Original Article

Genomic and phylogenetic analysis of the ORF7b gene from a Chinese feline infectious peritonitis virus isolate

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Abstract

FCoV viruses exhibit great genetic diversity, leading to the presence of FIPV-causing variants. Current molecular evolution analysis and genetic variation studies of FCoV in China are predominately focused on gene encoding the spike protein or other structural proteins, while few studies have evaluated genetic variations in nonstructural FCoV genes, which can play an important role in disease pathogenesis. In this study, the gene encoding the open reading frame (ORF) 7b nonstructural FCoV protein of the Chinese Fujian strain FJLY20201 was amplified from the ascitic fluid of a Chinese domestic cat infected with FIPV and compared with ORF 7b from previously published FCoV strains. Multiple sequence alignment revealed that FJLY20201 exhibited high identity with other Chinese FCoV strains. Phylogenetic analyses indicated that the Chinese strains did not differentiate between type I and type II serotypes of FCoV based on S proteins. In addition, they formed clades and differed genetically from strains originating outside China. This study provides the molecular epidemiology data about the *ORF 7b* genes of FCoV strains in China. Our results show that the identity of ORF 7b genes was closer between the Chinese isolates, and suggest that variation in *ORF 7b* is more dependent on geographical origin.

Keywords: feline coronavirus, feline infectious peritonitis virus, phylogenetic analysis, *ORF 7b*

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Introduction

Feline infectious peritonitis virus (FIPV) is a biotype of feline coronavirus (FCoV), which can cause fatal feline infectious peritonitis (FIP) in domestic and wild felids worldwide (Vennema *et al*. 1998). FIPV infection is most prevalent in cats under two years of age, males (Riemer *et al*. 2016), and certain breeds (Pesteanu-Somogyi *et al*. 2006,Rohrbach *et al*. 2001). FIPV arises from a mutation in the avirulent feline enteric coronavirus (FECV), which is another biological form of FCoV (Pedersen *et al*. 1981). FECV is globally distributed and has a nearly 100% prevalence in multi-cat environments (Hohdatsu *et al*. 1992). FECV is transmitted predominately through the fecal-oral route in cats and replicates in the intestinal epithelium. Most cats are asymptomatic after infection or exhibit only mild and transient diarrhea (Vogel *et al*. 2010). However, in some cases, FECV is mutated and transformed into FIPV, which then localizes in monocytes and macrophages. This transformation results in increased virulence and can lead to fatal disease (Kipar *et al*. 2001,Meli *et al*. 2004). In China, the number of domestic cats is on the rise, making FIPV infection an emerging serious problem, with increasingly high mortality rates resulting from FECV mutating to FIPV.

FCoV, which belongs to the *Alphacoronavirus* genus, is an enveloped virus with a large, capped and polyadenylated RNA genome of approximately 29,190 nucleotides (Dye and Siddell 2005). The FCoV genome encodes four structural proteins: (spike (S), envelope (E), membrane (M) and nucleocapsid (N)) and seven non-structural proteins (3a, 3b, 3c, 7a and 7b, and replicases 1a and 1b), which are thought to play a role in virus pathogenesis (Dye and Siddell 2005). The open reading frame 7b (ORF 7b) protein is a non-structural protein encoded at the 3'end of the FCoV genome. ORF 7b is species-specific and is observed exclusively in feline and canine strains (De Groot *et al*. 1988,Vennema *et al*. 1992). ORF 7b plays a known role in inducing antibody responses in naturally infected cats (Kennedy *et al*. 2008). Furthermore, ORF7b can be used as a phylogenetic marker to differentiate between FECV and FIPV strains as a result of the short deletions of *ORF 7b* that are often observed in avirulent FECV strains (Brown *et al*. 2009,Kennedy *et al*. 2001).

Despite the potential importance of *ORF 7b* in the pathogenicity of FCoV strains, most reports on the molecular epidemiology and phylogeny of FCoV in China have been based on sequence analysis of the genes encoding the S protein or other structural proteins. To date, there is little information on the molecular epidemiology of *ORF 7b* in China, which could have critical importance to understanding the evolution of pathological FCoV strains. In this study, we isolated ascitic fluid from a domestic cat with FIPV in Fujian, China, and analyzed the molecular sequence and genetic evolution of the *ORF 7b* gene in this strain in comparison with other previously published Chinese FCoV strains.

