

Research paper

A novel feline norovirus in diarrheic cats



Barbara Di Martino ^{a,*}, Federica Di Profio ^a, Irene Melegari ^a, Vittorio Sarchese ^a, Maria Assunta Cafiero ^b,
Serena Robetto ^c, Giovanni Aste ^a, Gianvito Lanave ^d, Fulvio Marsilio ^a, Vito Martella ^d

^a Faculty of Veterinary Medicine, Università degli Studi di Teramo, Italy

^b Experimental Zooprophyllactic Institute of Puglia and Basilicata, Foggia, Italy

^c Experimental Zooprophyllactic Institute of Piemonte, Liguria e Valle d'Aosta, Centro di Referenza Nazionale per le Malattie degli Animali Selvatici (CeRMAS), Italy

^d Faculty of Veterinary Medicine, Università Aldo Moro di Bari, Valenzano, Italy

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ABSTRACT

By screening a collection of fecal samples from young cats housed in three different shelters in South Italy, noroviruses (NoVs) were found in 3/48 (6.2%) specimens of animals with enteritis signs while they were not detected in samples collected from healthy cats (0/57). Upon sequence analysis of the short RNA-dependent RNA polymerase (RdRp) region, the three strains displayed the highest nucleotide (nt) and amino acid (aa) identities to the prototype GIV.2 strain lion/Pistoia/387/06/ITA (91.0–93.0% nt and 97.0–98.0% aa). The sequence of ~3.4-kb portion at the 3' end of the genome of a NoV strain, TE/77-13/ITA, was determined. In the full-length ORF2, encoding the VP1 capsid protein, the virus was genetically closest to the canine GVI.2 NoV strains C33/Visou/2007/PRT and FD53/2007/ITA (81.0–84.0% nt and 93.0–94.0% aa identities), suggesting a recombination nature, with the cross-over site being mapped to the ORF1–ORF2 junction. Based on the full-length VP1 amino acid sequence, we classified the novel feline NoV, together with the canine strains Visou and FD53, as a genotype 2, within the genogroup GVI. These findings indicate that, as observed for GIV NoV, GVI strains may infect both the canine and feline host. Unrestricted circulation of NoV strains in small carnivores may provide the basis for quick genetic diversification of these viruses by recombination. Interspecies circulation of NoVs in pets must also be considered when facing outbreaks of enteric diseases in these animals.

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1. Introduction

Noroviruses (NoVs), Caliciviridae family, have been identified as the most common cause of viral gastroenteritis in humans. NoV infections affect persons of all age groups and are predominantly transmitted through the fecal–oral route, either indirectly through contaminated food, water or surfaces or directly from person to person (Patel et al., 2008).

Drop virions are nonenveloped and approximately 30 to 35 nm in diameter. The RNA genome is organized into three open reading frames (ORFs) (Green, 2007). ORF1 encodes a polyprotein that is cleaved by the virus-encoded protease to produce several nonstructural proteins, including the RNA dependent RNA polymerase (RdRp), ORF2 encodes a major capsid protein (VP1) and ORF3 encodes a small basic protein (VP2) that has been associated with the capsid stability (Bertolotti-Ciarlet et al., 2003).

Based on the full-length VP1 amino acid sequence, NoVs have been divided into six genogroups (GI to GVI) and several genotypes (Zheng et al., 2006; Martella et al., 2009; Green, 2013). Only GI, GII, and GIV NoVs infect humans, with GII strains being the most prevalent

worldwide (Green, 2007). NoVs genetically similar to human NoVs have been recently found in dogs and cats (Martella et al., 2007, 2008; Summa et al., 2012; Pinto et al., 2012; Soma et al., 2014), raising public health concerns of potential cross-species transmission due to the strict social interaction between humans and pets.

