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Identification of feline calicivirus in cats with enteritis

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Abstract

Feline calicivirus (FCV) is a major pathogen of cats associated with either respiratory disease or systemic disease, but its possible role as an enteric pathogen is neglected. Using RT-PCR, the RNA of FCV was identified in 25.9% (62/239) of stools of cats with enteritis and in 0/58 (0%) of cats without diarrhoea or other clinical signs. Isolates of enteric origin were obtained and a large 3.2-kb portion of the genome was sequenced, encompassing the 3′ end of the RNA polymerase, the capsid protein precursor and the minor capsid protein. Also, the complete genome sequence of one such strain, the 160/2015/ITA, was determined. Upon sequence analysis, the enteric viruses were found to be genetically heterogeneous and to differ from each other and from isolates of respiratory origin. The enteric isolates were found to be more resistant to low pH conditions, to trypsin and to bile treatment than respiratory isolates. Overall, these findings are consistent with the hypothesis that some FCVs may acquire enteric tropism and eventually act as enteric pathogens. Whether this enteric tropism is maintained stably and whether it may affect, to some extent, the ability of the virus to trigger the classical and/or hypervirulent forms of disease should be assessed. Also, FCV should be included in the diagnostic algorithms of enteric diseases of cats to gain further information about FCV strains displaying enteric pathotype.

KEYWORDS

cats, enteritis, feline calicivirus (FCV)

1 | INTRODUCTION

Feline calicivirus (FCV) is a small non-enveloped virus of approximately 30–35 nm in diameter classified in the *Vesivirus* genus, within the *Caliciviridae* family (King, Adams, Carstens, & Lefkowitz, 2012). The icosahedral capsid surrounds an approximately 7.7-kb positive-sense single-stranded RNA genome organized in three open reading frames (ORFs). ORF1 encodes a 200-kDa polyprotein that is processed by a viral proteinase, the 3C-like cysteine proteinase, into p5.6, p32, p39 (NTPase), p30, p13 (VPg) and p76 (Pro-Pol) (Sosnovtsev, Garfield, & Green, 2002; Sosnovtseva, Sosnovtsev, & Green, 1999). ORF2 encodes a 73-kDa capsid precursor protein (pre-VP1) that is post-translationally processed to release a small protein of 124 amino acids designated the leader of the capsid and the 60-kDa mature capsid protein VP1 (Sosnovtsev, Sosnovtseva, & Green, 1998). The third ORF (ORF3) is located at the 3′ end of the genome and encodes a 106-amino acid (aa) protein (VP2) with a predicted molecular weight of 12 kDa (Di Martino & Marsilio, 2010; Sosnovtsev, Belliot, Chang, Onwudiwe, & Green, 2005). FCV is one of the most common viral pathogens of cats, especially in multi-cat environments such as shelters and catteries (Pedersen, Elliott, Glasgow, Poland, & Keel, 2000). The main clinical signs of FCV infection are upper respiratory tract symptoms, oral ulcerations and fever. Occasionally, FCV infection has been associated with acute febrile lameness syndrome, abortion

and severe pneumonia (Dawson et al., 1994; Ellis, 1981; Pedersen, Laliberte, & Ekman, 1983). Also, generalized and lethal forms of infection characterized by multi-organ involvement, subcutaneous oedema, and oral and cutaneous ulcers, referred to as virulent systemic disease (VSD), have been frequently described (Abd-Eldaim, Potgieter, & Kennedy, 2005; Coyne et al., 2006; Guo, Miao, Zhu, Yang, & Liu, 2018; Hurley et al., 2004; Ossiboff, Sheh, Shotton, Pesavento, & Parker, 2007; Pedersen et al., 2000; Pesavento, Maclachlan, Dillard-Telm, Grant, & Hurley, 2004; Reynolds et al., 2009; Schorr-Evans, Poland, Johnson, & Pedersen, 2003).

