

Discovery and Genomic Characterization of Noroviruses from a Gastroenteritis Outbreak in Domestic Cats in the US

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Abstract

Norovirus (NoV) RNA was detected in the stools of 6 out of 14 (42.8%) 8–12-week-old cats with enteritis from a feline shelter, in New York State. Upon sequence analysis of the complete capsid, the six NoVs were found to be identical, suggesting the spread of a unique NoV strain in the shelter. The full-length genomic sequence (7839 nt) of one feline NoV, CU081210E/2010/US, was determined. In the capsid protein VP1 region, the virus displayed the highest amino acid identity to animal genogroup IV genotype 2 (GIV.2) NoVs: lion/Pistoia-387/06/IT (97.9%) and dog/Bari-170/07/IT (90.4%). These findings document the discovery of a novel feline calicivirus, different from vesiviruses, and extend the spectrum of NoV host range. Epidemiological studies using feline NoV-specific diagnostic tools and experimental infection of cats are required to understand whether NoVs have a pathogenic role in this species.

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Introduction

Noroviruses (NoVs) are non-enveloped, polyadenylated, single-stranded, positive-sense RNA viruses and represent a distinct genus in the family *Caliciviridae* [1]. Human NoVs have a worldwide distribution and represent the major cause of non bacterial gastroenteritis. The genome of NoV possesses three open reading frames (ORFs) encoding for a large non-structural polyprotein, a major capsid protein (VP1) and a small basic structural protein (VP2). Based on the full-length VP1, NoVs have been classified into five distinct genogroups (GI to V) [2]. GI, GII and GIV NoVs infect humans, with GII NoVs accounting for the majority of human infections [3]. NoVs have also been detected in animals and classified as GII (swine), GIII (ruminants), GIV (lions and dogs) and GV (mice) [1,4].

Several studies have documented that NoVs also circulate in carnivores. NoVs were first identified in the feces of a captive lion cub with severe hemorrhagic enteritis in Italy [5]. Subsequently NoVs genetically related to the lion NoV were identified in the fecal samples of dogs with diarrhea in Italy and Greece [4,6]. Based on the full-length VP1, the lion and dog NoVs were classified as a distinct genotype (GIV.2) within GIV, along with human Alphantron-like NoVs (GIV.1) [2]. Some canine NoVs are

not classifiable within any established genogroups (GI-V), as they are distantly related to all other human and animal NoVs in the VP1 region [7–9].

Various viral agents including coronavirus, parvovirus, astrovirus, adenovirus, rotavirus and vesiviruses (feline calicivirus, FCV) have been identified in the stools of cats, although there is limited information on their role as enteric pathogens [10–13]. Small round structured viruses (SRSVs) have been detected occasionally in the feces of cats by using electron microscopy (EM) and have been mostly characterized as FCV [12,14]. Interestingly, Norwalk-like 27-nm virus particles, clearly distinguishable from FCVs, were detected from an outbreak of vomiting and diarrhea in adult cats in Germany in 1987, although the viruses were neither adapted to cultivation *in vitro* nor characterized further [15]. More recently, antibodies specific for the baculovirus-expressed VP1 of Lion/NoV/Pistoia-387/06/IT were detected in the sera of cats by ELISA. These previous findings suggest that NoVs infect cats [16].

In this study, the stools of cats with diarrhea were screened for norovirus by reverse transcription (RT)-PCR with several calicivirus universal primer sets followed by sequence confirmation. Also, the full-length sequence of a feline NoV was determined for the first time.

Materials and Methods

Collection of samples

A total of 24 fecal samples were collected in 2010 from domestic cats (*Felis catus*) by the veterinary clinics of The Ohio State University (OSU) (10 samples, collection A) and Cornell University (CU) (14 samples, collection B). Diarrheic specimens were collected from hospitalized cats with gastroenteric symptoms. The 14 CU fecal samples, collected in July and August 2010, originated from young cats (8–12 weeks old) housed in a New York State animal shelter. The OSU cat specimens were collected in three different periods (July, November and December 2010) from animals of different ages (4 months to 8 years old) (Table 1). During the period of hospitalization, cats were located in single cages and feces were collected directly from the cat litter and identified individually with an alpha-numeric code. Fecal samples were stored at -20°C until processing. The fecal samples, collected during this study, were negative for common feline parasites.

RNA extraction

A 10% fecal suspension in 0.01 M phosphate buffered saline (PBS, pH 7.2) was made and the debris were removed by centrifugation at $16000\times g$ for 3 min. The stool suspension was treated with DNase (RNase-Free DNase Set, Qiagen, Inc., Valencia, CA) and extracted using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA) and stored at -70°C .

RT-PCR screening for calicivirus

The samples were analyzed by using broadly reactive primers p289–p290 targeting the highly conserved motifs (DYSKWDST and YGDD) of the RNA-dependent RNA polymerase (RdRp) [17]. Primer pair p289–p290 amplifies a fragment of 319 bp for NoVs and of 331 bp for vesiviruses and sapoviruses. Amplicons of the expected size (319 bp) were obtained from three samples (CU081210E, CU081210J and CU081210M). The amplicons were purified after gel excision with the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA) and sequenced directly from both directions. Upon sequence analysis of the 275 nt fragment, the three samples displayed 94% nt identity to the lion NoV/GIV.2/Pistoia-387/06/IT, confirming the diagnosis of NoV infection.

