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Feline fecal virome reveals novel and prevalent enteric viruses

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Abstract

Humans keep more than 80 million cats worldwide, ensuring frequent contacts with their viruses. Despite such interactions the enteric virome of cats remains poorly understood. We analyzed a fecal sample from a single healthy cat from Portugal using viral metagenomics and detected five eukaryotic viral genomes. These viruses included a novel picornavirus (proposed genus “Sakobuvirus”) and bocavirus (feline bocavirus 2), a variant of feline astrovirus 2 and sequence fragments of a highly divergent feline rotavirus and picobirnavirus. Feline sakobuvirus A represents the prototype species of a proposed new genus in the *Picornaviridae* family, distantly related to human salivirus and kobuvirus. Feline astroviruses (mamastrovirus 2) are the closest relatives of the classic human astroviruses (mamastrovirus 1), suggestive of past cross-species transmission. Presence of these viruses by PCR among Portuguese cats was detected in 13% (rotavirus), 7% (astrovirus), 6% (bocavirus), 4% (sakobuvirus), and 4% (picobirnavirus) of 55 feline fecal samples. Co-infections were frequent with 40% (4/10) of cats shedding more than one

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of these viruses. Our study provides an initial unbiased description of the feline fecal virome indicating a high level of asymptomatic infections. Availability of the genome sequences of these viruses will facilitate future tropism and disease association studies.

Keywords

metagenomics; virome; enteric virus; *Felis catus*; sakobuvirus

Introduction

Cats are one of the most common pets with worldwide population estimated at 80-400 million. Interactions with humans presumably accelerated following domestication of cats ~9,500 years ago (Driscoll et al., 2007). Known feline viral pathogens that can infect humans include rabies virus causing encephalitis and rotaviruses from cats and dogs that may on occasion be transmitted to humans (Tsugawa and Hoshino, 2008). Feline panleukopenia parvovirus was a likely source of the highly pathogenic canine parvovirus 2 (Hoelzer and Parrish, 2010). Although more than 15 viral families have now been described in cats, our understanding of feline viral infections remains limited. Most feline viruses are currently identified by highly specific tests, such as PCR, antigens, or antibody assays, and highly divergent viruses likely remain undetected.

Here we performed a metagenomics analysis of nucleic acids from enriched viral particles in the feces of a single healthy cat from Portugal. We genetically characterized five enteric viruses and compared their features with those of human and animal viruses. PCR screening for these five viruses was then carried out in fecal samples from Portuguese cats collected in various households and facilities from both diarrheic and apparently healthy animals.

Overview of the Feline Fecal Virome

High throughput metagenomic sequencing of a feline fecal virome was performed using the following steps: filtration of fecal supernatant to enrich viral particles, depletion of host nucleic acid in filtrate using nucleases, unbiased sequence-independent amplification using random priming, and deep sequencing using Illumina MiSeq (Allander et al., 2005; Ng et al., 2012). Sequence data revealed five eukaryotic virus species (Table 1, Fig. 1). Non-viral reads consisted mostly of bacterial and unclassifiable sequence by BLAST. The eukaryotic viral sequences detected, in decreasing number of reads, were picornavirus > astrovirus > bocavirus > picobirnavirus > rotavirus (Table 1). The picornavirus generated the most sequence reads (~48,000 or 9% of total reads), while rotavirus generated the least reads (2 or <0.01% of total). The presence of all five viruses was confirmed by PCR following an independent nucleic acid extraction of the original cat fecal sample, confirming co-infections.

Picornavirus

Picornaviruses (prototype poliovirus) are non-enveloped vertebrate-infecting viruses with a single-stranded, positive-sense RNA genome. The family *Picornaviridae* currently consists

of at least 17 genera (Adams et al., 2013), with new genera being rapidly characterized. The first feline picornavirus (FePV) genome was described in 2012, from fecal and urine samples from stray cats in Hong Kong (Lau et al., 2012b). FePV is most closely related to members of the *Sapelovirus* genus and the unclassified bat picornavirus 3, and a high seroprevalence of FePV (33%) was recorded in stray cats. Recently, a feline kobuvirus, a species in the known *Kobuvirus* genus, was also reported in 15% of diarrhea samples from cats in South Korea (Chung et al., 2013). Here we characterize the third known picornavirus in cats.