In the literature, there are only sporadic reports on the identification of FCV from the enteric tract (Marshall et al., 1987; Mochizuki, 1992; Pinto et al., 2012; Soma, Nakagomi, Nakagomi, & Mochizuki, 2015), although FCV was first isolated from the intestinal content of cats in New Zealand (Fastier, 1957). Experimental infection with a FCV strain isolated from cats with neurological disorders and unrelated to the FCV vaccine strain F9 resulted in profuse watery diarrhoea in both non-vaccinated and vaccinated specific pathogen-free cats (Povey & Haley, 1974). Subsequently, small round viruses resembling FCV particles were detected in about 6% of stool specimens analysed by electron microscopy in a study performed in Australia (Marshall et al., 1987). Also, FCV was found in 2.2% of faecal samples from diarrhoeic and healthy cats during a survey conducted in Japan in the 1980s (Mochizuki, 1992). The differences in resistance to bile acids have been found between isolates of enteric and respiratory origins, suggesting that enteric FCVs have the ability to replicate actively in the enteric tract, whilst respiratory FCVs do not (Mochizuki, 1992). More recently, using a broadly reactive primer pair (p290-p289) for caliciviruses (Jiang et al., 1999), the RNA of FCV was found in 35.7% of diarrhoeic stools of 8- to 12-week-old kittens housed in a New York State animal shelter with an outbreak of gastroenteritis (Pinto et al., 2012) and in 9.6% of specimens collected from cats with enteritis in Japan (Soma et al., 2015). Despite these cumulative data, FCV is not included as a potential pathogen in the diagnostic evaluation of diarrhoea of cats. In this study, a collection of samples obtained from cats with acute enteritis was assessed to evaluate the possible association of FCV with enteric disease. Sequence information was gathered from FCV isolates of enteric origin. Also, attempts to evaluate the biochemical properties of the FCV isolates were made assessing the in vitro stability to low pH, trypsin and bile salts.

2 | MATERIALS AND METHODS

2.1 | Sampling

A total of 191 enteric samples (stool and rectal swabs) obtained from young and adult domestic cats with gastroenteric signs, collected during 2012–2015 at the Department of Veterinary Medicine, University of Bari (Italy), were screened for FCV (collection A). Moreover, a collection of 106 samples (collection B) from cats aged 2–12 months was stored during 2014–2015 at the Department of Veterinary Medicine, University of Teramo, Italy. Collection B included 48 samples from cats with gastroenteritis and 58 without clinical signs. Inclusion criteria for the definition of case with gastrointestinal disorders were anorexia, vomiting and diarrhoea. Each faecal sample was homogenized in 10% Dulbecco's modified Eagle's medium (DMEM) containing antibiotics, clarified by centrifugation at 5,000 *g* for 15 min and stored at −80°C until use.

2.2 | Molecular screening by RT-PCR

Total RNA was extracted by using QIAamp® Viral RNA Kit (Qiagen S.p.A.), according to the manufacturer's instructions. To assess the presence of FCV RNA, the samples were screened using a nested RT-PCR (external primer pair, Cali1: 5′-AACCTGCGCTAACGTGCTTA-3′ and Cali2: 5′-CAGTGACAATACACCCAGAAG-3′; internal primer pair, Cali3: 5′-TGGTGATGATGAATGGGCATC-3′ and Cali4: 5′-ACACCAGAGCCAGAGATAGA-3′), targeting a 467-bp portion of the ORF2 gene (Marsilio, Di Martino, Decaro, & Buonavoglia, 2005). The amplicons were excised from the gel and purified using a QIAquick Gel Extraction Kit (Qiagen S.p.A.). The fragment was then subjected to direct sequencing using BigDye Terminator Cycle Chemistry and 3,730 DNA Analyzer (Applied Biosystems). 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. Screening for other feline pathogens was done as described elsewhere (Di Martino et al., 2016).

2.3 | Virus isolation

For virus isolation, Crandell-Rees feline kidney (CRFK) (Crandell, 1974) cell line cultured in DMEM supplemented with 10% heat-inactivated foetal bovine serum (FBS) was used.

Briefly, 400 μl of each sample positive by RT-PCR was filtered with 0.22-μm filters, inoculated into 90% confluent CRFK cells in 24-well plates and incubated at 37°C in a 5% CO_2 incubator. After an adsorption period of 45 min, DMEM was added. Viral growth was evaluated daily monitoring the onset of cellular cytopathic effect (CPE) for 5 days. In the absence of CPE, the cryolysates were subcultured twice into fresh monolayers. In the presence of the CPE, the infected cells were examined by indirect immunofluorescence (IIF) assay using a rabbit hyperimmune serum raised against the FCV vaccine strain F9 (Di Martino, Marsilio, & Roy, 2007).

2.4 | Genome sequencing and phylogenetic analysis

For four isolates, the sequence of ~3.2-kb-long genomic fragment (the 3′ end of ORF1, the full-length ORF2, ORF3 and the non-coding region through the poly-A tail) was determined by 3′ RACE protocol as previously described (Scotto-Lavino, Du, & Frohman, 2006). cDNA was synthesized by SuperScript III