Full-length genome sequencing of the feline NoV strain CU081210E

The primers are listed in Table 2. The 3' end of the genome (~ 3500 bp) was amplified with a 3'-RACE protocol [18] using specific forward primers (FNoV-F1 and FNoV-F2) designed based on the sequence of the p290/289 amplicons and reverse primers QO and QI. The internal fragment (~ 2000 bp) was amplified using specific reverse primers (FNoV-R1 and FNoV-R2) and the forward primer P1210, targeting the NTPase region [19]. The 5' end fragment was obtained using specific reverse primers (FNoV-R5 and FNoV-R6) and the primer

Table 1. Feline sample collection and results for caliciviruses detection.

| | SAMPLE CODE | ANIMAL ID | COLLECTION DATE | AGE ¹ | SAMPLE TYPE | RT-PCR (p290-289) | RT-PCR (FNoV-F9 FNoV-R15) |
|---------------------------|-------------|-----------|-----------------|------------------|-------------|-------------------|---------------------------|
| OSU (COLLECTION A) | 020911H | H | December 2010 | 4 M | FECES | - | - |
| | 020911I | I | December 2010 | 1Y | FECES | - | - |
| | 020911K | K | December 2010 | 2Y | FECES | - | - |
| | 020911P | P | December 2010 | 1Y | FECES | - | - |
| | 121310A | A | November 2010 | 2Y | FECES | - | - |
| | 121310F | F | November 2010 | 8Y | FECES | - | - |
| | 121310G | G | November 2010 | 3Y | FECES | - | - |
| | 071510D | D | July 2010 | 8Y | FECES | - | - |
| | 071510F | F | July 2010 | 3Y 3 M | FECES | - | - |
| | 071510I | I | July 2010 | 1Y 4 M | FECES | - | - |
| CU (COLLECTION B) | 081210A | A | July 2010 | 8W | FECES | - | NoV |
| | 081210C | C | July 2010 | 8W | FECES | - | - |
| | 081210D | D | July 2010 | 12W | FECES | FCV | - |
| | 081210E | E | July 2010 | 8W | FECES | NoV | NoV |
| | 081210F | F | July 2010 | 8W | FECES | FCV | - |
| | 081210G | G | July 2010 | 8W | FECES | FCV | - |
| | 081210H | H | July 2010 | 8W | FECES | FCV | - |
| | 081210I | I | July 2010 | 8W | FECES | - | - |
| | 081210J | J | July 2010 | 8W | FECES | NoV | NoV |
| | 081210K | K | August 2010 | 8W | FECES | - | NoV |
| | 081210L | L | August 2010 | 8W | FECES | - | - |
| | 081210M | M | August 2010 | 8W | FECES | NoV | NoV |
| | 081210N | N | August 2010 | 8W | FECES | - | - |
| | 081210O | O | August 2010 | 8W | FECES | FCV | NoV |

¹Y: year; M: month; W: week.

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Table 2. Oligonucleotides used for cDNA synthesis and amplification in this study.

| Oligonucleotide | Polarity | Position ^a | Sequence (5' to 3') ^b | Note ^c | Reference |
|-----------------|----------|-----------------------|---|-------------------|------------|
| FNoV-F1 | + | 4480–4503 | GGTTATTAAGCCGCGCTTGACAT | A | This study |
| FNoV-R1 | – | 4659–4681 | GGCTGAGAGGGTGTAATCCAGT | A | This study |
| FNoV-F2 | + | 4507–4530 | GGTTAAATCTCTGCCGAACCCGA | A | This study |
| FNoV-R2 | – | 4626–4648 | GTTGACTTGAGAGGTGCAGGGAA | A | This study |
| P1210 | + | 1610–1632 | GGICMICCIGGIWKIGGIAARAC | A | [19] |
| FNoV-F3 | + | 5143–5163 | CGTGCCCAAGTTCGAAGCCAT | S | This study |
| FNoV-R3 | – | 7251–7274 | CATCCAGTCAACTAGCGTGGTCA | S | This study |
| FNoV-F4 | + | 3434–3455 | GTCAAACGTGCCAGTGGTGAAC | S | This study |
| FNoV-R4 | – | 2826–2846 | CACTGAGTCCTTACTGGAGA | S | This study |
| FNoV-R5 | – | 2797–2815 | CTTTCGCCACGACCCCTTC | A | This study |
| FNoV-R6 | – | 2787–2805 | CGACCCTCTTGCTCTTAC | A | This study |
| FNoV-R7 | – | 4197–4216 | CTTAAGGGCTCGCGTGAATC | S | This study |
| FNoV-R8 | – | 6525–6546 | GCAGATTATACCTCTGGTACTG | S | This study |
| FNoV-F5 | + | 5995–6014 | ATGAGGCCAGGTTGTGCAC | S | This study |
| FragAF | + | 1–21 | atattaattaaGTGAATGAAGATGGCGTCTAA | A | [20] |
| FNoV-F6 | + | 680–699 | GCACTCTACAACTCAATGG | S | This study |
| FNoV-R9 | – | 532–554 | CGAGGTAGATGGCGTAGTGGTAG | S | This study |
| FNoV-R10 | – | 498–518 | ACATCTCGAGGATGGAGCCAG | A | This study |
| FNoV-R11 | – | 424–446 | GTTTGACTTCACGCTCAGACAGG | A | This study |
| FNoV-R12 | – | 2099–2115 | GTGTTGCCAGCCTTGTC | S | This study |
| FNoV-F9d | + | 4655–4677 | GCCCACTGGATWTACACCCTCTC | A | This study |
| FNoV-R14d | – | 7067–7088 | CYT GGT TRT ACC CAA ACT CCA C | A | This study |
| FNoV-R15 | – | 4970–4993 | CTG ATG GTT GGG TCC TCT GGT CCA | A | This study |
| P290d | + | 4445–4467 | GATTACTCCASSTGGGAYTCMAC | A | [17] |
| P289d | – | 4742–4763 | TGACGATTCATCATCMCCRTA | A | [17] |
| Q _T | +/- | 3'/5' end | CCAGTGAGCAGAGTGACGAGGACT CGAGCTCAAGCTTTTTTTTTTTTTTTTTT | A | [18] |
| Q _o | +/- | 3'/5' end | CCAGTGAGCAGAGTGACG | A | [18] |
| Q _i | – | 3' end | GAGGACTCGAGCTCAAGC | A | [18] |
| AAP | + | 5' end | GGCCACGCGTCTGACTAGTACGGGI IGGGIIGGGIIG | A | [21] |
| AUAP | + | 5' end | GGCCACGCGTCTGACTAGTAC | A | [21] |

