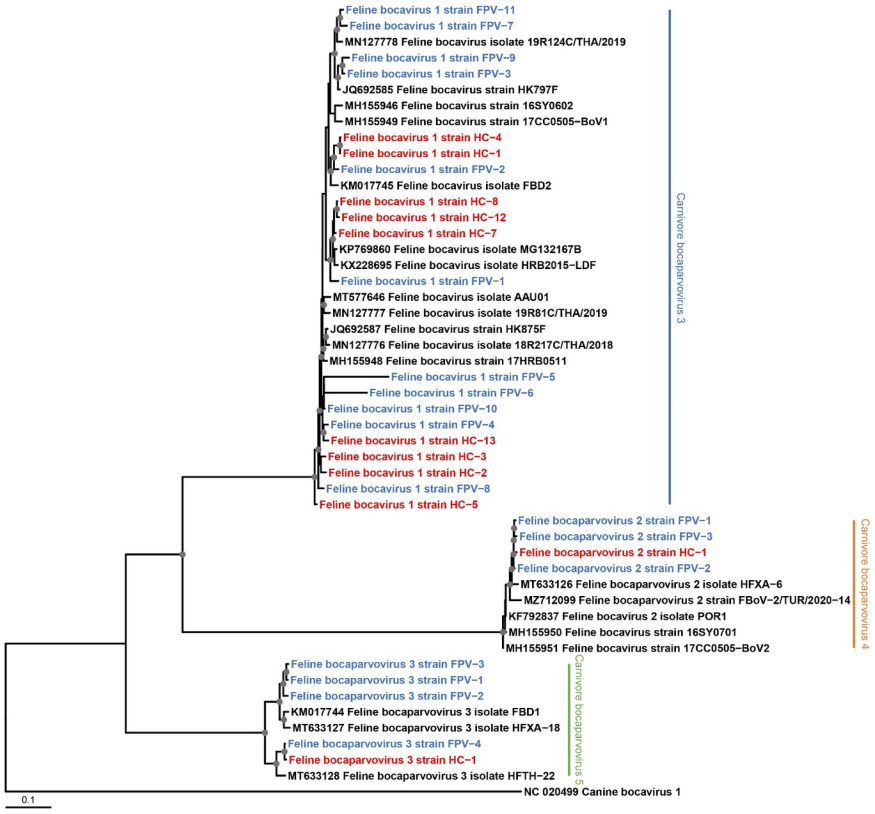