More than 48,000 sequence reads representing 9% of the total reads from that fecal sample (Table 1) were assembled into a picornavirus genome with some region coverage reaching up to 3500X. The genome was 7,807 nucleotides long (excluding the poly-A tail) with 55.5% GC content, and organized in form of 5' untranslated region (UTR)^{IRES-IV} [L/VP0-VP3-VP1/2A-2B-2C/3A-3B-3C-3D] 3'UTR-poly(A) (Fig. 2A).

This cat picornavirus was tentatively named feline sakobuvirus A (SakoV-A strain FFUP1; GenBank KF387721). Using amino acid pairwise comparison over the entire polyprotein, the feline sakobuvirus A is slightly more related to members of the *Kobuvirus* genus (41%) than to members of the *Salivirus* genus (40%) or the proposed “*Passerivirus*” genus (turdivirus 1, 33%) (Fig. 2A). Specifically, the SakoV-A genome shared 37%, 38%, and 45% identities to the human Aichi virus 1 (genus *Kobuvirus*) over the P1, P2, and P3 regions respectively, and lower identities to all other picornaviruses including salivirus and “*Passerivirus*” (Fig. 2A). When the 3D region only was analyzed the highest identity was to turdivirus 1 (60%) followed closely by Aichi virus B kobuvirus (59%). Current International Committee on Taxonomy of Viruses (ICTV) criteria states that members of the same picornavirus genus share greater than 40%, 40%, and 50% amino acid identities for the P1, P2, and P3 regions respectively. Thus the feline sakobuvirus A represents the prototype species for a novel picornavirus genus, for which we propose the name “*Sakobuvirus*” (*Salivirus*- and *Kobuvirus*-related *virus*).

Furthermore, sakobuvirus is phylogenetically related to three picornavirus genera: *Kobuvirus*, *Salivirus* and “*Passerivirus*” (Fig. 1A). While human kobuvirus (Aichi virus 1) is associated with human gastrointestinal diseases, salivirus and “passerivirus” were only recently discovered in human diarrheal cases and avian tracheal/cloacal swabs, respectively, but their pathogenicities remain unclear (Greninger et al., 2009; Li et al., 2009). The feline sakobuvirus A does not cluster with the recently described feline picornavirus from Hong Kong (FePV) nor the partially sequenced feline kobuvirus from South Korea (Chung et al., 2013). Therefore, feline sakobuvirus A represents a distinct species in a new genus.

SakoV-A encoded a 2,352 amino acids (aa) long polyprotein that is cleaved into smaller proteins. The proteolytic cleavage sites were predicted based on sequence alignments (Fig. 2A). The L protein was 106 aa long and did not contain the GxCG motif (where x represents a non-conserved amino acids) that is responsible for chymotrypsin-like protease activity in some other picornaviruses (Fig. 1A). Both 5' UTR and the L protein region contained polypyrimidine tracts, in which the longest sequence (36 nucleotides,

CCUUUCUCUUUCUCUACUCCUUCUCCCUUCCCUCCU) was located immediately after the initiation codon of the L protein.

The P1 region of SakoV-A was 2,643 nucleotides long encoding the capsid VP0 or 1AB (372 aa), VP3 or 1C (224 aa), and VP1 or 1D (285 aa) proteins. In some picornaviruses, VP0 is further cleaved into VP4-VP2, but an internal VP0 SakoV-A cleavage site could not be identified. The N terminus of VP0 contained a GxxxT myristoylation site.

The P2 region of SakoV-A encodes the non-structural 2A (113 aa), 2B (150 aa) and 2C (337 aa) proteins. The 2A protein of SakoV-A contained an H-box/NC motif conserved in kobuvirus and “passerivirus”, but not in salivirus. The H-box/NC proteins are related to cellular proteins involved in cell proliferation. The carboxyl terminus of the 2A protein contained a transmembrane domain related to those of kobuvirus and “passerivirus”. The 2C gene contained the conserved NTPase motif GxxGxGKS (GAPGVGKS). SakoV-A 2C also contain the helicase motif DDxxQ (DDIGQ), which resembles the kobuvirus/salivirus/ “passerivirus” motifs (DD[L/I/V]GQ).