Materials and Methods

Sample Collection: Ascitic fluid samples from a Chinese domestic indoor cat diagnosed with wet-type FIP were sent to the Longyan University Animal Hospital in November 2020. The samples collection was performed in accordance with the national guidelines (CNAS-CL06:2018) for the care and use of laboratory animals. FIP diagnosis was confirmed by a Rivalta test. Samples were stored at -80 °C until analysis. This study was approved by the Committee on the Ethics of Animal Experiments of Longyan University (November, 2020). Informed consent was obtained from the cat's owners prior to sample collection. The sampling and data publication were approved by the cats' owners.

RNA extraction and PCR amplification: Total RNA was extracted from 200 µL of ascitic fluid using the Simply P Virus RNA Extraction Kit (Hangzhou Bioer Technology Co. Ltd, China). Reverse transcription polymerase chain reaction (RT-PCR) was carried out at 42° °C for 1 h using 3 µg of total RNA, 0.5 µg random primer (Promega, Madison, WI, USA) and 5 U AMV reverse transcriptase (Promega). Two pairs of primers were used to amplify the complete *ORF 7b* gene, as previously described (Lin *et al*. 2009).

Cloning and sequencing: The amplified PCR product was subjected to gel electrophoresis, excised and purified using an agarose gel DNA purification kit (Takara Biomedical Technology (Beijing) Co. Ltd, China). The purified PCR product was sent to Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China) for sequencing.

Sequence and phylogenetic analysis: Multiple sequence alignment and sequence analysis were performed using the Multiple Sequence Alignment tool of the DNAMAN 6.0 software (Lynnon BioSoft, Point-Claire, Quebec, Canada). Sequences across different viral strains were compared using the pairwise distances of the untitled ClustalW (weighted) method. Phylogenetic trees derived from the nucleotide sequences were constructed by MEGA version 5.2 using the neighbor-joining method with the p-distance model, 1,000 bootstrap replicates. (Tamura *et al*. 2013). Phylogenetic trees were displayed and annotated using the online tool ITOL v5 (https://itol.embl.de/).

Results

Identity and sequence analysis: RT-PCR of *ORF 7b* in samples from the FIP-positive cat yielded the expected 766 base pair product (Fig 1). The complete *ORF7b* (621 bp) of the Chinese Fujian strain was named FJLY20201. The amino acid sequence of FJLY20201 was compared with *ORF 7b* sequences from other Chinese FCoV strains in addition to international strains previously published in GenBank (Table 1). Multi-sequence alignment analysis revealed that the identity between FJLY20201 and other strains collected in our study was 66.6-98.5%, while identity between FJLY20201 and other Chinese strains was 91.8-98.5%. Among all strains that we evaluated, we observed the highest identity (98.5%) between FJLY20201 and HLJ-DQ-2016-01. The identity between FJLY20201 and FCoV-

Black, a classical Black strain of the type I FCoV originating in the United States, was 91.8%, while the identity between FJLY20201 and the two type II FCoV strains (WSU 79-1146 and WSU 79-1683) was 89.3% and 66.6%, respectively. Multiple alignment of amino acid sequences demonstrated that there were two amino acid differences between FJLY20201 and HLJ-DQ-2016-01 (E→G at p.110 and N→S at p.158) and these differences also occurred in other Chinese strains (Fig 2).

Phylogenetic analysis: Phylogenetic analysis of the *ORF 7b* genes showed that FJLY20201 and two Chinese strains were included in the same clade, while the other five Chinese strains were included in another clade with a Netherlands isolate FCoV UU54. Our results also indicate that the *ORF 7b* genes from mainland China are genetically distinct from most other foreign strains. Lastly, the results revealed that FJLY20201 and other Chinese strains were clearly distinct from CCoV strains (Fig 3).

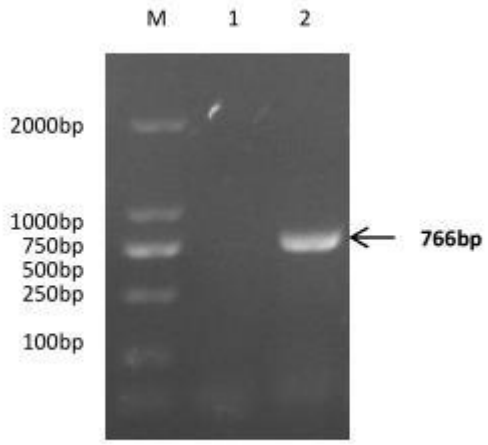

Figure 1 **Amplification of ORF7b of FJLY20201.** Amplification of 766 base pair (bp) DNA fragment from the ascitic fluid specimen. M: 2000 bp molecular weight ladder, 1: negative control, 2: FJLY20201.