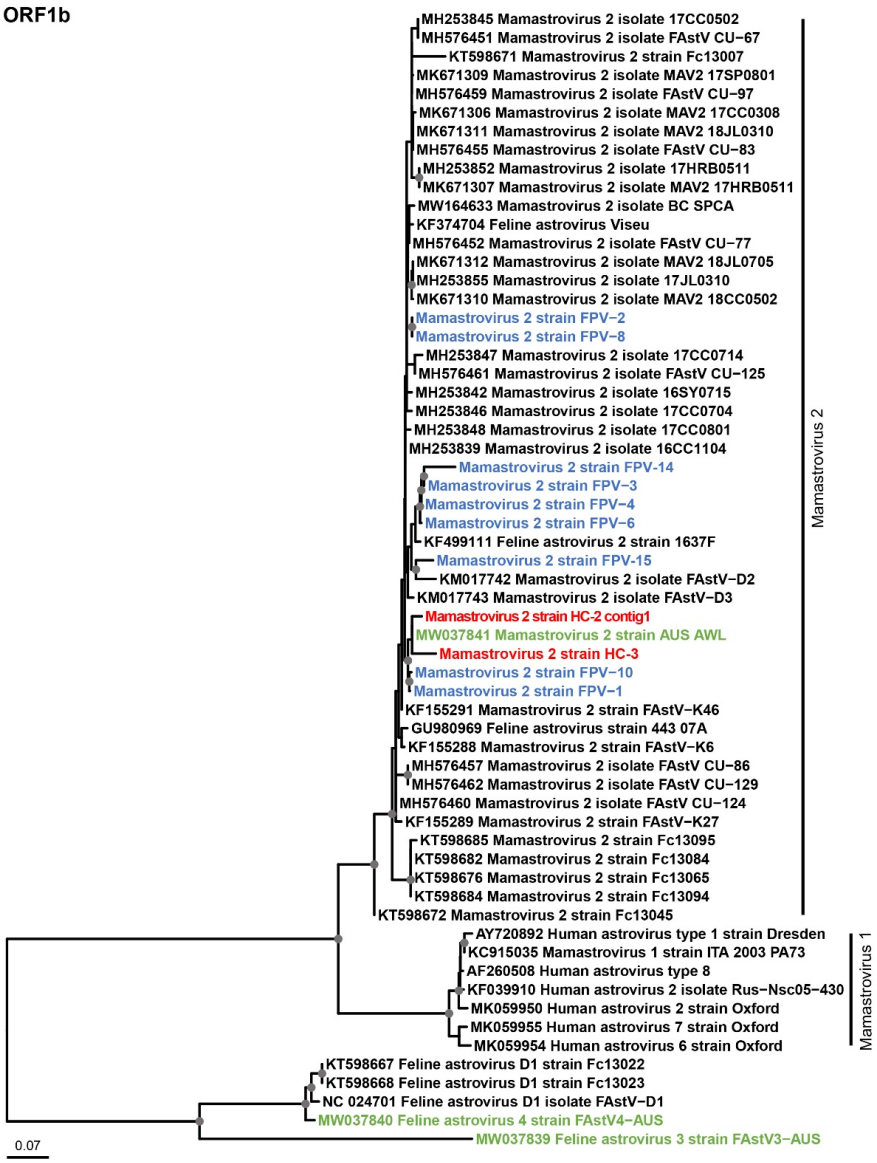