^aNucleotide position refers to the complete genome sequence of the feline NoV CU081210E (GenBank accession no. JF781268).

^bNon viral sequences are indicated in lowercase.

^cA: amplification; S:sequencing.

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FragAF [20]. The actual 5'-end of the genome was generated with the 5'-RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen, Corp., Carlsbad, CA) using a 5'-RACE protocol with minor modifications [21] and specific reverse primers (FNoV-R10 and FNoV-R11). The cDNA was synthesized using SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen, Corp., Carlsbad, CA) and PCR was performed using TaKaRa Ex TaqTM polymerase (TaKaRaMirus Bio, Madison, WI).

Cloning and sequencing

The RT-PCR or PCR products were purified using the gel purification kit (Qiagen, Inc., Valencia, CA) and cloned into pCR-XL-TOPO vector (Invitrogen, Corp., Carlsbad, CA) for sequencing. DNA sequencing was performed using BigDye Terminator Cycle chemistry and an automated sequencer ABI Prism 3100XL (Applied Biosystems, Foster, CA).

Sequence analysis

Sequence editing, assembling and alignment were performed using BioEdit Sequence Analysis Editor (version 7.0.9.0) [22]. Basic Local Alignment Search Tool (BLAST, <http://www.ncbi.nlm.nih.gov>) was used to find homologous hits in the sequence databases. Phylogenetic analysis (Neighbor-Joining) with bootstrap (1,000 replicates) was conducted using MEGA version 5.03 [23]. Identity matrices were calculated without removing the gaps and with no distance correction.

Pair-wise identity in the full-length VP1 of strain CU081210E to 180 NoV strains was determined using multiple alignments generated with Bioedit software package vers. 2.1 [22]. The values were calculated by the uncorrected distance method using a 181-sequence alignment without removing the gaps, following the outlines of Zheng et al. [2].

Pair-wise identity in the partial polyprotein (~250 aa) was also calculated using a selection of 72 reference NoV strains for whom

either partial (the C-terminus of the polyprotein) or complete ORF1 sequences are available. In addition, the full-length polyprotein was aligned to cognate sequences of five reference NoV strains and the cleavage sites were predicted on the basis of conserved aa motives [24,25].

The full length genome sequence of the feline GIV NoV CU081210E/US was deposited in GenBank with accession number JF781268.

RT-PCR for the diagnosis of feline NoVs

An RT-PCR specific for feline NoV was developed based on the cat and lion NoV sequences. Primers FNoV-F9 and FNoV-R15 (Table 2) were designed to amplify a 338-bp amplicon at the 3' end RdRp region. The assay was performed using the QIAGEN one-step RT-PCR kit (Qiagen, Inc., Valencia, CA). The amplification program included 50°C for 30 min, 95°C for 15 min, 35 cycles at 94°C for 30 sec, 57°C for 30 sec and 72°C for 60 sec and a final extension of 10 min at 72°C.

RT-PCR amplification of the ORF2 of feline NoVs

Primers FNoV-F9 and FNoV-R14 were designed to amplify a 2433 bp fragment encompassing the 3' end of ORF1, the full-length ORF2 and the 5' end of ORF3. The amplification was performed using the QIAGEN one-step RT-PCR kit (Qiagen, Inc., Valencia, CA). The thermal conditions consisted in 50°C for 30 min, 95°C for 15 min, 35 cycles at 94°C for 30 sec, 53°C for 30 sec and 72°C for 2 min and 30 sec and a final extension of 10 min at 72°C. The primers are listed in Table 2.