First-Strand cDNA Synthesis Kit (Invitrogen Ltd.) with primer QT. PCR was then performed with Takara LA PCR Kit Ver. 2.1 (Takara Bio) with forward primer p290 (Jiang et al., 1999) and reverse primers QO and QI (Scotto-Lavino et al., 2006). Finally, the amplicons were purified and cloned by using TOPO® XL Cloning Kit (Invitrogen Ltd.). Furthermore, the complete genome of one additional isolate was generated using consensus primers p1277 GGCCGCCGGGTTATTGTAAAAGAAATTTGAGACAA) and p1278 (CCGAAGTTGGGGGGGTTTTTTTTTTTTTTTTTTTTTTTTTTCCCTGGGGTTAGGCGCA), designed, respectively, at the very 5′ end and 3′ end of FCV genomic RNA. Briefly, the RNA was reverse-transcribed with the reverse primer 1,278 using the SuperScript III First-Strand cDNA Synthesis Kit (Invitrogen Ltd.). PCR was then performed as described above, in a 50 µl reaction containing 1 mmol/l of primers. The thermal file conditions started with activation at 94°C for 2 min, followed by 10 cycles at 94°C for 30 s, 44°C for 30 s and 68°C for 10 min, and by 30 cycles at 94°C for 30 s, 48°C for 30 s and 68°C for 10 min, with a final extension at 68°C for 10 min. The amplicon was gel-purified, and the DNA was quantified by using the Fluorometric Qubit dsDNA High-Sensitivity Assay Kit (Thermo Fisher Scientific). A genomic DNA library was prepared by using the Nextera DNA Flex Library Prep Kit (Illumina) according to the manufacturer's protocol, and a size-selection step was done manually by using AMPure XP magnetic beads (Beckman Coulter). Quality control analysis was performed on the sample library by using the QIAxcel Advanced System with QIAxcel ScreenGel Software 1.4.0 (Qiagen S.p.A.). We normalized library samples as suggested by Qiagen and performed sequencing by using a MiSeq instrument, version 2 (Illumina), and a MiSeq Reagent Kit (Illumina). The total paired reads obtained by next-generation sequencing (NGS) were checked for quality by using FastQC (Andrews, 2019). Sequence trimming, assembly of NGS reads and genome annotation were performed using the Geneious platform version 10.1.3 (Biomatters Ltd.). Sequence editing and multiple alignments were carried out with the Geneious software package version 10.2.4 (Biomatters Ltd). The alignment of the sequences was conducted using the MAFFT multiple alignment program version 7.388 plugin of the Geneious software. Multiple alignment of the aa sequence of the capsid was inspected to identify hallmark mutations, on the basis of the literature (Brunet, Sigoillot-Claude, Pialot, & Poulet, 2019).

2.5 | Evaluation of susceptibility to pH, trypsin and bile salts

The in vitro stability to low pH, trypsin and to bile salts treatment of enteric FCVs (E-FCVs) was investigated and compared with that of five strains of respiratory origin (R-FCVs) (Di Martino, Di Francesco, Meridiani, & Marsilio, 2007) (Table 1), using protocols previously described with some minor modifications (Mochizuki, 1992). Briefly, virus stocks were prepared by two plaque purification steps in CRFK cells. Plaques were obtained by inoculation of freshly seeded cells (24–48 hr) at a multiplicity of infection of 0.1 plaque-forming units (PFU)/cell. The viral suspensions, harvested after 24–36 hr by three cycles of freezing/thawing and clarified by centrifugation at 10,000 *g* for 15 min, were aliquoted and frozen at −80°C until used. In the low pH test, hydrochloric acid (HCl) 0.1 N was added to each aliquot of virus suspension in order to yield a final pH of 3.0. Each mixture was kept at room temperature for 60 min. Susceptibility to trypsin treatment was performed by adding 400 µl of each viral suspension to an equal amount of trypsin diluted 1% in distilled water to obtain a final concentration of 0.5% (vol/vol). The mixture was then incubated for 1 hr at 37°C in 5% CO_2 atmosphere. To test resistance of FCVs to bile salts, 250 µl of virus suspensions was mixed with an equal volume of bile salt mixture (Sigma-Aldrich) at final concentration of 0.5%. The mixtures were placed at 37°C for 1 hr in a 5% CO_2 incubator. For each reaction, a negative control (DMEM + virus suspension) was included. After contact/exposure, the different viral mixtures were titrated in quadruplicates in 96-well plates containing CRFK cells. The plates were incubated for 72 hr at 37°C in 5% CO_2 and the resulting titre was calculated by the Reed and Muench endpoint method, which identifies the final infectivity at 50% tissue culture infectious doses ($TCID_{50}$). For each test, the results are expressed as the change in titre (log_{10} reduction).