The P3 region of SakoV-A encodes the 3A (78 aa), 3B (29 aa), 3C (190 aa), and 3D (468 aa) proteins. The 3C protein, which encodes for a protease, contains the conserved catalytic triad H-E-C, conserved active site motif GxCG (GLCG), and lacks the RNA-binding motif KFRDI. All of these 3C^{Pro} features are also found in kobuvirus, salivirus and “passerivirus” (Fig. 1). The 3D protein, which encodes the RdRp, contained the conserved KDELR, GGNPSG, YGDD and FLKR motifs.

Four clusters of picornaviruses, the sakobuvirus, kobuvirus, salivirus and “passerivirus”, form a monophyletic clade (shaded, Fig. 1A). These 4 groups share common genomic features including a high GC content, L protein, uncleaved VP0, and an H-E-C catalytic triad in the 3C protease (Fig. 1A).

Analysis of the picornavirus non-coding elements

As determined by RACE, the 5' UTR of SakoV-A was 591 nucleotides long and contained a type IV internal ribosomal entry site (IRES) (Hellen and de Breyne, 2007). It is unknown whether any of the 5' UTR may still be missed by 5' RACE. The AUG initiation codon was postulated to be at position 592-594. The first 21 nucleotides of 5' UTR were 95% identical to those at the same location of canine kobuvirus (GenBank JN088541). The first 80 nucleotides exhibit potential secondary and tertiary structure (hairpins A, B, and C, data not shown) found in kobuviruses (Reuter et al., 2009). High sequence identity was found between the 3' end of the 5' UTR and similar regions in porcine kobuvirus (GenBank EU787450, genus *Kobuvirus*) and duck hepatitis A virus 1 (GenBank EU395440, genus *Avihepatovirus*) (73% and 89% identity to position 462- 582 and 506-562 of the SakoV-A genome respectively).

The predicted secondary RNA structure of the 5' UTR IRES showed a potential hepacivirus/ pestivirus-like (HP) type IV IRES with pseudoknots and conserved elements in an upstream bent hairpin-like structure in domain II (II₁, II₂, II₃) and in domain III (III₁, III₂, III₃, III₄, IIIa, IIIb, IIIc, IIId, IIIe and IIIf) (Fig. 3). The predicted lengths of the pseudoknot stem 1, stem 2,

linker, and spacer are 11, 8, 2, and 9 nucleotides, respectively. The conserved nucleotide motifs of branching hairpins III_d and III_e are: UUGGAAA₅₁₀₋₅₁₇ and GCCUGAUAGGGU₅₃₈₋₅₄₉ (Fig. 3).

The 3' UTR was 157 nucleotides long. Position 7718-7769 shared 85% sequence identity with duck hepatitis A virus 1 strain FZ05 (position 7603-7653 of GenBank JX390983). The unexpected region of RNA sequence similarities to duck hepatitis A virus 1 in genus *Avihepatovirus* at both extremities of the sakobuvirus genome may reflect past recombinations seen even between different picornavirus genera (Hellen and de Breynne, 2007). The SaKoV-A 3' UTR is predicted to form a “barbell-like” secondary RNA structure also identified in members of the genera *Avihepatovirus*, *Kobuvirus*, “Gallivirus” and “Passerivirus” (Fig. 3).

Astrovirus

Using deep sequencing, PCR and RACE, we obtained a complete feline astrovirus genome (FeAstV Viseu). Astroviruses are known to infect a wide variety of mammals and birds, and feline astrovirus has been shown to induce enteritis by experimental infection of specific-pathogen-free cats (Harbour et al., 1987). Feline astrovirus (FeAstV) was observed by electron microscopy from cat feces with or without diarrhea (Hoshino et al., 1981; Marshall et al., 1987), and FeAstV sequences have been reported in Europe and in Asia (Lau et al., 2013) (Harbour et al., 1987).