Table 1 The information for the reference strains in this study

Strains	ID number	Country or region	Year
FCoV strain HLJ DQ 2016 01	KY292377	China	2016
FCoV isolate XXN	MN165107	China	2018
FCoV strain HLJ HRB 2016 13	KY566211	China	2016
FCoV strain HLJ HRB 2016 11	KY566210	China	2016
FCoV strain HLJ HRB 2016 10	KY566209	China	2016
CCoV HLJ-072	KY063617	China	2016
FCoV strain ZJU1709	MT239440	China	2020
FCoV strain ZJU1617	MT239439	China	2020
FCoV NTU156 P 2007	GQ152141	Taiwan	2007
FCoV NTU2	DQ160294	Taiwan	2003
FCoV NTU8	DQ675419	Taiwan	2003
FCoV NTU6	DQ675417	Taiwan	2003
FCoV NTU5	DQ675416	Taiwan	2003
FCoV NTU44	DQ675452	Taiwan	2005
FCoV NTU43	DQ675451	Taiwan	2005
FCoV NTU42	DQ675450	Taiwan	2005
FCoV NTU4	DQ675415	Taiwan	2003
FCoV NTU37	DQ675446	Taiwan	2005
FCoV NTU3	DQ675414	Taiwan	2003
FCoV NTU19	DQ675430	Taiwan	2004
FCoV NTU17	DQ675428	Taiwan	2004
FCoV NTU13	DQ675424	Taiwan	2004
FCoV NTU10	DQ675421	Taiwan	2004
FCoV NTU1	DQ648122	Taiwan	2002
FCoV isolate UG-FH8	KX722529	Belgium	2015
FCoV isolate Cat3 withoutdeletion	KU215428	Belgium	2013
FCoV isolate Cat3 deletion	KU215427	Belgium	2013
FCoV isolate Cat1	KU215424	Belgium	2013
FCoV isolate FB6E	JX467670	Brazil	2009
FCoV isolate 781	JX239139	Brazil	2004
FCoV isolate 579	JX239119	Brazil	2003
FCoV isolate 1476	JX239091	Brazil	2010
FCoV isolate 1462	JX239090	Brazil	2008
FCoV isolate 1341	JX239089	Brazil	2007

FCoV isolate 1055	JX239101	Brazil	2005
FCoV isolate Cat 2 Holstebro	KX722531	Denmark	2015
FCoV isolate Cat 1 Karlslunde	KX722530	Denmark	2015
CCoV 23 03	AY307020	Italy	2003
CCoV 174 06	EU856362	Italy	2006
FCoV strain NLD UU88 2010	KF530123	Netherlands	2010
FCoV UU11	FJ938052	Netherlands	2007
FCoV UU10	FJ938059	Netherlands	2007
FCoV UU54	JN183883	Netherlands	2010
FCoV UU9	FJ938062	Netherlands	2009
FCoV UU30	HQ392472	Netherlands	2008
FCoV UU34	HQ012372	Netherlands	2007
FCoV UU8	FJ938055	Netherlands	2009
FCoV UU7	FJ938053	Netherlands	2009
FCoV UU5	FJ938056	Netherlands	2009
FCoV UU47	JN183882	Netherlands	2010
FCoV UU40	HQ392469	Netherlands	2008
FCoV UU4	FJ938054	Netherlands	2007
FCoV UU31	HQ012371	Netherlands	2008
FCoV UU24	HQ012370	Netherlands	2008
FCoV UU21	HQ012369	Netherlands	2007
FCoV UU20	HQ392471	Netherlands	2007
FCoV UU18	HQ012368	Netherlands	2007
FCoV UU17	HQ012367	Netherlands	2007
FCoV UU16	FJ938058	Netherlands	2007
FCoV UU15	FJ938057	Netherlands	2007
FCoV mRNA for 7a and 7b protein	X90571	Netherlands	1995
FIPV 79-1146	DQ010921	UK	2005
FCoV isolate 80F	KP143511	UK	2013
FCoV isolate 65F	KP143509	UK	2013
FCoV isolate 27C	KP143507	UK	2013
FCoV isolate 26M	KP143512	UK	2013
FCoV black	EU186072	USA	2007
FCoV RM	FJ938051	USA	2002
FIPV	NC_002306	USA	2005
FCoV strain WSU 79-1683	JN634064	USA	2011
FIPV-UCD18a	FJ917528	USA	2008
FIPV-UCD17	FJ917527	USA	2008
FIPV-UCD16	FJ917526	USA	2008
FIPV-UCD15b-1	FJ943770	USA	2008
FIPV-UCD15a	FJ917525	USA	2008
FIPV-UCD14	FJ917524	USA	2007
FIPV-UCD13 3a	FJ943764	USA	2008
FIPV-UCD13	FJ917523	USA	2008
FIPV-UCD12-1	FJ943766	USA	2007
FIPV-UCD12	FJ917521	USA	2008
FIPV-UCD11b-2b	FJ917535	USA	2008
FIPV-UCD11b-2a	FJ917534	USA	2008
FIPV-UCD11b-1a	FJ917532	USA	2008
FIPV-UCD11b	FJ917520	USA	2008
FIPV-UCD11a-1a	FJ917530	USA	2008
FECV-UCD6-1	FJ943772	USA	2008
FECV-UCD5	FJ917522	USA	2008
FECV-UCD4	FJ943763	USA	2008
FECV-UCD3a	FJ943761	USA	2007
FCoV UU3	FJ938061	USA	1998
FCoV UU2	FJ938060	USA	1993
FCoV strain OH11927	MF457591	USA	2014
FCoV isolate T	FJ943773	USA	2008
FCoV isolate 1	FJ943768	USA	2007
CCoV A76	JN856008	USA	1976
CCoV S378	KC175341	USA	1978