TABLE 1 List of FCV strains used in this study to evaluate the in vitro sensitivity to pH, bile and trypsin

FCV strain	Isolation source	Reference
81/2014/ITA	Enteritis: faecal swab	This study
148/2015/ITA	Enteritis: faecal swab	This study
160/2015/ITA	Enteritis: faecal swab	This study
180/2015/ITA	Enteritis: faecal swab	This study
182/2015/ITA	Enteritis: faecal swab	This study
185/2002/ITA	Upper respiratory tract disease: oral swab	Di Martino, Di Francesco, et al., (2007)
236/2002/ITA	Upper respiratory tract disease: oral swab	Di Martino, Di Francesco, et al., (2007)
260/2003/ITA	Upper respiratory tract disease: oral swab	Di Martino, Di Francesco, et al., (2007)
293/2004/ITA	Upper respiratory tract disease: oral swab	Di Martino, Di Francesco, et al., (2007)
299/2004/ITA	Upper respiratory tract disease: oral swab	Di Martino, Di Francesco, et al., (2007)

3 | RESULTS

3.1 | Molecular investigations and virus isolation

Out of 239 diarrhoeic samples, 62 contained FCV RNA with an overall prevalence of 25.9%. More specifically, FCV RNA was detected in 54/191 (28.3%) of the cats of the collection A and in 8/48 (16.7%) diarrhoeic animals of collection B, whilst it was not found in specimens (0/58) collected from asymptomatic cats (collection B). By the chi-square test, positivity to FCV was significantly correlated with the enteric symptoms (p = 0,004; OR = 8,17; CI = [2,48; 27]). Also, young animals (less than 3 months of age) were apparently, but not significantly more susceptible to FCV enteric infection (OR = 2,74; CI = [1,55; 4,8]). The diarrhoeic samples of the collection B were also screened for the presence of other pathogens causing enteric signs. In this subset, 16/48 samples (33.3%) were positive for feline panleukopenia virus (FPV), 5/48 (10.4%) for feline coronavirus (FECV), 4/48 (8.3%) for feline kobuvirus (FeKoV) and 3/48 (6.2%) for GIV.2 noroviruses (NoVs). FCV was found alone (50.0%; 4/8) or in mixed infection with FECV (37.5%; 3/8) or with FPV, FeKoV and NoVs (12.5%; 1/8). Virus isolation was successful for five strains designated as FCVs 81/2014/ITA, 148/2015/ITA, 160/2015/ITA, 180/2015/ITA and 182/2015/ITA. After initial CRFK inoculation, all isolates produced CPE within 2–4 days, and FCV antigens were observed by indirect immunofluorescence assay.

3.2 | Sequence analysis

Partial ORF2 sequences obtained with the primer pair Cali3/Cali4 (Marsilio et al., 2005) were determined from 10 samples and compared with cognate sequences available in the databases. By FASTA and BLAST analyses, all the sequences shared 78.0%–95.0% nucleotide (nt) identities to each other, whilst identities to FCVs currently available in the databases were 72.1%–84.3%.

The sequence of ~3.2-kb fragment at the 3′ end of the genome, including the partial RNA-dependent RNA polymerase (RdRp) and the complete ORF2 and ORF3 of four isolates, FCVs 81/2014/ITA, 148/2015/ITA, 180/2015/ITA and 182/2015/ITA, was generated and then submitted to GenBank under accession no. MT008246-9. Also, the 7722-nt complete genome sequence of the strain 160/2015/ITA (MT00824650) was determined by NGS analysis. Three ORFs were predicted from computer analysis of the nucleotide sequence and by comparing the results with the genomic organization and ORFs of other FCVs. ORF1 was 5,289 nt long (nt 20–5308) and encoded a polyprotein of 1762 aa. ORF2 was 2016 nt in length (nt 5305–7320) and encoded for a capsid protein of 671 aa. ORF3 was 321 nt long (nt 7317–7637) and encoded a protein of 106 aa. In the complete genome, the overall nt identity of the strain 160/2015/ITA to FCV full sequences currently available in the databases ranged from 76.9% to 81.8%, whilst the identity of the deduced aa ranged from 87.3% to 94.5%, for ORF1, 82.5%–89.3% for ORF2 and 88.7%–98.1% for ORF3. Upon phylogenetic analysis based on the complete genome sequence, the strain 160/2015/ITA grouped along with FCVs previously detected in cats with upper respiratory tract disease in the United States (Abd-Eldaim et al., 2005) and with Chinese isolates obtained from cats with respiratory signs or VSD (Guo et al., 2018). The identity within this group was 80.6–81.4 nt (Figure 1). To further investigate the genetic heterogeneity of the E-FCVs identified in this study, analyses of the partial polymerase region and of the full-length capsid protein were performed. By sequence comparison in the 0.8-kb portion of the RdRp region, the five viruses shared 85.2%–96.2% nt and 93.6%–99.0% aa to each other, whilst identities to FCV RdRp sequences available in the databases were 75.2%–83.1% nt and 84.9%–92.2% aa, respectively. Upon sequence analysis of the full-length capsid gene, the E-FCV strains displayed 87.3%–94.5% aa identities to each other.

The overall aa identities to FCV capsid sequences available in the databases were 81.6%–92.3%.