Feline NoVs were first detected in the stools of 8–12-week-old kittens from a feline shelter with an outbreak of diarrhea in New York State (Pinto et al., 2012). In the VP1 encoding gene, the feline NoVs displayed the highest amino acid (aa) identity (97.9%) to the prototype NoV strain GIV.2/Pistoia/387/06/ITA, detected in a captive lion cub with severe hemorrhagic enteritis (Martella et al., 2007) and to the canine strain GIV.2/Bari-170/07/IT (90.4% aa), detected in a young dog with diarrhea (Martella et al., 2008). Using baculovirus-expressed VP1 of the lion NoV strain GIV.2/Pistoia/387/06/ITA, antibodies specific for GIV NoVs have been identified in 16.1% of cats in Italy (Di Martino et al., 2010), providing indirect evidence for the circulation of these NoVs in felines.

In addition, the RNA of GIV.2 NoVs has been detected in 1.2% of fecal samples of cats with enteritis in Japan (Soma et al., 2014). Upon genome sequencing, the feline NoV strain cat/GVI.1/JPN/2012/M49 (Takano et al., 2015) was found to be more similar (87.0% aa identity) in the full-length ORF2 to the canine NoV GVI.1/Bari/91/07/IT (Martella et al., 2009).

* Corresponding author.

E-mail address: bdimartino@unite.it (B. Di Martino).

Altogether these findings indicate that diverse NoV strains may infect cats, as observed in dogs, and that the feline and canine host may be infected by the same NoV strains, thus constituting an enlarged host reservoir for these animal NoVs. In order to draw a more complete picture of NoVs molecular epidemiology in cats, in this study a collection of fecal specimens from diarrheic and healthy animals was screened using either broadly-reactive primers for caliciviruses and primers specific for NoVs.

2. Materials and methods

2.1. Sampling

A total of 105 stool samples from domestic cats aged 2–12 months were collected from April to July 2013 in three different shelters located in South Italy. The fecal panel consisted of 48 samples from cats with signs of mild to severe gastroenteritis and 57 samples from asymptomatic animals. All the samples were stored at -80°C until use.

2.2. RNA and DNA extraction

Fecal specimens (10%) were re-suspended in phosphate-buffered saline pH 7.2, and the debris were removed by centrifugation at $8000 \times g$ for 5 min. DNA and RNA extracts were prepared using the DNeasy® and QIAamp® viral RNA kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions and stored at -80°C until use.

2.3. Screening by RT-PCR and PCR

To assess the presence of NoV RNA, the samples were screened using a broadly reactive primer pair, p289–p290, targeted to highly conserved motifs DYKWDST and YGDD of the RNA-dependent RNA polymerase (RdRp) region of the polymerase complex (Jiang et al., 1999). In the samples yielding amplicons of the expected sizes, the presence of NoV was confirmed using norovirus-specific primer pair JV12Y–JV13I (Vennema et al., 2002). All the fecal samples were also tested by PCR or RT-PCR for feline parvovirus (FPV) (Buonavoglia et al., 2001), feline enteric coronavirus (FECV) (Gunn-Moore et al., 1998) and feline kobuviruses (FeKoV) (Di Martino et al., 2015).

2.4. Sequence and phylogenetic analysis of the NoV strains

The amplicons were excised from the gel and purified using a QIAquick gel extraction kit (Qiagen GmbH, Hilden, Germany). The fragment was then subjected to direct sequencing using BigDye Terminator Cycle chemistry and 3730 DNA Analyzer (Applied Biosystems, Foster, CA). Basic Local Alignment Search Tool (BLAST; <http://www.ncbi.nlm.nih.gov>) and FASTA (<http://www.ebi.ac.uk/fasta33>) with default values were used to find homologous hits. The sequence of ~3.4-kb fragment at the 3' end of the genome of one such strain, TE/77-13/ITA, including the partial RdRp gene and the complete ORF2 and ORF3 genes, was determined by 3' RACE protocol, as previously described (Scotto-Lavino et al., 2006). cDNA was synthesized by SuperScript III First-Strand cDNA synthesis kit (Invitrogen Ltd., Milan, Italy) with primer QT. PCR was then performed with TaKaRa La Taq polymerase (Takara Bio Europe S.A.S. Saint-Germain-en-Laye, France) with forward primer p290 and reverse primers QO and QI. Finally, the amplicons were purified and cloned by using TOPO® XL Cloning Kit (Invitrogen Ltd., Milan, Italy). Additional primers were designed to determine the complete 3.4 kb sequences by an overlapping strategy (Table 1). Sequence editing and multiple alignments were performed with the BioEdit software package, version 2.1 (Hall, 1999). Phylogenetic trees were generated using Bayesian analysis with MrBayes (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). The appropriate substitution model settings were derived using jModelTest (Posada, 2009). The sequence obtained was analyzed with Simplot (Lole et al., 1999) using a window size of 200 and step size of 20, with gap strip off and Hamming correction on. Additionally, recombination analysis was carried out with different algorithms implemented in the Recombination Detection Program v.4.43 (RDP4) (Martin et al., 2010), with default settings.