The genome of feline astrovirus Viseu was 6,780 nucleotides in length, with 49.8% GC content (GenBank KF374704). Consistent with other astroviruses, three ORFs were identified, including ORF1a and ORF1b that encode non-structural proteins, and ORF2 that encodes the structural capsid proteins. In the ORF1a/1b overlap region of the FeAstV genome, the conserved “slippery” ribosomal frame shift signal (AAAAAAC) was identified. In addition, at the ORF1ab/ORF2 junction, the highly conserved promoter sequence UUUGGAGNGGGACCNAAN₇ AUGNC was identified, in which the start codon initiating ORF2 is underlined.

Phylogenetically, FeAstV Viseu belongs to the mamastrovirus genogroup 1 (Fig. 1B), and is closely related to a feline astrovirus (FeAstV2) reported in September 2013 in cats from Hong Kong (Lau et al., 2013) and more distantly to another feline astrovirus from the United Kingdom (FeAstV Bristol, GenBank AF056197). Using pairwise comparison of the entire capsid protein, FeAstV Viseu shared 92.6% identity to FeAstV2 from HK, 70.8% to FeAstV Bristol, and 58.7% to human astrovirus 1. Among feline astroviruses, FeAstV Viseu and FeAstV2 differ from FeAstV Bristol in the C-terminal half of the capsid protein, sharing <40% identity.

According to the ICTV, the ORF2 capsid proteins are used to distinguish genotypes and species of astroviruses. Noticeably, the N-terminal half of the feline astrovirus ORF2s shared high amino acid identities among themselves (FeAstV Viseu and FeAstV 2, 96%, Fig. 2B) and with human astroviruses (FeAstV Viseu and HAstV1, 89%). The FeAstVs are most closely related to HAstVs in the capsid amino terminal half, compared to the third closest relative, the porcine astroviruses (FeAstV Viseu and PoAstV2, 65%). In addition,

long stretches of near-identical amino acid residues are observed between feline and human astroviruses (Fig. 2B, black arrow)

The carboxyl terminal half of the ORF2 capsid protein showed much weaker conservation (Fig. 2B). A conserved motif (SRGHAE) was recognized. The 3' UTR was 84 nucleotides long, and shares most nucleotide identities (94%) to human astrovirus SH1 (GenBank FJ375759). RNA folding analysis revealed a double stem loop structure in the 3' UTR sequence typical for astroviruses (Fig. 3).

Based on currently available genome information, the most closely related viruses to the classic human astrovirus (mamastrovirus 1 genotype 1-8) are therefore feline astroviruses, with the Bristol strain slightly closer than Viseu or the Honk Kong FeAstV2 strains (Fig. 1B and Fig. 2B). Past cross-species astrovirus transmission may have led to this close phylogenetic relationship.

Bocavirus

A new bocavirus was also detected, represented by 230 sequence reads that shared <80% nucleotide and amino acid identity to other bocaviruses. The near-complete genome sequence of this virus, tentatively named feline bocavirus 2 strain POR1 (FBoV2-POR1), was 5,479 nucleotides long, with a GC content of 43.3%.

Bocaviruses are small, non-enveloped, ssDNA viruses belonging to the family *Parvoviridae*, which usually infect human and animals through the fecal-oral route and they can cause a broad spectrum of diseases. *Bocavirus* is a genus of *Parvoviridae*, some members of which have been associated with diarrheal and respiratory illnesses in human and other mammals such as dogs, cattle, and pigs (Allander et al., 2005; Kapoor et al., 2012; King et al., 2011), but its pathogenicity remains largely unknown (Martin et al., 2009). Feline bocavirus (FBoV) was recently reported in a survey of 364 stray cats in Hong Kong and detected in fecal, nasal, urine, kidney, and blood samples, respectively (Lau et al., 2012a). Similar to other bocaviruses, FBoV2 encodes four major ORFs: NS1, NP1, VP1, and VP2 (Fig. 2C). The 5' and 3' UTRs were 319 and 157 nucleotides long, respectively. The genome extremities are incomplete, as inverted repeats typical of parvoviruses could not be identified.

Phylogenetically, FBoV2 clustered with, but was distinct from, the three closely related FBoVs reported from Hong Kong (Fig. 1C). FBoV2 shared 58% and 70% identities with these Honk Kong FBoVs in their NS1 and VP1 proteins, respectively. Based on proposed species criterion of NS1 divergence of >15% to its closest relative, FBoV2 is therefore a species distinct from FBoV. The clade of feline bocaviruses (FBoV and FBoV2) was most closely related to canine minute virus, canine bocaviruses, and California sea lion bocaviruses (Fig. 1C).