Results

Full-length genome sequencing of the feline NoV strain CU081210E

In RT-PCR with primers p289/p290, 8 out of the 24 feline samples (all originated from CU collection B), were positive, but only 3 samples (CU081210E, CU081210J and CU081210M) showed a band of the expected size for NoV (319 bp), while the other 5 samples showed a band of 331 bp and were confirmed as FCV by sequence analysis (Table 1). Upon sequence analysis, the feline NoV strains were highly similar to the lion GIV NoV Pistoia-387/06/IT (94% nt and 98% aa identity) in the 275 nt fragment. By the 3' RACE protocol, a ~3.5 kb fragment (from the 3' end of ORF1 through the poly-A tail) was obtained only for

strain CU081210E. The 7839-bp complete genome sequence of the cat NoV was determined by a primer walking strategy. By comparison with a selection of full-length genomic sequences of GI, GII, GIII and GV NoV strains available in the databases, the highest nt identity was found to GII NoVs (56.4–57.6% nt). Nucleotide identity to GI, GIII and GV NoV was $\geq 47.1\%$, while identity to other caliciviruses (sapovirus, vesivirus, lagovirus, nebovirus, recovirus and valovirus) ranged from 29.3 to 34.5% (Table 3, Figure 1).

Three ORFs were identified in the genome of strain CU081210E, as described for other NoVs [1]. The ORF1 was 5241 nt long and encodes the non-structural polyprotein with a predicted size of 1747 aa. In the polyprotein, 5 cleavage sites were predicted: Gln³⁶⁷/Gly³⁶⁸, Glu⁷³⁵/Gly⁷³⁶, Glu⁹²²/Gly⁹²³, Glu¹⁰⁵⁵/Ala¹⁰⁵⁶ and Glu¹²³⁶/Gly¹²³⁷ located between the N-terminal protein, NTPase, “3A-like” protein, VPg, protease and RdRp, respectively [24,25].

As most diagnostic primers target highly conserved motives in the RdRp (ORF1), partial sequences of the 3' end of the RdRp are available in the databases. A selection of 72 partial sequences of the RdRp (~250 aa) of NoVs was retrieved from GenBank and used to compare in detail the feline NoV strain. Identity was 98.9% aa and 94.5% nt to the strain lion/Pistoia-387/06/IT and 98.4% aa and 85.7% nt to strain dog/Bari-170/07/IT. Identity to human GIV NoVs was 76–78% aa and 69.2–70.3% nt (Table 3).

The ORF2 was 1737 nt long and the predicted size of VP1 was 579 aa. The identity was calculated on a selection of 181 NoV capsid sequences. The feline NoV displayed 97.9% aa and 94.1% nt identity to the strain lion/Pistoia-387/06/IT, 90.4% aa and 82% nt to the NoV dog/Bari-170/07/IT and 64.6–68.5% to human GIV (Alphatron-like) NoVs. Identity to non-GIV NoVs was 36.7–54.5% aa (Table 3, Figure 2). In the VP1, four regions were identified, namely the NH2-terminal arm (residues 10–45), the S-domain (residues 46–221), the P1-subdomain (residues 222–274 and 451–569) and the P2-domain (residues 275–450) [26]. In the highly variable P2-domain, identity to the lion and to the dog strains (Bari-170/07/IT) was 97.1% and 86.3% aa, respectively, while identity to human GIV NoVs was much lower (~44% aa). A 20-aa insertion was present in the P2-domain of animal GIV NoVs, with respect to human GIV NoVs.

The ORF3 and the predicted minor structural protein (VP2) were 765 nt and 255 aa long. In the ORF3, identity was high to the lion NoV (96.8% aa and 93.9% nt) and the canine strain Bari-

Table 3. Noroviruses identity (%) to the new feline strain fe/CU081210E/10/USA.

| | GI | GII | GIII | GIV.1 | GIV.2 lion (EF450827) | GIV.2 dog (EU224456) | GV | GVI | | |
|--|--------------|-----------|-----------|-----------|-----------------------|----------------------|------|------|-----------|---------------------|
| feline/NoV/GIV.2/CU081210E/US/2010 (JF781268) | ORF1* | 60,8–64,4 | 62,2–69,5 | 59,8–64,2 | 69,2–70,3 | 94,5 | 85,7 | 55,7 | 79,0–86,3 | Nucleotide sequence |
| | | 62,5–65,1 | 67,3–75,3 | 62,5–65,1 | 76,0–78,0 | 98,9 | 98,4 | 51,2 | 95,3–98,4 | Amino acid sequence |
| | ORF2 | 43,3–46,2 | 50,2–53,9 | 41,4–43,9 | 64,6–65,2 | 94,1 | 82 | 46,2 | 52,8–59,7 | Nucleotide sequence |
| | | 37,6–41,5 | 45,5–49,8 | 36,7–37,4 | 68,2–68,5 | 97,9 | 90,4 | 36,9 | 50,2–54,5 | Amino acid sequence |
| | ORF3 | 26,6–28,2 | 39,0–47,8 | 31,9–32,9 | 58,4–59,3 | 93,9 | 72,9 | 25,2 | 46,3–47,2 | Nucleotide sequence |
| | | 25,4–30,3 | 34,7–44,3 | 20,9–26,1 | 58,8–60,7 | 96,8 | 77,6 | 20,2 | 41,5–44,7 | Amino acid sequence |

*The ORF1 identity (%) was calculated on the partial RdRp nucleotide (~750 nt) and amino acid (~250 aa) sequence of 72 representative strains available in GenBank. doi:10.1371/journal.pone.0032739.t003