3. Results

Out of 105 samples, 3 (2.8%) contained NoVs RNA, either alone (0.9%, 1/105) or in mixed infections with FeKoV or FECV (1.9%, 2/105). Sixteen samples (15.2%) were found to contain FPV DNA alone. All the NoV positive samples were identified from diarrheic cats with a prevalence rate of 6.2% (3/48), while they were not detected from asymptomatic animals (0/57). By sequence comparison in the short RdRp fragment, the viruses TE/68-13, ME/78-13 and TE/77-13/ITA shared 89.9–93.2% nt and 90.8–95.3% aa identities to each other and displayed the highest

Table 1

Oligonucleotides used for cDNA synthesis and amplification in this study. Nucleotide position refers to the sequence of the feline NoV cat/TE/77-13/ITA (GenBank accession no. KT245136).

Oligonucleotide	Position	Sequence (5' to 3')	Sense	Reference
p290	2–24	GATTACTCCAAGTGGGACTCCAC	+	Jiang et al. (1999)
p289	299–317	TGACAATGTAATCATCACATA	–	Jiang et al. (1999)
JV12Y	40–60	ATACCACCTATGATGCAGAYTA	+	Vennema et al. (2002)
JV13I	292–312	TCATCATCACCATAGAAGAG	–	Vennema et al. (2002)
FeNoV-201	201–221	TCAACAGCATCGCCCACTGGA	+	This study
FeNoV-550	550–572	GCATCATCGTCTCGTTGGGGTCC	–	This study
FeNoV-852	852–870	CCAGAGAGTCAACAAGAGG	+	This study
FeNoV-1026	1026–1043	AGGGCCAAGCTCGAGATC	–	This study
FeNoV-1084	1084–1114	TGGAGGGATGGAAGTGCAGAT	+	This study
FeNoV-1581	1581–1601	ATCCAGGGTGCACCTGCCATT	–	This study
FeNoV-1581	1581–1601	AATGGCAGGTGCACCCTGGAT	+	This study
FeNoV-2094	2094–2113	TACAACGGGGCCATAGGGGA	+	This study
FeNoV-2094	2094–2113	TCCCCTATGGCCCCGTGTA	–	This study
FeNoV-2452	2452–2472	CAGTCCCACAGGGCTGAGTG	–	This study
FeNoV-2729	2729–2749	GAAGCCGCCCTTGCGCAACGC	–	This study
FeNoV-2729	2729–2749	GCGTTGCGCAAGGGCGGCTTC	+	This study
FeNoV-2851	2851–2872	GGTGGCCATGCCAGATACCTT	–	This study
FeNoV-3198	3281–3297	TTGGACTCACCTCTGCC	–	This study
Q _r	3'/5' end	CCAGTGAGCAGGTGACGAGACTCGAGCTCAAGCTTTTTTTTTTTTTTTT	+/-	Scotto-Lavino et al. (2006)
Q _o	3'/5' end	CCAGTGAGCAGAGTGACG	+/-	Scotto-Lavino et al. (2006)
Q _I	3' end	GAGGACTCGAGCTCAAGC	–	Scotto-Lavino et al. (2006)

identity (91.0–93.0% nt and 97.0–98.0% aa) to the prototype GIV.2 strain lion/Pistoia/387/06/ITA.