Rotavirus

Rotaviruses, members of the family *Reoviridae*, can cause severe diarrhea in young or immunosuppressed subjects of various host species. Their genome consists of 11 segments

of double-stranded RNA. Based on the antigenic VP6 protein, rotaviruses have been subdivided into eight serological species A-H by the ICTV (Matthijnssens et al., 2012).

Here a novel rotavirus, feline rotavirus Viseu (GenBank KF792839), was detected and its presence was confirmed by PCR and Sanger sequencing. We obtained a 206-base-pair sequence fragment belonging to the segment 6 encoding intermediate capsid VP6. This partial sequence shared its highest amino acid identity (53%) to the group H rotaviruses (Matthijnssens et al., 2012) and <47% identity to group B rotaviruses, reflecting the presence of a highly divergent virus. Phylogenetically, the feline rotavirus Viseu shared a deep branch with rotavirus group B, G and H (Fig. 1D). Therefore, the feline rotavirus Viseu may represent a new species of rotavirus; however, sequences of all of its 11 segments will be required to determine its exact phylogenetic relationship to other rotaviruses.

Picobirnavirus

Picobirnaviruses (PBVs) are small non-enveloped viruses with a bisegmented double-stranded RNA genome (King et al., 2011). Picornaviruses are highly diverse and can be grouped in two major clades (GI and GII). Picobirnaviruses have been reported in fecal and respiratory samples in humans, non-human mammals, reptiles, and birds, but the pathogenicity of picobirnavirus remains unknown. Until now, no picobirnaviruses had been reported from cats or the family *Felidae* (King et al., 2011).

A feline picobirnavirus genome was partially characterized, in which we obtained 1,236 base pairs of Segment 2 that encodes the RdRp (GenBank KF792838). The feline picobirnavirus was most closely related to a GII human picobirnavirus (ACY01868, Fig. 1E), sharing 65% amino acid identity.

Prevalence and Co-infections of Enteric Viruses in Feline Feces

Viral shedding for each of the five viruses was PCR tested in the feces from 55 cats (including the original fecal sample) from the city of Viseu, Portugal. The presence of all five viruses was confirmed in the original sample. Nine other animals were found to be shedding one to three of these feline viruses. The most common detection was rotavirus (7/55), astrovirus (4/55), bocavirus (3/55), feline sakobuvirus (2/55), and picobirnavirus (2/55) and co-infections were frequent (Fig. 4). Although the samples were collected from different households, clinics, and facilities within the same city (Suppl. Table 1), the resulting virus sequences shared >99% amino acid identities in the short targeted amplicons, suggesting that the same strains were circulating in this city. Because highly specific PCR primers were designed to target only the genomes identified in the original animal, divergent strains may not have been amplified. Other viruses not found in the original case would also have gone undetected. Of the 55 samples, eight were from cats with diarrhea, one of which was positive for sakobuvirus (Suppl. Table 1).

Discussion

The global distribution of cats and their close contacts with humans underscore the need to better understand the composition of their enteric virome. Five viruses were detected in a

single cat feces sample, four of which were highly divergent from previously reported viruses, demonstrating that the enteric feline virome is still largely uncharacterized. It is formally possible that some of these viral sequences are from ingested meat and do not reflect viruses replicating in these cats' digestive systems, although multiple precedents exist for related viruses' detection in cats and other carnivorans. Viral amplifications in vitro, animal inoculations, and antibody testing of cats will be required to confirm the feline tropism of these fecal viruses.

Our data showed that shedding of enteric virus(es) is very common, as 18% (10/55) of the cats tested PCR-positive for at least one of these five viruses (Fig. 4 and Suppl. Table 1). Nine out of the ten cats positive for enteric viruses appeared healthy (Suppl. Table 1.) and co-infections were common (Fig. 4 and Suppl. Table 1). The lack of defined clinical symptoms in cats shedding one of the viruses supports prior studies reporting frequent enteric viral infections in healthy animals (Sabshin et al., 2012; Shan et al., 2011) and humans (Ayukekong et al., 2011; Kapusinszky et al., 2012; Witsø et al., 2006). In order to determine whether these feline viruses may cause disease in a subset of animals (e.g. certain breeds or immunodeficient cats), disease association studies comparing their prevalence in diseased versus epidemiologically matched healthy control, or laboratory inoculation of these viruses, will be required.