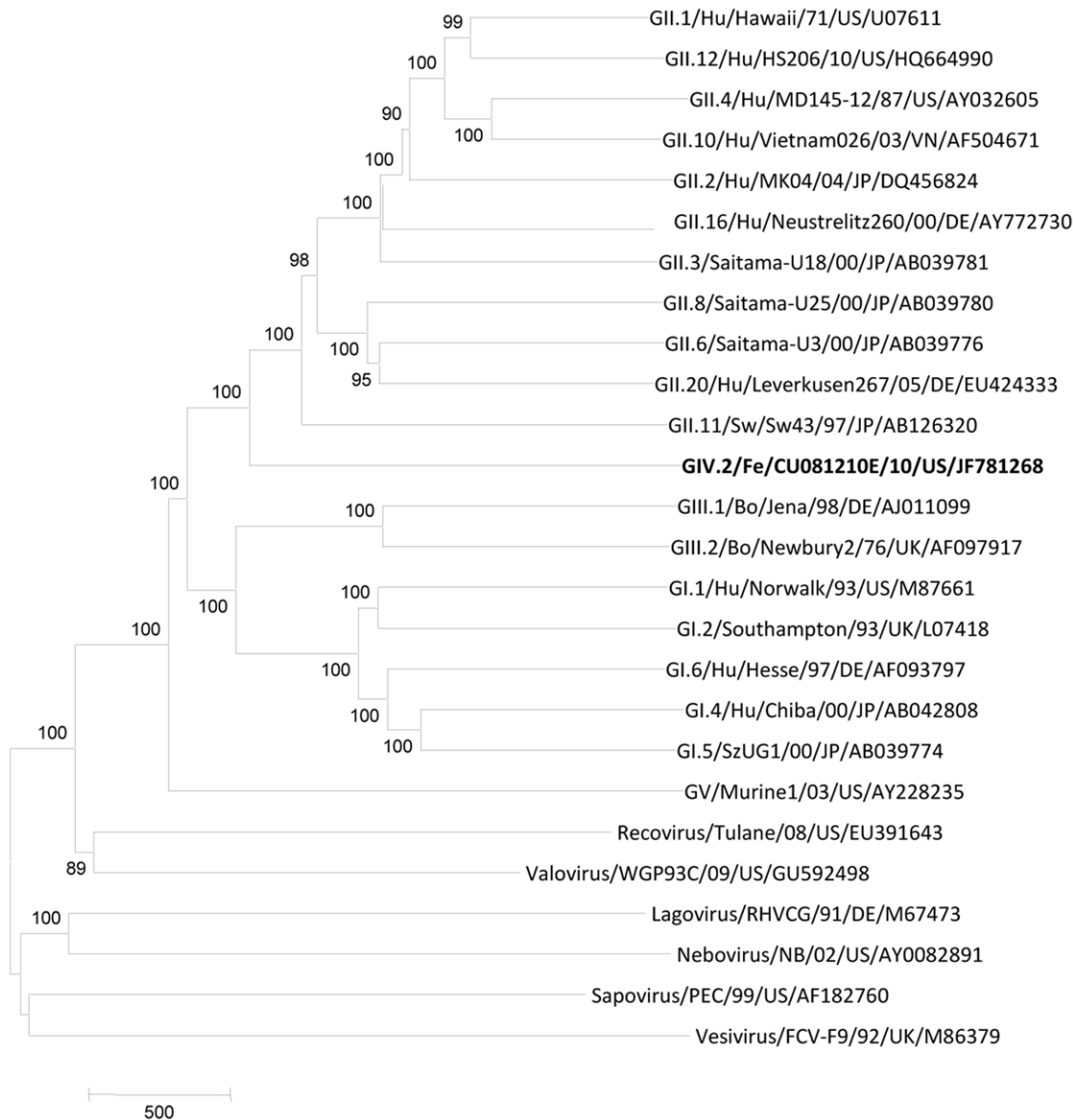


Figure 1. Neighbor-Joining phylogenetic tree of caliciviruses based on the complete genomes (nucleotide). The newly identified feline NoV CU081210E/10/USA is in bold. Bootstrap values are shown near branches. doi:10.1371/journal.pone.0032739.g001

170/07/IT (77.6% aa and 72.9% nt), while it was lower to the other NoVs strains (Table 3).

A 17-nt overlap was present between the end of ORF1 and the beginning of ORF2, while there was a 1-nt overlap between ORF2 and ORF3. The 5' untranslated region (UTR) was 10 nt-long, while the 3' UTR was 104 nt long.

RT-PCR for the detection of feline NoVs

Using the sequences of the feline NoVs obtained in the study, a new diagnostic RT-PCR was developed. By re-screening the sample collections A and B with primers FNoV-F9 and FNoV-R15, 3 additional samples tested positive to NoV from the collection B, but none from collection A. Accordingly, 6 out of the 14 feline diarrheic samples (42.8%) from Cornell University veterinary clinic were positive for NoV. These samples were obtained from 8–12 weeks old kittens with enteric signs housed in a New York shelter (Table 1).

The sequences of a 2.4 kb-genomic fragment of three additional feline NoV strains (CU081210J, CU081210M, CU081210O) were obtained and compared with the sequence of strain CU081210E. The four feline NoV strains displayed 100% nt identity to each other, suggesting a clonal origin.