For the strain TE/77-13/ITA the sequence of ~3.4-kb fragment at the 3' end of the genome, including the partial RdRp (0.8 kb) and the complete ORF2 and ORF3 (GenBank accession number KT245136), was sequenced and the genome organization was determined. Phylogenetic analysis was based on the 750-nt sequence of the COOH terminus of the polymerase complex of the carnivore NoV strains available in the databases. Also, RdRp sequences of human GIV.1 NoVs were included in the analysis and used to calculate a nt identity matrix. By visual inspection of the tree, the carnivore NoVs segregated in at least three different genetic clusters (Fig. 1). The strain cat/TE/77-13/ITA was grouped with the feline NoV strains lion/GIV.2/Pistoia/387/06/ITA, cat/GIV.2/CU081210E/USA/2010 and cat/GVI.1/JPN/2012/M49 (Martella et al., 2007; Pinto et al., 2012; Takano et al., 2015), with a nt identity of 90.8–94.0%. This group shared identity of 82.3–85.4% to the recombinant NoV dog/GVI.1/91/2007/ITA and to the GIV.2 strains dog/170/07/ITA and dog/Thessaloniki/30/08/GR (Martella et al., 2008, 2009; Ntafis et al., 2010), which in turn segregated in a second cluster (91.6–97.8% nt identity). A minor group, distantly related to the feline NoVs (78.2–79.6% nt identity) and to the canine NoVs GVI.1/91/2007/ITA, GIV.2/170/07/ITA and GIV.2/Thessaloniki/30/08/GR (80.4–82.6%), included the strains dog/GVI.2/C33/Viseu/2007/PRT (Mesquita et al., 2010) and dog/GVI.2/FD53/2007/ITA (unpublished data). The nt identity within this group was 98.8%. One strain, the recombinant dog/GVI.1/FD210/2007/ITA (unpublished data), did not cluster within any three established groups. This strain shared a nt identity of 83.4–85.9% to the feline NoV group, 80.4% to the strains GVI.2/C33/Viseu/2007/PRT and dog/GVI.2/FD53/2007/ITA, 87.6–88.6% to the other canine viruses. Two additional clusters were resolved in the tree that included, respectively, the human GIV.1 strains detected from stool and sewage samples in different geographic settings (Fankhauser et al., 2002; La Rosa et al., 2008; Eden et al., 2012; Ao et al., 2014; Han et al., 2014) and GIV.1

NoVs found in sewage samples in Italy in 2008–2009 (La Rosa et al., 2010). The nt identity between these two groups was 87.0–91.5%.

The ORF2 of strain TE/77-13/ITA was 1719 nt in length and encoded a VP1 capsid protein with a predicted size of 572 aa. ORF3 was 795 nt long and encoded a VP2 protein of 264 aa. A 16-nucleotide (nt) overlap was present in the ORF1-ORF2 junction region.

In the complete VP1, the strain TE/77-13/ITA was most closely related (81.0–84.0% nt and 93.0–94.0% aa) to the strains dog/GVI.2/C33/Viseu/2007/PRT and dog/GVI.2/FD53/2007/ITA, while identity to the feline strain cat/NoV/GVI.1/JPN/2012/M49 and to the canine strain GVI.1/Bari/91/2007/ITA (Martella et al., 2009) was 70.0–71.0% nt and 80.0–81.0% aa. Strain TE/77-13/ITA displayed <54.0% aa identity to animal and human GIV NoVs. Phylogenetic analysis was performed with a selection of complete capsid sequences representative of the *Norovirus* genus. In the VP1-based tree (Fig. 2), strain TE/77-13/ITA segregated with the canine NoVs GVI.2/C33/Viseu/2007/PRT and GVI.2/FD53/2007/ITA into genogroup GVI, genotype 2. A nucleotide identity plot of the genome of strain TE/77-13/ITA was elaborated, in comparison with the canine strain dog/NoV/GVI.2/C33/Viseu/2007/PRT and the feline strain cat/NoV/GVI.2/CU081210E/USA/2010. By SimPlot (Fig. 3) and RDP (Fig. S1) analyses, a putative recombination break-point event was mapped to ORF1-ORF2 junction region at nt 703 with a significant statistical support ($P < 0.05$).