Figure 3. Phylogenetic analysis of the feline bocaparvovirus sequences identified in this study using the VP1 nucleotide alignment and the GTR+F+R8 nucleotide substitution model. Feline bocaparvovirus sequences from the FPV-case libraries are coloured in blue and sequences from the healthy control libraries are coloured in red. The scale bar represents the number of nucleotide substitutions per site. The tree was midpoint rooted for clarity only.



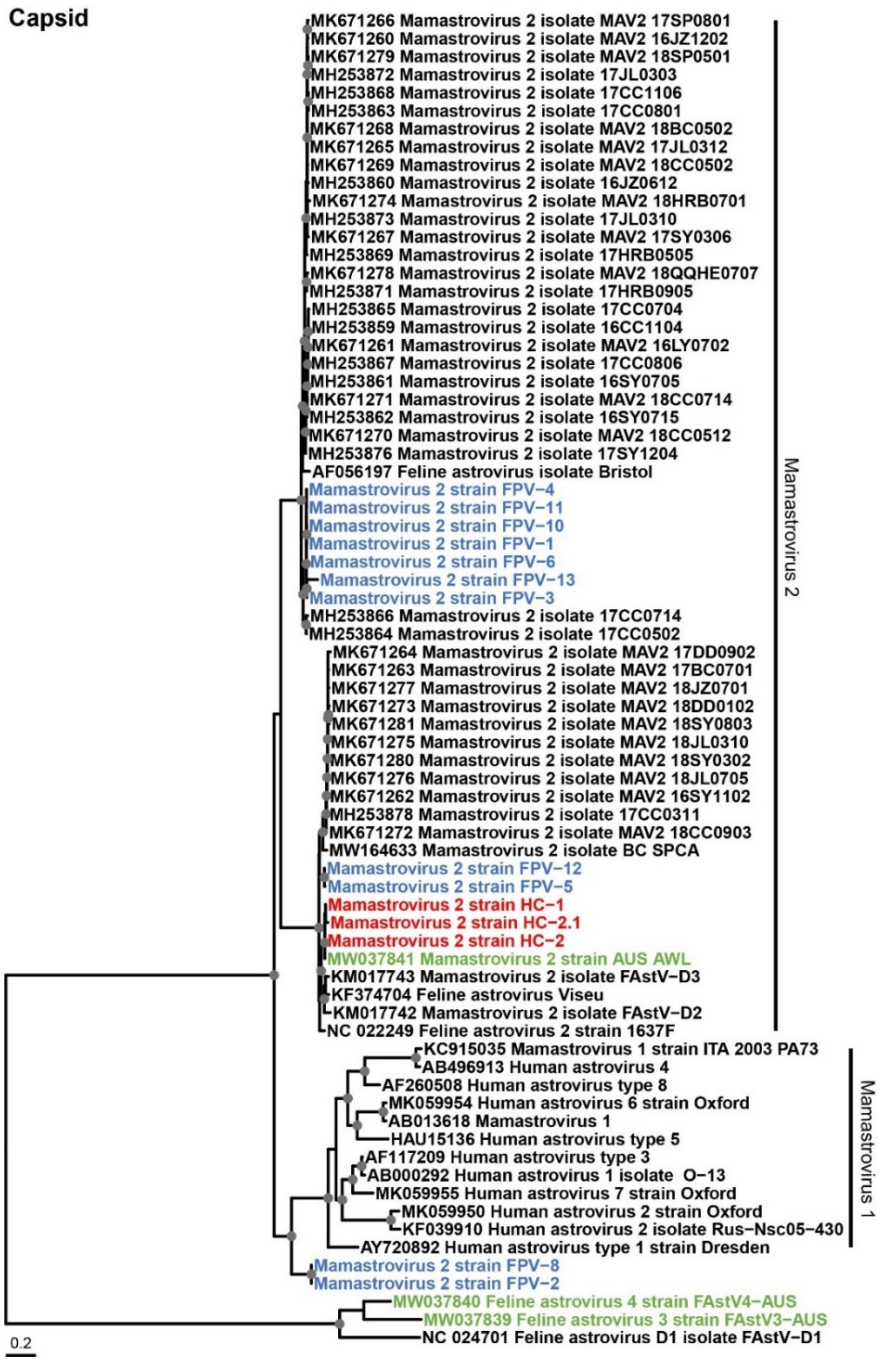


Figure 4 . Phylogenetic analysis of the *Mamastrovirus2* sequences identified in FPV-cases and healthy controls using the ORF1b and capsid protein amino acid alignment and LG+R3 and LG+F+R8 amino acid substitution model, respectively. The *Mamastrovirus 2*sequences from the FPV-case libraries are presented in blue, the sequences from the healthy control libraries in red and the feline astrovirus species and *Mamastrovirus 2* sequence identified in the pilot study in green. The scale bar represents the number of amino acid substitutions per site. The tree was midpoint rooted for clarity only.