This initial characterization of the feline virome provides candidate pathogens for future studies of gastrointestinal or other diseases in cats. Feline sakobuvirus is distantly related to the genera *Kobuvirus* and *Salivirus* and the proposed avian genus "Passerivirus". Aichi virus in the *Kobuvirus* genus has been associated with human gastroenteritis, and animal kobuviruses have been detected in pigs and cows with diarrhea as well as in mouse feces, canine feces, and raw sewage (Kapoor et al., 2011; Li et al., 2011; Phan et al., 2011). Human salivirus (also known as klassevirus) has been associated with diarrhea and detected in human feces from both gastroenteritis patients and healthy people (Greninger et al., 2010; Greninger et al., 2009; Li et al., 2009). The characterization of a new feline picornavirus will allow further investigation into its pathology using animal models.

Viral pathogens can originate from reassortment and recombination events among and/or between human and animal viral species. Some rotaviruses infecting humans are likely to have originated from animals, and feline rotaviruses have been implicated in reassortments with human viral strains (Martella et al., 2010; Tsugawa and Hoshino, 2008). Our analysis indicates that the feline astroviruses (FeAstV Viseu, FeAstV2 and FeAstV Bristol) are the phylogenetically closest astroviruses to the main human astrovirus group (Mamastrovirus 1). In addition, the closest known relative to the first feline picobirnavirus genome reported here is one of the numerous variants of human picobirnaviruses. These results suggest the occurrence of past astroviruses and picobirnaviruses cat-human zoonoses.

This study detected an unexpectedly high number of previously unknown and recently described viruses in the feces of a single healthy cat. An even greater number of viruses may have been detected if the virion size limit imposed by the use of 220 nm pore size filters (Materials and Methods) was increased to allow passage of larger viral particles. Given the number of new viral species in a single cat sample, further viral metagenomics and PCR

prevalence studies in this globally distributed carnivore are likely to rapidly increase the number of viruses associated with cats. The frequent contacts of cats with humans, as well as birds, rodents, and dogs, likely expose those cats to numerous viruses with zoonotic potential. The emergence of canine parvovirus 2 from feline panleukopenia in the late 1970s provides one of the best understood examples of such cross-species adaptation (Hoelzer and Parrish, 2010), while other parvovirus zoonoses do not appear to result in frequent onward transmission in their new hosts (Allison et al., 2013). Comparisons of the viromes in closely interacting animal species, including humans, will indicate which viruses were likely transmitted among them allowing further mutations to fully adapt them to a new host.

Materials and Methods

Sample Collection

A total of 55 stool samples were collected between September and December 2012 from cats housed in catteries, municipal pounds, non-profit rescue shelters, veterinary hospitals, and veterinary clinics across the city of Viseu, Portugal (Suppl. Table 1). Feces were kept at -20°C until processing.

Metagenomic Sequencing

Viral particles were first enriched from the feces of one healthy non-diarrheal cat. Frozen feces were resuspended in PBS buffer, clarified by brief centrifugation and semi-purified by 200 nm filter (Millipore). The filtrate was incubated with a cocktail of DNase and RNase for 1.5 hours (Ng et al., 2012). Purified viral nucleic acids were extracted using QIAamp Viral RNA Mini Kit. Reverse transcription reactions (RT) were performed, using an arbitrarily designed 20-base oligonucleotide followed by a randomized octamer sequence at the 3' end as previously described (Ng et al., 2012). After denaturation, the complementary strand was synthesized using the Klenow fragment DNA polymerase (New England Biolabs), followed by 15-20 rounds of PCR amplification using a primer consisting of only the 20-base fixed portion of the random primer. The library was sequenced using the MiSeq platform (Illumina). Raw reads were trimmed for quality and primer, and assembled *de novo* into contigs. Sequences and contigs were compared to the GenBank non-redundant protein database using BLASTx with an E-value cutoff of 10^{-4} .