4. Discussion

In this study direct evidence was collected for circulation of NoVs in cats. NoVs were detected in cats with enteric signs while they were not identified in samples collected from healthy animals used as control study group. Experimental inoculation of specific pathogen free cats with the feline GVI.1 strain JPN/2012/M49 can induce enteritis signs, diarrhea and vomiting (Takano et al., 2015). Although the pathogenic role of NoV in cats should be confirmed in larger epidemiological studies

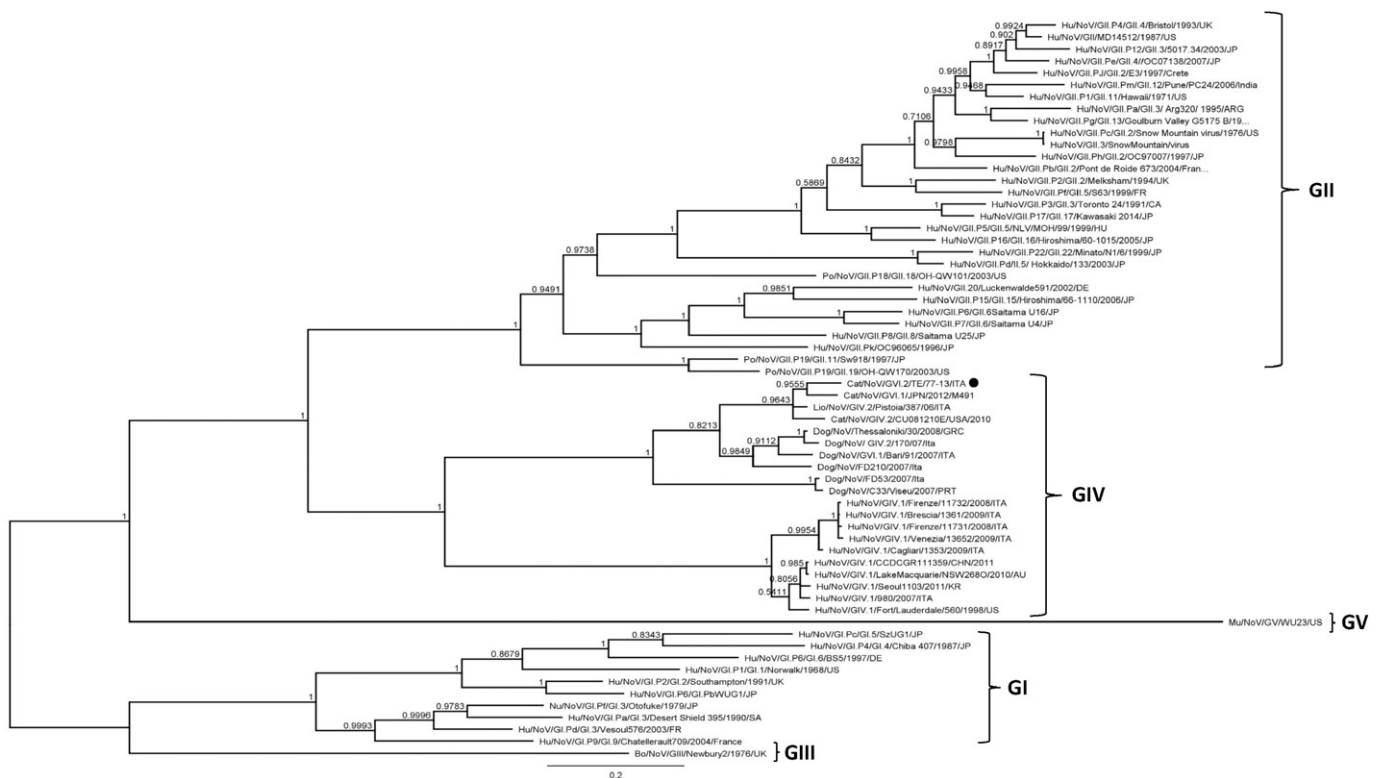


Fig. 1. Phylogenetic tree based on the 750-nt sequence of the COOH terminus of the polymerase complex. Tree was generated using the Bayesian inference with Generalized Time-Reversible (GTR) model and gamma rate variation and supplying statistical support with subsampling over 200 replicates. Numbers on the tree branches indicate the posterior probability values. The scale bar indicates the number of nt substitutions per site. Black circle indicates the NoV strain detected in this study.