The complete capsid sequence from 58 FCV strains was retrieved from the databases and used to perform the phylogenetic analysis. In the tree (Figure 2), three of the five E-FCVs (148/2015/ITA, 180/2015/ITA and 182/2015/ITA) formed a well-defined cluster (bootstrap value 99%), sharing 92.4%–94.5% aa identity. The isolate 81/2014/ITA displayed an overall aa identity of 87.3%–88.1% to the other E-FCVs analysed in this study and showed the highest genetic relation to the FCV strain LS015 (92.3% aa identity) previously detected in a cat with chronic stomatitis in the UK (Glenn et al., 1999). The E-FCV strain 160/2015/ITA (aa identities 88.2%–89.2%) appeared in the tree more distantly related to the other isolates (aa identities 88.6%–89.4%) and resulted genetically more similar to FCV strains George, Kaos and Jengo found in cats with VSD in the United States (Ossiboff et al., 2007).

By alignment of the region E of capsid protein (aa residues 426–523) (Seal, 1994), the variation among the E-FCVs was 14.4%–38.9% nt and 13.34%–36.0% aa. When compared in the 5′ hypervariable region (5′ HV), the nt distance among strains 148/2015/ITA, 180/2015/ITA and 182/2015/ITA ranged from 14.7% to 17.6%, whilst the nt distance between those 3 strains and the isolates 160/2015/ITA and 81/2014/ITA was 32.4%–52.0%. The strains 160/2015/ITA and 81/2014/ITA differed from each other by 44.2% nt.

Residues 438, 440, 448, 452 and 455 in the N-portion, residue 465 in the central-conserved part and residue 492 in the C portion of capsid region E are statistically relevant to define the VSD- or R-pathotype (Brunet et al., 2019). Upon alignment and visual inspection of this capsid region, the E-FCVs showed either VSD- or R-pathotype constellation of aa residues (Table 2).

3.3 | In vitro *sensitivity to pH, trypsin and bile*

The results obtained for each E-FCV strain are summarized in Table 3. In the acid lability test, with the exception of strains 180/2015/ITA and 182/2015/ITA, which were completely stable in low pH conditions (pH 3.0), the other three E-FCVs showed a loss of infectious titre ranging from 0.25 to 0.5 $\log_{10}$. Conversely, the R-FCV strains