Acquisition of Viral Genomes

In order to obtain full viral genomes, primers were designed to amplify and sequence gaps between metagenomic sequences. PCRs were performed as follows: 95°C for 5 min, 45 cycles of [94 °C for 30 sec, 58°C minus 0.2°C per cycle for 30 sec, 72°C for 1 to 5 min], followed by 72°C for 10 min. The 5' and 3' genome extremities were amplified using RACE amplification kits (Invitrogen) according to the manufacturer's instructions and the detailed protocols previously described (Ng et al., 2012). PCR amplicons were directly sequenced in their entirety using specific primers and Sanger sequencing.

Screening of Feline Fecal Viruses by PCR

Stool samples were diluted (10%) in sterile PBS and centrifuged at 8,000 g for 5 min to remove solids. Nucleic acid was extracted from 140 µl of the supernatant with spin column (Viral RNA mini kit, Qiagen, Germantown, USA) according to the manufacturer's instructions. Primer sequences were described in Table 2. For screening feline picornavirus, primers SakobuAF and SakobuAR targeting 304 nucleotides of the 5' UTR was used. For screening feline astrovirus, primers FeAstVAF and FeAstVBF targeting 418 nucleotides of the ORF1b were used. For bocavirus, primers FBoV2AF and FBoV2AR targeting 301 nucleotides of the nonstructural gene were used. For rotavirus, primers FeRVAF and FeRVAR targeting 151 nucleotides of the Segment 6/ intermediate capsid were used. For picobirnavirus, primers FePBVAF and FePBVAR targeting 1327 nucleotides of the RdRP were used.

For feline sakobuvirus, astrovirus, rotavirus and picobirnavirus RNA detection RT-PCR kits were used (Qiagen One-Step RT-PCR Kit, Hilden, Germany). Reverse transcription was performed at 42°C for 30 min followed by 95°C for 15 min. Touchdown PCR cycling conditions consisted of: i) for sakobuvirus, 8 cycles [30 sec at 94°C, 1 min at 63°C minus 1°C per cycle, 1 min at 72°C] and 32 cycles [30 sec at 94°C, 1 min at 55°C, 1 min at 72°C] followed by a final extension for 10 min at 72°C; ii) for astrovirus and rotavirus, 5 cycles [30 sec at 94°C, 1 min at 55°C minus 1°C per cycle, 1 min at 72°C] and 35 cycles [30 sec at 94°C, 1 min at 50°C, 1 min at 72°C] followed by a final extension for 10 min at 72°C; iii) for picobirnavirus, 2 cycles [30 sec at 94°C, 1 min at 52°C minus 1°C per cycle, 1 min at 72°C] and 38 cycles [30 sec at 94°C, 1 min at 50°C, 1 min at 72°C] followed by a final extension for 10 min at 72°C. For feline bocavirus DNA detection, PCR was performed with KAPA Taq DNA Polymerase (Kapabiosystems, MA, USA) with the cycling conditions consisting of 35 cycles of 30 sec at 94°C, 30 sec at 50°C, and 1 min at 72°C, followed by a final extension for 10 min at 72°C.

Amplified products of appropriate size were visualized under UV, excised from the electrophoresis gel, purified with the GRS PCR & Gel Band Purification Kit (GRISP Research Solutions, Porto, Portugal) and sequenced in both directions using the BigDye Terminator v1.1 Cycle Sequencing kit (PE Applied Biosystems, Foster City, Warrington, UK).

Genomic and Phylogenetic Analyses

For picornavirus, the RNA secondary structures of the internal ribosomal entry site (IRES) of the 5' UTR was predicted using the Mfold program with manual correction for picornavirus (Reuter et al., 2009). For astrovirus, the DNA secondary structure of the 3' UTR was also analyzed with the Mfold program.