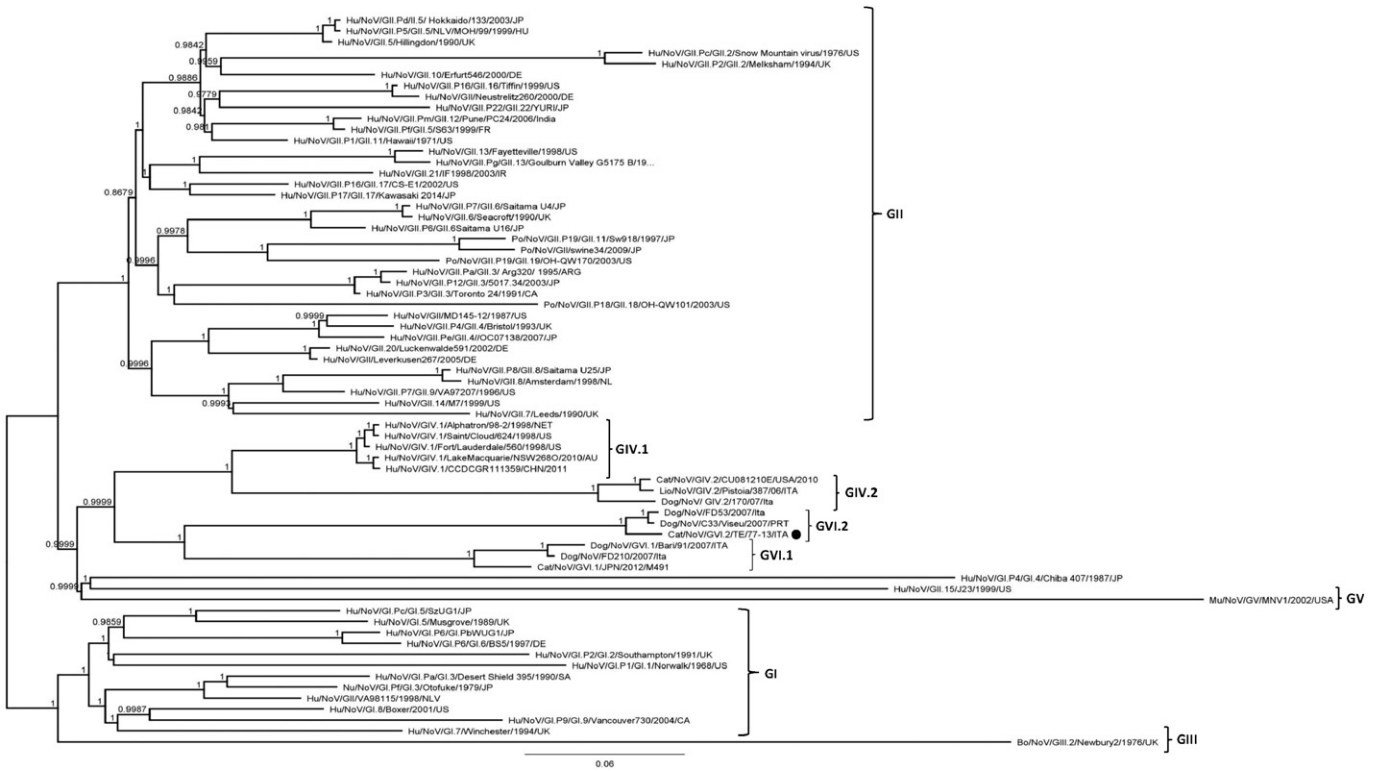


Fig. 2. Phylogenetic tree based on the full-length aa sequence of the VP1 protein of NoVs. Tree was generated using the Bayesian inference with GTR model with gamma rate variation and supplying statistical support with subsampling over 200 replicates. Numbers on the tree branches indicate the posterior probability values. The scale bar indicates the number of aa substitutions per site. Black circle indicates the NoV strain detected in this study.

and, possibly, in experimental infections with other NoV genotypes, these findings seem to indicate a possible role of these viruses as feline enteric pathogens.

Based on sequence and phylogenetic analysis of the 3' partial sequence of ORF1 spanning 750 nt, at the COOH terminus of the polymerase complex, within the genogroup GIV different genetic lineages could be distinguished. The feline strain TE/77-13/ITA possessed a polymerase