Discussion

Feline vesiviruses, commonly referred to as FCV, are widespread in cats and they are associated with mild to severe disease of the upper respiratory tract [27]. FCV may be detected from conjunctival, nasal and oropharyngeal swabs and from internal organs [12,28]. Although it is not clear whether FCV also plays a role as an enteric pathogen or if it is shed in the feces after primary localization in other regions, it has been occasionally identified in the feces of cats and associated with enteric disease [28–31]. Also, differences in resistance to bile acids have been found between

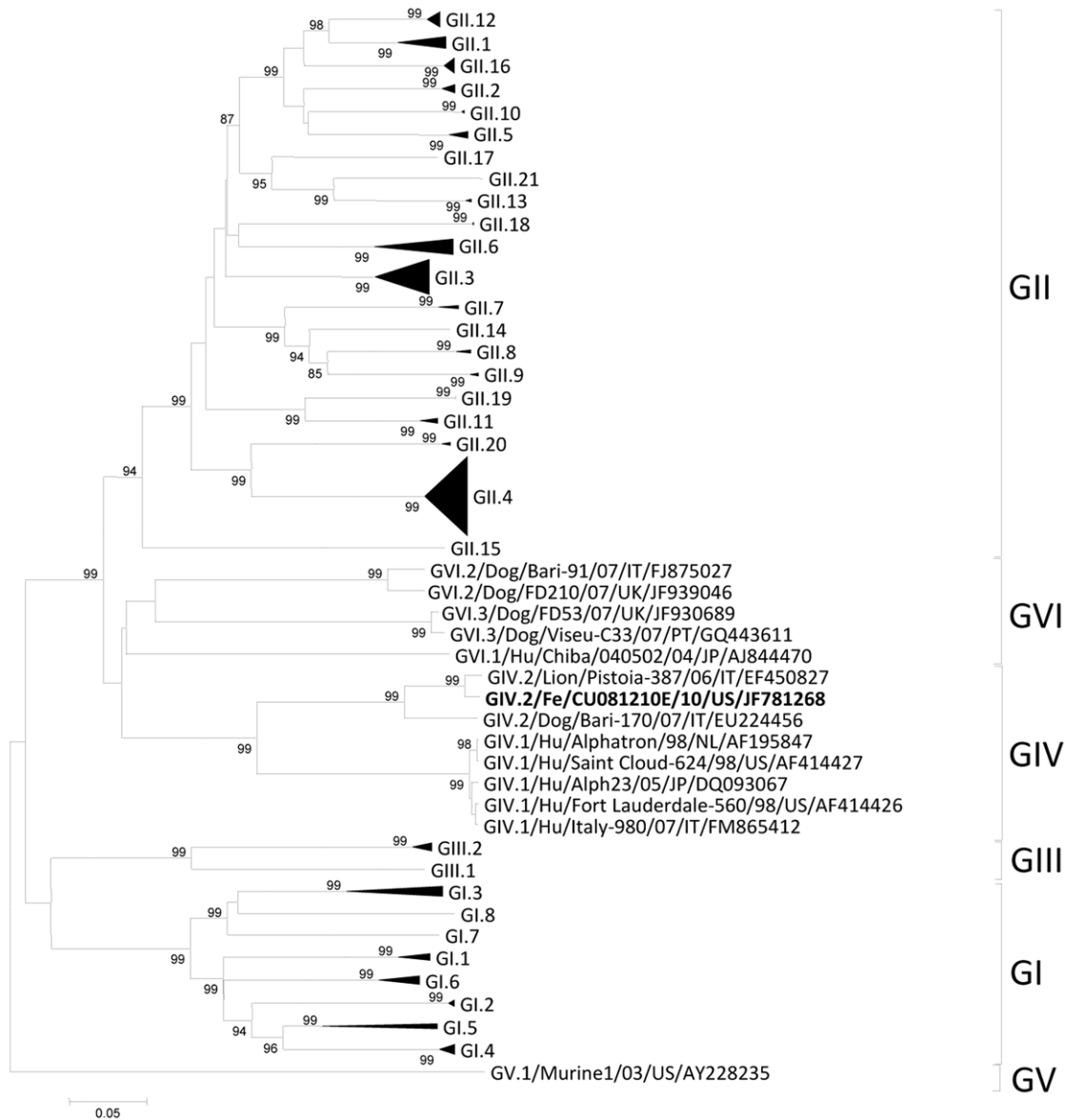


Figure 2. Neighbor-Joining phylogenetic tree of noroviruses based on the complete capsid sequence (amino acid). The newly identified feline NoV CU081210E/10/US is in bold. Bootstrap values are shown near branches. doi:10.1371/journal.pone.0032739.g002

FCV strains of enteric and respiratory origin, thus suggesting that enteric FCVs have the ability to replicate actively in the enteric tract while respiratory FCVs do not [14]. By EM observation, SRSVs have been detected in 6% of the stools of both symptomatic and asymptomatic cats [12]. In the present study FCVs were identified by RT-PCR in 5 out of 14 (35.7%) diarrheic fecal samples of group B while they were not detected from animals of group A (Table 1).

Also, in this study, a novel calicivirus was discovered in cats and characterized as a member of the Norovirus genus by sequence analysis of the complete genome. This novel feline calicivirus was detected in 6/14 samples collected from cats housed in a shelter in New York State (collection B) (Table 1). These findings are in agreement with previous evidence that cats can be infected with NoVs [15,16]. It is of relevance that animals from group B showed enteric symptoms and were young kittens almost of the same age (8 to 12 weeks old). This age period is critical for kittens, since

maternally derived antibodies tend to wane and the animals become fully susceptible to various pathogens [32]. On the other hand, NoVs were not detected in animals from group A. This might reflect either geographic or temporal variations, age-related patterns of susceptibility or merely the relatively small number of samples included in the analysis.