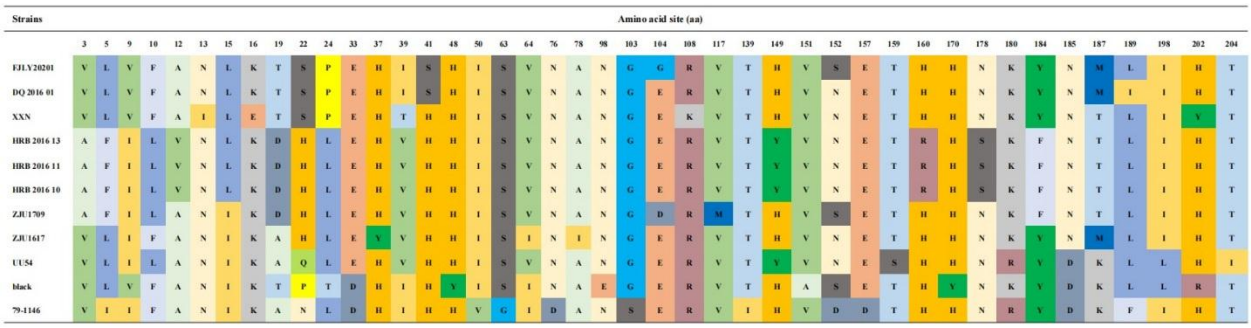

Figure 2 **Amino acids variations in the ORF 7b among Chinese strains and the classical strain.** The same amino acids are denoted in the same color.