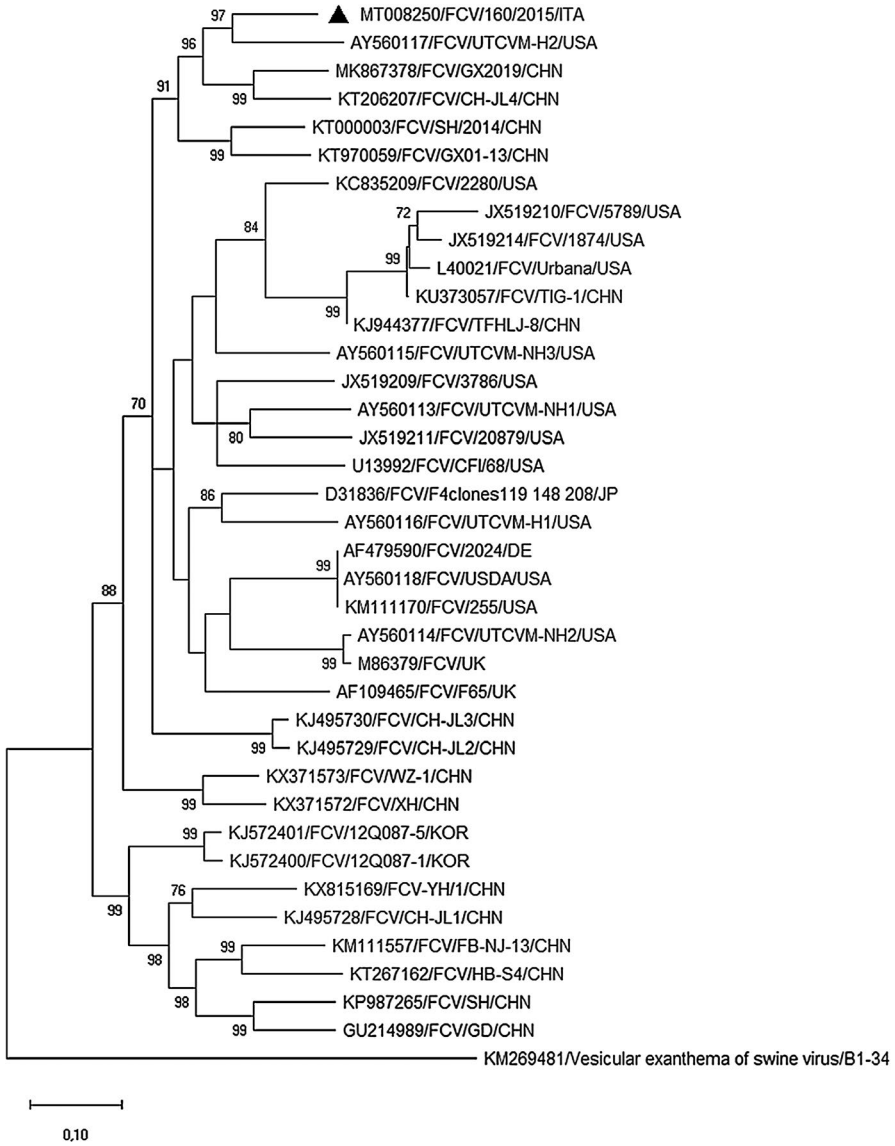

FIGURE 1 Phylogenetic tree constructed on complete genome of FCV. Tree was generated using the maximum likelihood with Tamura–Nei model and supplying statistical support with bootstrapping of 1,000 replicates. The scale bar indicates nucleotide substitutions per site. Black triangles indicate the FCV strain 160/2015/ITA. Evolutionary analyses were conducted in MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018)

were more susceptible to low pH with a titre reduction from 1.0 to 2.0 $\log_{10}$, with the exception of strain FCV-260/FA (0.5 $\log_{10}$). When analysing susceptibility to trypsin, the five E-FCVs were either highly stable (180/2015/ITA and 182/2015/ITA) or showed a limited reduction in $TCID_{50}$, of 1.0 $\log_{10}$ (81/2014/ITA), 1.5 $\log_{10}$ (148/2015/ITA) and 2.0 $\log_{10}$ (160/2015/ITA). All the R-FCV strains were susceptible to trypsin, which decreased the $TCID_{50}$ by 2.75–4.0 $\log_{10}$. Exposure to bile salts decreased the $TCID_{50}$ of the E-FCV strains 148/2015/ITA, 180/2015/ITA, 182/2015/ITA and 160/2015/ITA by 0.25–1.0 $\log_{10}$, whilst for strain 81/2014/ITA the $\log_{10}$ reduction was of 2.25. The $TCID_{50}$ was decreased by 1.25–2.5 $\log_{10}$ for four R-FCV strains, whilst for strain FCV-293/FA the titre was only slightly affected (0.5 $\log_{10}$).

4 | DISCUSSION

Feline calicivirus has been identified only occasionally from the intestinal tract or stool samples of cats, and information on its role as an enteric pathogen is still unclear (Marshall et al., 1987; Mochizuki, 1992; Pinto et al., 2012; Soma et al., 2015). In our analysis, using specific primer sets (Marsilio et al., 2005), FCV RNA was detected in a total of 62 faecal samples with an overall prevalence of 25.9%. Positivity was found in diarrhoeic samples of two collections, A and B, with rates, respectively, of 28.3% and 16.7%, whilst FCV RNA was not identified in samples collected from animals without diarrhoea used as a control group. The diarrhoeic samples of collection B were also screened for other enteric viruses. FCV was detected as a single virus pathogen in 50.0% of the positive cats, whilst co-infections were found in conjunction with FECV in three samples (37.5%) or with FPV, FeKoV and NoV in one sample (12.5%). Interestingly, only one of the FCV-infected cats showed co-infection with FPV, despite the high prevalence of FPV (33.3%). These findings seem to indicate that FCVs are a common component of the feline enteric virome. Whether FCV can act as a primary causative agent of gastrointestinal disease or whether it can trigger mechanisms of synergism in co-infections with other enteric pathogens should be demonstrated in experimental infections. It is also possible that

FIGURE 2 Phylogenetic tree based on the aa sequence of the full-length capsid protein. The tree was generated using the maximum likelihood with Tamura–Nei model and supplying statistical support with bootstrapping of 1,000 replicates. Black circles indicate the FCV strains detected in this study. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018)

TABLE 2 Amino acidic residues of region E of the capsid protein VP1 precursor (ORF2) hypothesized to control FCV pathotype shift form respiratory (R) to virulent systemic disease (VSD) (Brunet et al., 2019)

	aa residues and physicochemical properties associated with VSD pathotype						
Strain	438 hydrophobic aliphatic	440 not small	448 polar positive charged	452 not small	455 not negative	465 polar	492 small
VSD FCV	**V_9T_8**	**$Q_6G_5E_4SK$**	**$K_7A_2E_2G_2P_2R_2$**	**$E_{11}D_6$**	**$T_6D_4M_2I_2NES$**	**$S_{14}G_3$**	**$V_{16}R$**
R-FCV	T_{37}**V_2**I	$G_{22}S_6$**Q_4R_2**A_2**E**NDT	$A_{30}P_4G_3$**K_3**	D_{36}**E_3**N	D_{27}**$T_5S_3V_2$**G**R**E	G_{26}**S_{14}**	**V_{18}**$L_8I_7R_5K_2$
E-ITA/2013/160	**V**	**Q**	**R**	**E**	**T**	G	**V**
E-ITA/201X/81	**V**	S	A	D	E	**S**	**V**
E-ITA/201X/I48	T	R	A	D	D	G	L
E-ITA/201X/I80	T	G	A	D	D	G	L
E-ITA/201X/I82	T	G	A	D	D	G	L

Note: The residues of strains with enteric (E) pathotype have been mapped. Residues in bold indicate correspondence with the VSD pathotype configuration. Subscript numbers indicate the frequencies from the original alignment (Brunet et al., 2019).