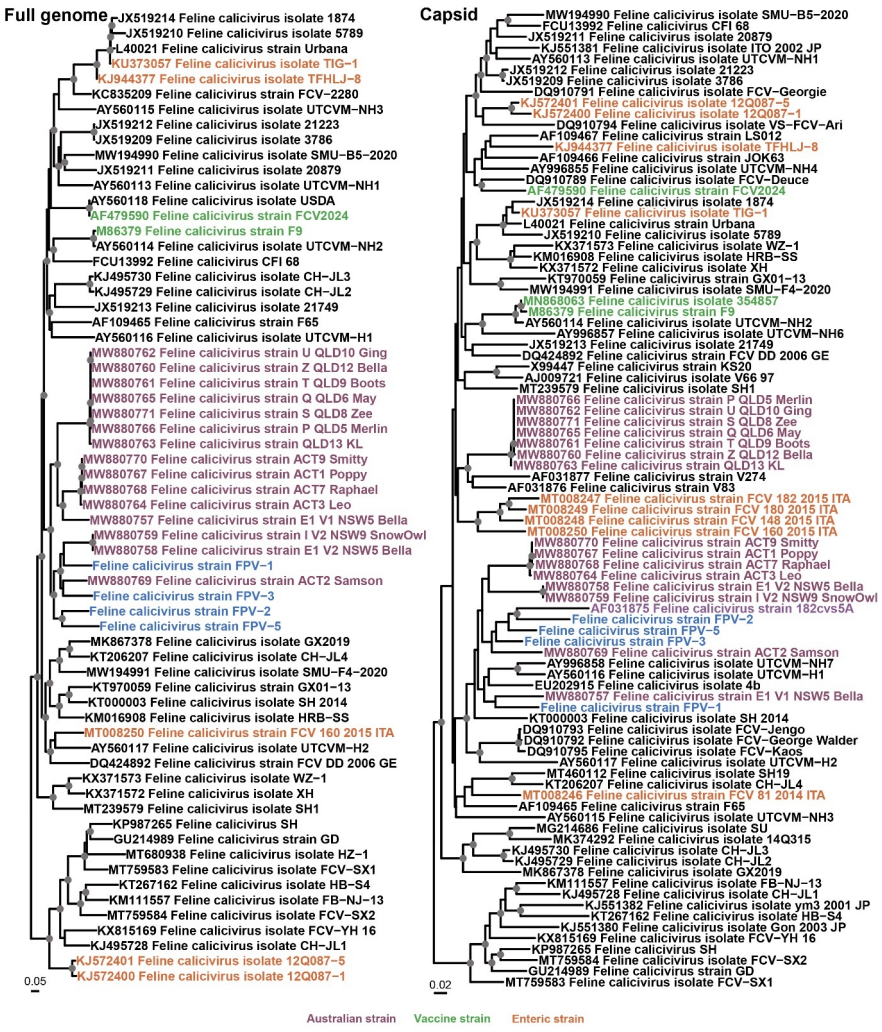


Figure 5. Phylogenetic analysis of the *Feline calicivirus* enteric sequences identified in FPV-cases and healthy controls using the full genome nucleotide alignment and the GTR+F+R4 nucleotide substitution model and capsid protein amino acid alignment and the LG+F+R8 amino acid substitution model, respectively. The*Feline calicivirus* sequences from the FPV-case libraries are coloured in blue. The scale bar represents the number of nucleotide and amino acid substitutions per site. The tree was midpoint rooted for clarity only.