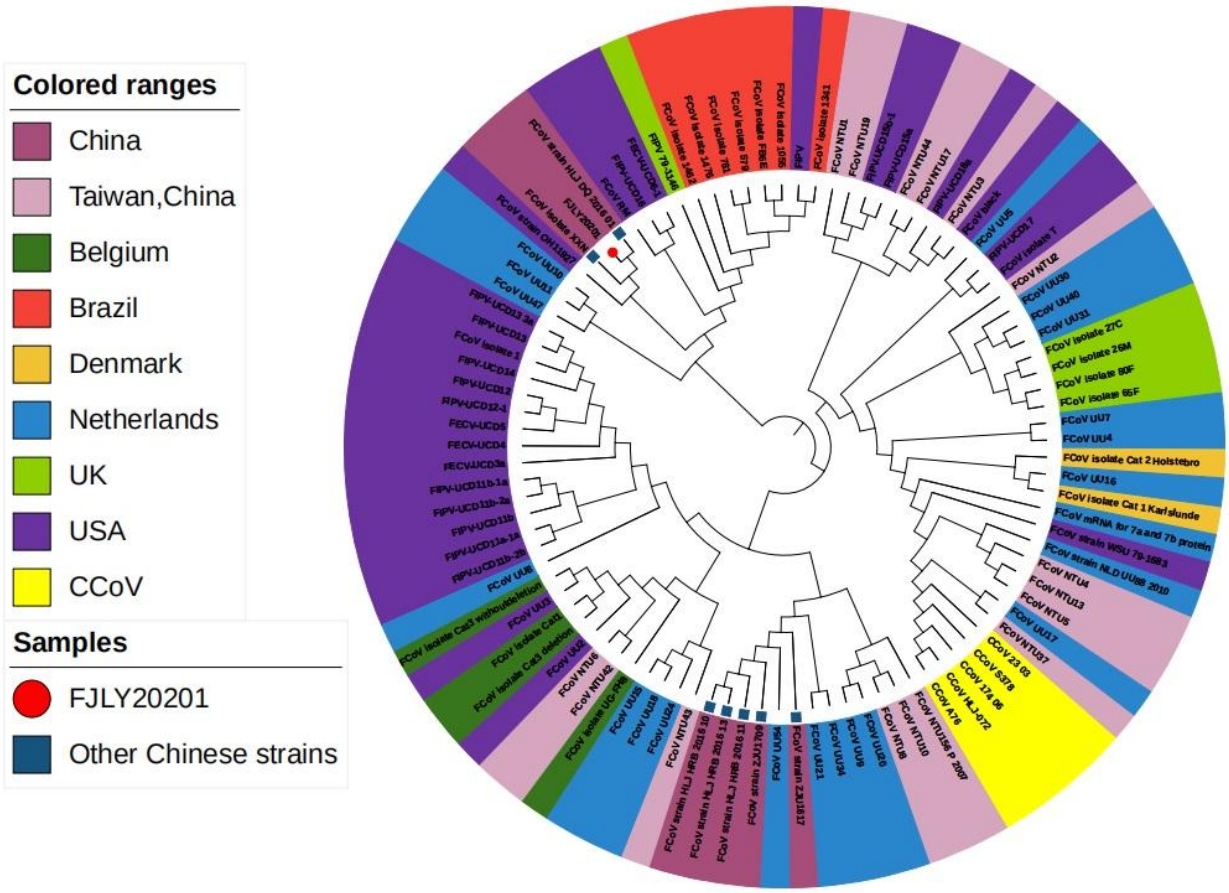

Figure 3 **ORF7b gene phylogenetic analysis.** The phylogenetic tree was generated using the neighbor-joining method and supported by 1000 bootstraps. Strains from different countries or regions are denoted in different colors. The ● and ■ represent FJLY20201 identified in our study and the Chinese strain collected respectively.

Discussion

Both FCoV serotypes (I and II) can occur in two antigenically and morphologically indistinguishable biotypes (or pathotypes): FECV and FIPV. However, our phylogenetic analysis revealed that ORF 7b sequence did not differentiate between type I or type II serotypes of FCoV, consistent with a previous report (Herrewegh *et al*. 1995). Additionally, our analyses indicated that ORF7b sequences from all FCoV strains were distinct from those of both type I and II CCoV strains and exhibited phylogenetic divergence. However, in previous reports of sequence analysis of S genes, type I FCoV was shown to be more similar to type I CCoV and type II FCoV was more closely related to type II CCoV (Whittaker *et al*. 2018).

Deletions in *ORF7b* have been previously reported in laboratory strains and avirulent strains (Herrewegh *et al*. 1995). In our study, no deletions were observed in FJLY20201 or the other seven published Chinese FCoV strains, providing evidence for the absence of deletions in the majority of field strains (Lin *et al*. 2009). However, deletions have been reported in some virulent strains (Lin *et al*. 2009), highlighting that the relationship between *ORF 7b* deletions and pathogenicity requires further investigation.

A previous study evaluating Brazilian, North American and Taiwanese isolates suggested that the phylogeny of the *ORF 7b* gene sequence is more dependent on geographical origin than pathological serotypes (Myrrha *et al*. 2019). Consistently, we observed that strains from mainland China formed two