TABLE 3 Effects of low pH, trypsin and bile salts on infectivity of enteric and respiratory FCVs

	Titre of FCV (log10 TCID50) and log10 reduction resulted from the treatment indicated								
	HCL (pH 3.0)			Trypsin (0.5%)			Bile (0.5%)		
FCV strain	Control	Treated	Δ	Control	Treated	Δ	Control	Treated	Δ
E-81/2014/ITA	6.5	6.25	0.25	7.25	6.25	1.0	5.75	3.5	2.25
E-148/2015/ITA	5.75	5.25	0.5	5.75	4.25	1.5	5.0	4.75	0.25
E-160/2015/ITA	7.0	6.75	0.25	6.0	4.0	2.0	4.75	4.0	0.75
E-180/2015/ITA	6.5	6.5	0.0	6.5	6.25	0.25	5.5	4.5	1.0
E-182/2015/ITA	4.0	4.0	0.0	4.5	4.25	0.25	3.25	3.0	0.25
R-185/2002/ITA	4.5	3.5	1.0	4.5	1.0	3.5	4.0	2.75	1.25
R-236/2002/ITA	6.75	5.75	1.0	6.25	3.0	3.25	3.75	1.25	2.5
R-260/2003/ITA	6.5	6.0	0.5	6.25	2.25	4.0	4.25	2.75	1.5
R-293/2004/ITA	6.5	4.5	2.0	5.5	2.75	2.75	4.75	4.25	0.5
R-299/2004/ITA	7.0	5.25	1.75	6.5	2.5	4.0	4.0	2.5	1.5

Note: Resistant phenotype (null or modest decrease in the infectious titre) is evidenced in grey shadow.
Abbreviations: E, enteric; R, respiratory; Δ, log10 reduction.

occasionally cats with classical respiratory disease shed the virus in their faeces and that the virus is passively transported through the gastroenteric tract. In experimental studies with respiratory FCV isolates, the virus has been detected in the faeces of a few infected kittens and of a cat in contact with experimentally infected animals (Bartholomew & Gillespie, 1968), although enteric signs were not described in those subjects.

Five strains were successfully adapted to grow in a feline cell line (CRFK) and used to further investigate their genetic features. Sequence analysis of the complete genome of the strain 160/2015/ITA and analysis of ~3.2-kb fragment at the 3′ end of the genome of 4 E-FCV isolates revealed a marked genetic diversity, as outlined in the phylogenetic analyses based on the complete genome (Figure 1) or on the ORF2 (capsid) gene (Figure 2) and as described elsewhere (Geissler et al., 1997; Glenn et al., 1999). Also, in those analyses, it was clear that 5 E-FCV strains did not display a common clustering pattern. In the complete capsid-based tree (Figure 2), only 3 isolates (180/2015/ITA, 182/2015/ITA and 148/2015/ITA), all which were obtained from collection A, formed a monophyletic cluster, suggesting an epidemiological link, whilst strains 160/2015/ITA (collection A) and 81/2014/ITA (collection B) were more distantly related (Figure 2). Analysis of the 5′ HV region E of the capsid gene, containing the major B-cell epitopes (Neill, Sosnovtsev, & Green, 2000; Radford, Coyne, Dawson, Porter, & Gaskell, 2007; Radford, Willoughby, Dawson, McCracken, & Gaskell, 1999; Tohya, Yokoyama, Maeda, Kawaguchi, & Mikami, 1997), has been used to investigate the genetic relationships among various FCV isolates. The nucleotide sequences of this region revealed a degree of variation that reached about 8% among variants of a single isolate (Radford et al., 1998), up to 18% in multi-cat environments (Coyne, Gaskell, Dawson, Porter, & Radford, 2007), and up to 21%–43% among unrelated

isolates (Radford et al., 1997; Radford, Dawson, Wharmby, Ryvar, & Gaskell, 2000). In our analysis, applying this cut-off values and following the phylogenetic reconstruction (Figure 2), the clustering observed for strains 148/2015/ITA, 180/2015/ITA and 182/2015/ITA, coupled with the nt variation (14.7%–17.6%) in the 5′ HV region, could indicate that the 3 isolates may have evolved from a common ancestral leading to a geographical co-circulation of strains with enteric tropism.