gene of the same lineage as the feline NoV strains cat/NoV/GVI.1/JPN/2012/M49 and cat/NoV/GIV.2/CU081210E/USA/2010, and as the lion NOV strain GIV.2/Pistoia/387/06/ITA.

Interestingly, inconsistencies were observed between the RdRp- and capsid-based phylogeny, that are suggestive of a potential recombinant nature. Strain TE/77-13/ITA, in the full-length VP1 capsid gene, closely resembled the canine GVI.2 strains C33/Viscu/2007/PRT and FD53/

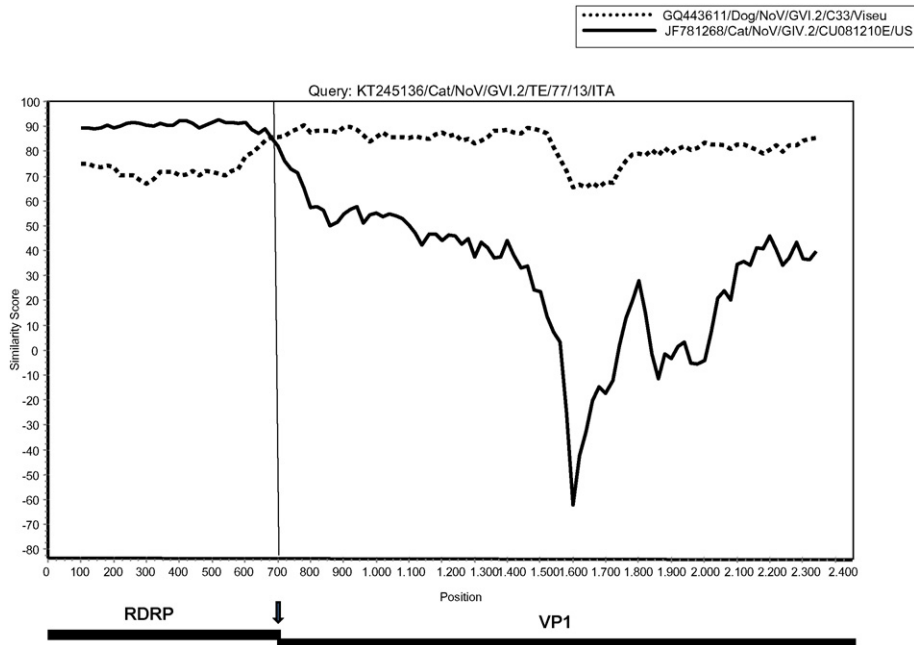


Fig. 3. Nucleotide identity plot performed using SimPlot (Lole et al., 1999). The genome of strain cat/NoV/GVI.2/TE/77-13/ITA (the 3' end of ORF1 and the complete ORF2) was compared with those of the NoV strains dog/GVI.2/C33/Viscu/2007/PRT and cat/GIV.2/CU081210E/USA/2010. The arrow indicates the crossover site at the ORF1-ORF2 junction region.