The complete sequence of one strain (CU081210E) and partial (~2.4 kb) sequences of three additional strains (CU081210J, CU081210M, CU081210O) were analyzed. All the NoV strains displayed 100% nt identity to each other. The clonal origin of these viruses suggests that a single NoV strain was spreading quickly in group B animals, consistent with the highly infectious nature of NoVs [33].

In the VP1, the feline NoV displayed high aa identity to the GIV.2 NoV strain lion/Pistoia-387/06/IT (97.9%) and to the dog strain Bari-170/07/IT (90.4%) while the aa identity was ~68% to human GIV.1 (Alphatron-like) NoVs (Table 3). Based on the

currently accepted classification system (2), the feline NoV can be classified as a GIV.2 genotype (Figure 2). In the hypervariable P2-domain, the feline NoV strain was highly conserved with respect to the NoV strains lion/Pistoia-387/06/IT and dog/Bari-170/07/IT (97.1% and 86.3% aa, respectively) while it was less related to human GIV NoVs (~44% aa). The P2-domain represents the protruding region of the capsid protein and is the binding site for cells and protective antibodies [34,35].

A fragment of the RdRp (the 3' end of the ORF1) was used for analysis and comparison with a large selection of NoV strains belonging to all genogroups. This part of the NoV genome can be sequenced easily using the 3'-RACE protocol and forward primers designed in conserved aa motives of the RdRp. Therefore, sequence data spanning this region is available for most NoV strains. In the RdRp the feline NoV displayed high identity to animal GIV.2 NoV strains and to other unusual canine NoV strains that are highly divergent in the VP1 and likely constitute a novel NoV genogroup (GVI) [7,9].

Although a number of GIV NoVs have been detected in humans and sewage samples thus far [36,37], the full-length genome sequence of GIV NoVs is not available for detailed comparison with other NoV genogroups. In this study the complete genome sequence of a GIV NoV was determined and compared with full-length genomic sequences of NoV strains available in the databases, revealing the highest nt identity to GII NoVs (56.4–57.6% nt), whilst nt identity to GI, GIII and GV NoV was $\leq 47.1\%$ (Table 3, Figure 1). This confirms that NoV genogroups GII and GIV are genetically much more related to each other than to other NoV genogroups. The GIV includes both animal (carnivores) GIV.2 and human GIV.1 viruses [6,7].