The in vitro characteristics of stability to low pH, trypsin and bile salts of the E-FCVs were compared to those of five R-FCV strains in order to evaluate whether there are biological differences among FCV strains obtained from different sites of replication/shedding. Overall, E-FCV strains showed a higher stability than R-FCVs to all the treatments applied in this study. Based on the characteristic of stability to bile salts, it has been previously proposed that FCVs could be divided into two phenotype groups, respiratory (R) type and enteric (E) type (Mochizuki, 1992). In our analysis, these differences were less marked when testing the strains with bile salts and with low pH conditions, whilst they were more evident when treating the viruses with trypsin and this was likely accounted for by different standardization of the assays among the various laboratories. When using trypsin, we observed a reduction in the infectious titre as high as 2.75–4.0 $\log_{10}$ for R-FCV strains, whilst no significant titre loss was observed for E-FCV strains. Trypsin plays an important role in modulating viral properties in vivo and in vitro. For instance, several enteric viruses require trypsin to replicate in vitro (Brinker, Blacklow, & Herrmann, 2000; Estes, Graham, & Mason, 1981). Also, trypsin-like serine proteases modulate the pathogenesis of influenza viruses (Kido, Takahashi, & Kimoto, 2019). Orally transmitted viruses have to resist the extreme conditions of the gastrointestinal environment of the host, especially proteolysis of their structural proteins. Losing and masking protease-sensitive residues on the structural proteins are the likely mechanisms adopted to survive the harsh gastrointestinal conditions (Wei et al., 2018). It is possible that FCV strains are able to develop resistance to trypsin, low pH and bile salts in the gut environment, whilst this is not required for replication in the upper respiratory tract. Serial passaging of a bile-sensitive R-FCV in vitro in the presence of bile salts selected a bile-resistant variant (Mochizuki, 1992), demonstrating the biological plasticity of FCV. Overall, however, our findings confirm the existence of two distinct phenotypes of FCV correlated to the source of isolation/shedding. FCV adopts several strategies/mechanisms to ensure its long-term survival in the host population, such as progressive evolution of a given strain variant through the accumulation of punctate mutations within an individual, sequential reinfection with either a variant of the same strain or a different strain, and mixed infection, eventually coupled with recombination events (Coyne et al., 2007). These mechanisms relentlessly mould the genetic/antigenic/biological properties of FCV, leading eventually to dramatic phenotype changes, as observed in VSD (hypervirulent) FCV strains (Ossiboff et al., 2007). However, defining the molecular bases of the observed pathotype changes has been, thus far, challenging. Analysis of FCV sequences from capsid region E, responsible for interaction with the cellular receptor, on the basis of their aa physicochemical properties, was able to differentiate VSD and classical R-FCVs and identified seven key residue positions (438, 440, 448, 453, 455, 465 and 492) statistically significant for pathotype differentiation, mainly located in the N-terminal hypervariable part of region E (Brunet et al., 2019). This analysis, however, confirmed that there is no evident genetic marker of the VSD pathotype. When analysing our E-FCV strains in the capsid region E, three strains showed aa patterns found in R-FCVs, whilst a unique isolate, 160/2015/ITA, matched the residue configuration found in VSD strains and another strain (81/2014/ITA) showed an intermediate configuration (Table 2). It will be interesting to extend this kind of analysis on a larger set of VP1 sequences of E-FCVs to understand whether those residues also significantly vary or not with the enteric pathotype.

The existence/distinction of two distinct FCV pathotypes, referred to as classical (or respiratory) and VSD (or hypervirulent) forms or pathotypes, has now been largely accepted by veterinary practitioners, animal care operators and researchers/scientific literature. Although not supported by experimental infections, our study demonstrates that FCV may also be associated with enteric disease in cats, implying the existence of a third FCV pathotype, as proposed previously (Mochizuki et al., 1992). Also, we demonstrated that E-FCVs clearly differ from classical R-FCVs in terms of resistance to some biochemical treatments. Implementation of the diagnostic algorithms for feline enteric pathogens with FCV-specific assays will be pivotal to dissect the role of these viruses in enteritis of cats and eventually to devise more effective prophylactic strategies. Likewise, collection of faecal samples from cats with respiratory and VSD signs should be considered in order to establish whether and to what extent faecal shedding of FCV occurs. Genome sequencing of enteric FCVs will be useful to understand whether there are genetic hallmarks accounting for the different biological properties of these viruses. Also, it will be important to understand whether the enteric tropism of FCV is maintained stably and whether it may affect the ability of the virus to trigger the classical and/or hypervirulent forms of disease.

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CONFLICT OF INTEREST

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.

ETHICAL APPROVAL

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No

ethical approval was required, and ethical statement is not applicable as sample collection from animals has been gathered.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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