2007/ITA. Following strictly the outlines of Zheng's classification (Zheng et al., 2006), we classified the novel feline NoV, together with the canine strains Viseu and FD53, as a genotype 2 (>85% pairwise aa identity intergenotypes), within the genogroup GVI (>55% pairwise aa identity intergenogroups). Accordingly, cats and dogs may harbor NoVs of the same genotypes, GIV.2, GVI.1 and GVI.2. Circulation of NoVs genetically related in different host species has been already demonstrated. Porcine NoVs cluster in GII (Wang et al., 2005), but within different genotypes (GII.11, GII.18, and GII.19) from those infecting humans. GIII NoVs have been detected in large and small ruminants, with GIII.1 and GIII.2 (Liu et al., 1999; Oliver et al., 2007) found in cattle and GIII.3 in sheep (Wolf et al., 2009). However, unlike small carnivores, circulation of NoV strains belonging to the same genotypes in heterologous species has not been reported thus far.

This intriguing finding poses several questions. Binding of GVI.2 and GVI NoVs in dog tissues seems to be mediated by the presence of the H and A antigens of the histo-blood group antigen (HBGA) family (Caddy et al., 2014) and therefore to be genetically determined, as observed in humans (Marionneau et al., 2002). This may suggest that dogs and cats share a similar pattern of HBGAs as attachment factor for NoV infections. Also, virus-like particles of seven different human NoV genotypes (GI.1, GI.2, GI.3, GII.3, GII.4, GII.6, and GII.12) have been shown to be able to bind to canine gastrointestinal tissues (Caddy et al., 2015). It will be interesting to assess whether cats may also be infected by human NoVs, as observed in dogs (Summa et al., 2012; Caddy et al., 2015), as this may have implications for the transmission of human NoVs.

Recombination among NoVs of domestic carnivores has been already described. The canine NoV strain Bari/91/07/ITA, resembles GIV.2 NoVs in its polymerase gene while it is genetically unrelated in the VP1 gene to GIV NoV (Martella et al., 2009). The feline NoV strain, JPN/2012/M49 (Takano et al., 2015), possesses a GIV.2 RdRp region, and a GVI.1 ORF2 related to the canine virus Bari/91/07/ITA (87.0% aa identity). In all the cases, the site of recombination was mapped to the ORF1/ORF2 junction region. This part of NoV genome is highly conserved and has been individuated as a preferential recombination site (Bull et al., 2005). Recombination has been shown to strongly influence the evolution and epidemiology of human NoVs (Ambert-Balay et al., 2005; Reuter et al., 2006) and surely poses a challenge for the development of specific diagnostic tools for NoV of carnivores. Analysis of the RdRp fragment cannot be used to characterize unequivocally these animal NoVs and a definitive characterization should rely on the ORF2.

5. Conclusions

The development of molecular assays for caliciviruses and for NoVs has allowed gathering epidemiological information about these viruses in several animal species, including domestic carnivores. It is now clear that cats and dogs may harbor NoVs of several genotypes and genogroups, although the clinical relevance of these viruses remains to be investigated.

Gathering information on the genetic diversity of animal NoVs will be useful to optimize/develop direct and indirect diagnostic tools, and to investigate more effectively the epidemiology of NoVs in carnivores. In addition, as NoVs of carnivores are suspected to have a zoonotic relevance (Peasey et al., 2004; Mesquita et al., 2013; Di Martino et al., 2014; Caddy et al., 2015), this will be useful to understand the extent of inter-species transmission from cat to dogs, and vice versa, from carnivores to humans.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.meegid.2015.12.019>.

Conflict of interest statement

All Authors declare that there are no financial or other relationships that might lead to a conflict of interest. All authors have seen and approved the manuscript and have contributed significantly to the work.

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