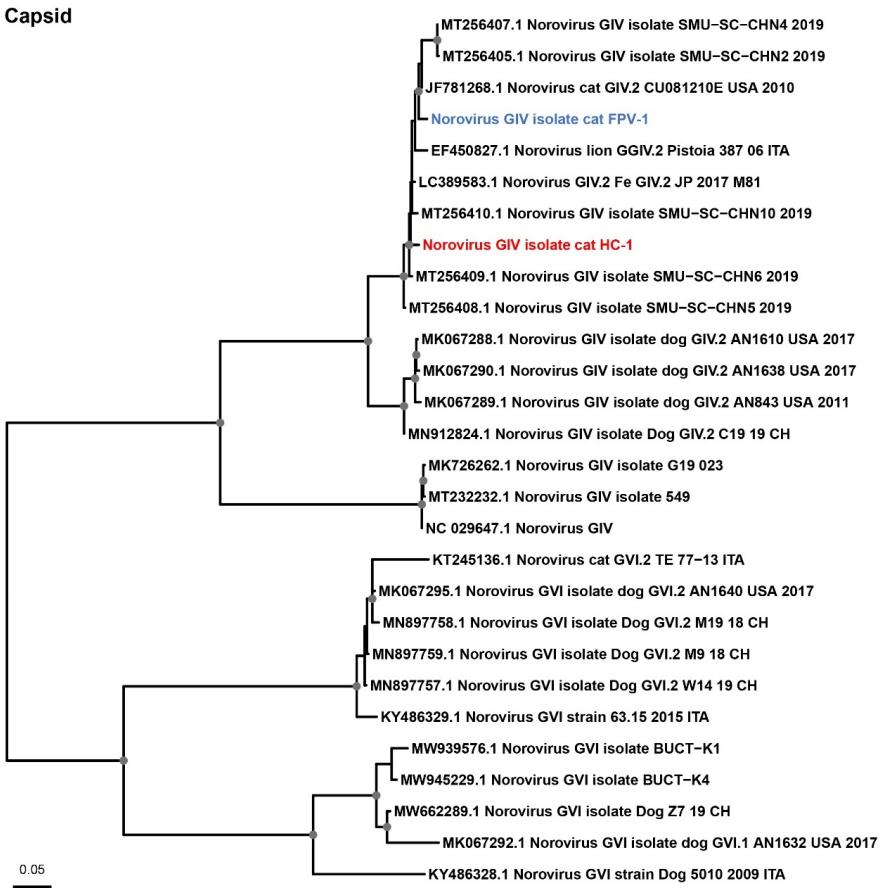


Figure 6. Phylogenetic analysis of the norovirus sequences from FPV-cases and healthy controls using the capsid protein amino acid alignment and the LG+F+R3 amino acid substitution model. The norovirus sequence from the FPV-case cat #202 is coloured in blue and from the healthy control cat #CPS73 in red. The scale bar represents the number of amino acid substitutions per site. The tree was midpoint rooted for clarity only.

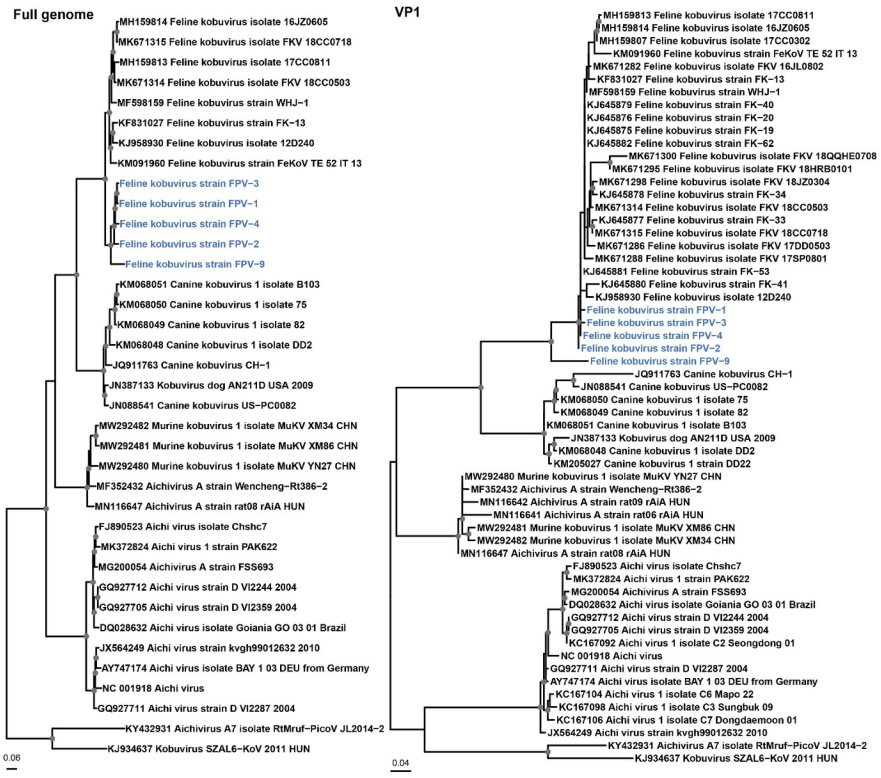


Figure 7. Phylogenetic analysis of the feline kobuvirus sequences identified FPV-cases and healthy controls. The full genome nucleotide alignment and GTR+F+R4 nucleotide substitution model and VP1 amino acid alignment and JTT+G4 amino acid substitution model. The feline kobuvirus sequences from this study are presented in blue. The scale bar represents the number of nucleotide and amino acid substitutions per site. The tree was midpoint rooted for clarity only.