For sequence alignment, sequences were aligned using Mafft 5.8 with the E-INS-I alignment strategy and previously described parameters (Ng et al., 2011). Bayesian inference trees were constructed using MrBayes. The Markov chain was run for a maximum of 1 million generations. Every 50 generations were sampled and the first 25% of mcmc samples were discarded as burn-in. Mid-point rooting was conducted using MEGA. Identity plots were

created from sliding window analyses using pairwise amino acid sequence alignments, with protein identities calculated between translated sequential fragments of 32 in-frame codons, incrementing by 8 codons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Phylogenetic and genomic analysis of five feline enteric viruses. The Bayesian inference trees were conducted based on A) 3D RdRp protein depicting relationships among representative members of the family *Picornaviridae*, a table detailing genomic features of the reference genomes based on previously published literature (Lau et al., 2012b; Sweeney et al., 2012). Symbols for protease were noted as follows: Y, presence of a protease motif in L; Y/N, presence of L but L is not protease; N, absence of L. Other motifs are described in the main text. B) the complete ORF2 capsid protein of representative astroviruses C) the

complete non-structural protein (NS1) of bocaviruses, D) the partial intermediate capsid protein of rotavirus Segment 6, and E) the partial RdRp protein of picobirnavirus Segment 2. Posterior probabilities of the Bayesian analysis (>95%) are shown next to each node. Scale bar indicates amino acid substitutions per site.

A) Feline Sakobuvirus A - FFUP1 **B) Feline Astrovirus Viseu** **C) Feline Bocavirus 2 POR1** **Fig. 2.**

Genome organization, identity plot and pairwise sequence comparison analyses of three feline virus genomes: A) feline sakobuvirus A, B) feline astrovirus Viseu, and C) feline bocavirus 2. Cleavage site predictions of the sakobuvirus are shown below its genome. For each virus, three identity plots are shown below the genome organization, comparing the coding regions for each virus with three related viruses based on phylogenetic analyses in Fig. 1. The identity values were color-coded: green, 100%; light green, 30-100%, red, <30%.

5' UTR IRES analysis of Feline Sakobuvirus A

3' UTR analysis of Feline Sakobuvirus A

3' UTR of Feline Astrovirus Viseu

Fig. 3.

Predicted RNA secondary structure of the 5' and 3' UTR of the feline sakobuvirus A (FeAstV-A) and the 3' UTR of the feline astrovirus Viseu. For FeAstV-A 5' UTR, the type IV IRES has been annotated according to picornavirus conventions. Domains are labeled II and III; individual helical segments are labeled II₁, II₂, III₁, and III₂, etc.; and individual hairpins are labeled IIIa and IIIb, etc. to maintain the continuity of the current nomenclature. The positions of conserved domains and the polyprotein AUG start codon are indicated by shaded boxes. For FeAstV-A 3' UTR, detailed structure of the 'barbell-like' formation between nucleotide 7712 and 7775 was shown. Grey boxes indicate identical nucleotides in members of genera *Avihepatovirus*, *Kobuvirus*, "Gallivirus" and "Passerivirus". The presence of poly(Y) tract characteristic of this barbell-like structure is indicated.

Fig. 4.

Venn diagram analysis of the five enteric viruses in fecal samples from 55 cats. Co-infections of more than one virus were annotated.

Table. 1

Read distribution of the feline virome. The number of reads and percentage of total reads of the 5 feline enteric viruses were shown.

	# of Reads	% of Total Reads
Picornavirus	48,094	9.11%
Astrovirus	423	0.08%
Bocavirus	348	0.07%
Rotavirus	2	<0.01%
Picobirnavirus	13	<0.01%
Other Sequences	479,278	90.75%
Total	528,158	100.00%

Table. 2

Primers used for the PCR screening.

SakobuAF	CTG GTC GTG GTG ACC GGC TG
SakobuAR	AGC CGC GAC CCT ATC AGG CA
FeAstVAF	GAA ATG GAC TGG ACA CGT TAT GA
FeAstVBF	GGC TTG ACC CAC ATG CCG AA
FBoV2AF	TCG TTC GTC TTG GAA CAT AGC
FBoV2AR	CAG AGC GTG GAT CTG TCT GA
FeRVAf	ATC ACC AAC GTGT TGT CTA CTG A
FeRVAR	TTT TGT GAC TTC CGG ATC AGC
FePBVAF	AGA CCA CCG ACC ATG TTC TC
FePBVAR	CTC AAA TGT GCAA ACC GAA A