References

- Green KY (2007) Caliciviridae: the noroviruses. In: Knipe DM, Howley PM, eds. *Fields Virology*. Philadelphia: Wolters Kluwer Health/Lippincott Williams and Wilkins. pp 949–979.
- Zheng D-P, Ando T, Fankhauser RL, Beard RS, Glass RI, et al. (2006) Norovirus classification and proposed strain nomenclature. *Virology* 346(2): 312–323.
- Siebenga JJ, Vennema H, Zheng D-P, Vinjé J, Lee BE, et al. (2009) Norovirus illness is a global problem: emergence and spread of norovirus GII.4 variants, 2001–2007. *J Infect Dis* 200(5): 802–812.
- Martella V, Lorusso E, Decaro N, Elia G, Radogna A, et al. (2008) Detection and molecular characterization of a canine norovirus. *Emerging Infect Dis* 14(8): 1306–1308.
- Martella V, Campolo M, Lorusso E, Cavicchio P, Camero M, et al. (2007) Norovirus in captive lion cub (*Panthera leo*). *Emerging Infect Dis* 13(7): 1071–1073.
- Ntasis V, Xylouri E, Radogna A, Buonavoglia C, Martella V (2010) Outbreak of canine norovirus infection in young dogs. *J Clin Microbiol* 48(7): 2605–2608.
- Martella V, Decaro N, Lorusso E, Radogna A, Moschidou P, et al. (2009) Genetic heterogeneity and recombination in canine noroviruses. *J Virol* 83(21): 11391–11396.
- Mesquita JR, Barclay L, Nascimento MSJ, Vinjé J (2010) Novel norovirus in dogs with diarrhea. *Emerging Infect Dis* 16(6): 980–982.
- Martella V, Pinto P, Buonavoglia C (2011) Canine noroviruses. *Vet Small Animal Clinics* In Press.
- Kipar A, Kremendahl J, Jackson ML, Reinacher M (2001) Comparative Examination of Cats with Feline Leukemia Virus-associated Enteritis and Other Relevant Forms of Feline Enteritis. *Veterinary Pathology Online* 38(4): 359–371.
- Rice M, Wilks CR, Jones BR, Beck KE, Jones JM (1993) Detection of astrovirus in the faeces of cats with diarrhoea. *N Z Vet J* 41(2): 96–97.
- Marshall JA, Kennett ML, Rodger SM, Studdert MJ, Thompson WL, et al. (1987) Virus and virus-like particles in the faeces of cats with and without diarrhoea. *Aust Vet J* 64(4): 100–105.
- Kennedy FA, Mullaney TP (1993) Disseminated adenovirus infection in a cat. *J Vet Diagn Invest* 5(2): 273–276.
- Mochizuki M (1992) Different stabilities to bile among feline calicivirus strains of respiratory and enteric origin. *Vet Microbiol* 31(2–3): 297–302.
- Herbst W, Lange H, Krauss H (1987) 27-nm virus particles found in the faeces of a cat with vomiting and diarrhoea. *Zentralblatt Veterinärmedizin Reihe B* 34(4): 314–316.
- Di Martino B, Marsilio F, Di Profio F, Lorusso E, Friedrich KG, et al. (2010) Detection of antibodies against norovirus genogroup GIV in carnivores. *Clin Vaccine Immunol* 17(1): 180–182.
- Jiang X, Huang PW, Zhong WM, Farkas T, Cubitt DW, et al. (1999) Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR. *J Virol Methods* 83(1–2): 145–154.
- Scotto-Lavino E, Du G, Frohman MA (2006) 3' end cDNA amplification using classic RACE. *Nat Protoc* 1(6): 2742–2745.
- L'Homme Y, Sansregret R, Plante-Fortier E, Lamontagne A-M, Ouardani M, et al. (2009) Genomic characterization of swine caliciviruses representing a new genus of Caliciviridae. *Virus Genes* 39(1): 66–75.
- Yun S-I, Kim J-K, Song B-H, Jeong A-Y, Jee Y-M, et al. (2010) Complete genome sequence and phylogenetic analysis of a recombinant Korean norovirus, CBNU1, recovered from a 2006 outbreak. *Virus Res* 152(1–2): 137–152.
- Scotto-Lavino E, Du G, Frohman MA (2006) Amplification of 5' end cDNA with «new RACE». *Nat Protoc* 1(6): 3056–3061.
- Hall TA (1998) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In: *Nucl Acids Symp Ser*. pp 95–98.
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 24(8): 1596–1599.
- Belliot G, Sosnovtsev SV, Mitra T, Hammer C, Garfield M, et al. (2003) In vitro proteolytic processing of the MD145 norovirus ORF1 nonstructural polyprotein yields stable precursors and products similar to those detected in calicivirus-infected cells. *J Virol* 77(20): 10957–10974.
- Sosnovtsev SV, Belliot G, Chang K-O, Prikhodko VG, Thackray LB, et al. (2006) Cleavage map and proteolytic processing of the murine norovirus nonstructural polyprotein in infected cells. *J Virol* 80(16): 7816–7831.
- Prasad BV, Hardy ME, Dokland T, Bella J, Rossmann MG, et al. (1999) X-ray crystallographic structure of the Norwalk virus capsid. *Science* 286(5438): 287–290.
- Radford AD, Addie D, Belák S, Boucraut-Baralon C, Egberink H, et al. (2009) Feline calicivirus infection. ABCD guidelines on prevention and management. *J Feline Med Surg* 11(7): 556–564.
- Povey RC, Hale CJ (1974) Experimental infections with feline caliciviruses (picornaviruses) in specific-pathogen-free kittens. *J Comp Path* 10(6): 1307–1314.
- Spradbrow PB, Bagust TJ, Burgess G, Portas B (1970) The isolation of picornaviruses from cats with respiratory disease. *Aust Vet J* 46(3): 105–108.
- Love DN, Baker KD (1972) Sudden death in kittens associated with a feline picornavirus. *Aust Vet J* 48(11): 643.
- Wardley RC, Povey RC (1977) The pathology and sites of persistence associated with three different strains of feline calicivirus. *Res Vet Sci* 23(1): 15–19.

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Author Contributions

Conceived and designed the experiments: PP QW CB VM LS. Performed the experiments: PP QW NC ED JD LM VM. Analyzed the data: PP QW VM CB LS. Contributed reagents/materials/analysis tools: QW VM LS. Wrote the paper: PP QW VM CB LS.

32. Johnson RP, Povey RC (1983) Transfer and decline of maternal antibody to feline calicivirus. *Can Vet J* 24(1): 6–9.
33. Karst SM (2010) Pathogenesis of Noroviruses, *Emerging RNA Viruses*. *Viruses* 2: 748–781.
34. Lindesmith LC, Donaldson EF, Baric RS (2011) Norovirus GI.4 strain antigenic variation. *J Virol* 85(1): 231–242.
35. Lochridge VP, Hardy ME (2007) A single-amino-acid substitution in the P2 domain of VP1 of murine norovirus is sufficient for escape from antibody neutralization. *J Virol* 81(22): 12316–12322.
36. Kitajima M, Haramoto E, Phanuwat C, Katayama H, Ohgaki S (2009) Detection of genogroup IV norovirus in wastewater and river water in Japan. *Lett Appl Microbiol* 49(5): 655–658.
37. La Rosa G, Iaconelli M, Pourshaban M, Fratini M, Muscillo M (2010) Molecular detection and genetic diversity of norovirus genogroup IV: a yearlong monitoring of sewage throughout Italy. *Arch Virol* 155(4): 589–593.
38. Cheetham S, Souza M, Meulia T, Grimes S, Han MG, et al. (2006) Pathogenesis of a genogroup II human norovirus in gnotobiotic pigs. *J Virol* 80(21): 10372–10381.
39. Souza M, Azevedo MSP, Jung K, Cheetham S, Saif LJ (2008) Pathogenesis and immune responses in gnotobiotic calves after infection with the genogroup II.4-HS66 strain of human norovirus. *J Virol* 82(4): 1777–1786.