clades and were genetically distinct from other foreign isolates, providing further support for the correlation between geographical origin and *ORF 7b* gene genealogy. In addition, a previous study indicated that the whole genomes of three Chinese strains (HLJ-HRB-2016-10, HLJ-HRB-2016-11 and HLJ-HRB-2016-13) form a cluster and may represent a new type I FCoV (Li *et al*. 2019). Our phylogenetic analysis of ORF7b confirmed that these three strains were closely related, supporting these previous observations. In addition, two Chinese strains ZJU1617 and ZJU789 isolated in 2020 were also located in the same clade, indicating that this new type I FCoV could already be circulating in China. Furthermore, it was found that Brazilian FCoV isolates may have originated from North America because the ORF 7b genes of the Brazilian isolates are closely related to the two North American isolates (Myrrha *et al*. 2019). Interestingly, according to the phylogenetic tree, five Chinese isolates (ZJU1617, ZJU1709, HLJ-HRB-2016-13, HLJ-HRB-2016-11 and HLJ-HRB-2016-10) showed a high phylogenetic relationship with a Dutch isolate. This finding provides support for further investigation of the origin, evolution and recombination of field strains in China.

In conclusion, FJLY20201 isolated in this study had a higher identity with Chinese strains than other foreign strains. There were no deletions in FJLY20201 or the other seven published Chinese FCoV strains, providing evidence for the absence of deletions in the majority of field strains. The phylogenetic tree in this study shows that the phylogeny of the *ORF 7b* gene sequence is more dependent on geographical origin than pathological serotypes.

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References

Brown MA, Troyer JL, Pecon-Slattery J, Roelke M.E and O'Brien SJ 2009. Genetics and pathogenesis of feline infectious peritonitis virus. Emerg Infect Dis. 15: 1445-1452.

Brown MA, Troyer JL, Pecon-Slattery J, Roelke M.E and O'Brien SJ 2009. Genetics and pathogenesis of feline infectious peritonitis virus. Emerg Infect Dis. 15: 1445-1452.

De Groot RJ, Andeweg AC, Horzinek MC and Spaan WJ 1988. Sequence analysis of the 3'-end of the feline coronavirus FIPV 79-1146 genome: comparison with the genome of porcine coronavirus TGEV reveals large insertions. Virology. 167: 370-376.

Dye C, Siddell SG 2005. Genomic RNA sequence of Feline coronavirus strain FIPV WSU-79/1146. J Gen Virol. 86: 2249-2253.

Herrewegh AA, Vennema H, Horzinek MC, Rottier PJ and de Groot RJ 1995. The molecular genetics of feline coronaviruses: comparative sequence analysis of the ORF7a/7b transcription unit of different biotypes. Virology. 212: 622-631.

Hohdatsu T, Okada S, Ishizuka Y, Yamada H and Koyama H 1992. The prevalence of types I and II feline coronavirus infections in cats. J Vet Med Sci. 54: 557-562.

Kennedy M, Boedeker N, Gibbs P and Kania S 2001. Deletions in the 7a ORF of feline coronavirus associated with an epidemic of feline infectious peritonitis. Vet Microbiol. 81: 227-234.

Kennedy MA, Abd-Eldaim M, Zika S.E, Mankin JM and Kania SA 2008. Evaluation of antibodies against feline coronavirus 7b protein for diagnosis of feline infectious peritonitis in cats. Am J Vet Res. 69: 1179-1182.

Kipar A, Köhler K, Leukert W and Reinacher M 2001. A comparison of lymphatic tissues from cats with spontaneous feline infectious peritonitis (FIP), cats with FIP virus infection but no FIP, and cats with no infection. J Comp Pathol. 125: 182-191.

Li C, Liu Q, Kong F, Guo D, Zhai J, Su M and Sun D 2019. Circulation and genetic diversity of Feline coronavirus type I and II from clinically healthy and FIP-suspected cats in China. Transbound Emerg Dis. 66: 763-775.

Lin CN, Su BL, Huang HP, Lee JJ, Hsieh MW and Chueh LL 2009. Field strain feline coronaviruses with small deletions in ORF7b associated with both enteric infection and feline infectious peritonitis. J Feline Med Surg. 11: 413-419.

Meli M, Kipar A, Müller C, Jenal K, Gönczi E, Borel N, Gunn-Moore D, Chalmers S, Lin F, Reinacher M and Lutz H 2004. High viral loads despite absence of clinical and pathological findings in cats experimentally infected with feline coronavirus (FCoV) type I and in naturally FCoV-infected cats. J Feline Med Surg. 6: 69-81.

Myrrha LW, Silva FMF, Vidigal PMP, Resende M, Bressan GC, Fietto JLR, Santos MR, Silva LMN, Assao VS, Silva-Jú Nior A and de Almeida MR 2019. Feline coronavirus isolates from a part of Brazil: insights into molecular epidemiology and phylogeny inferred from the 7b gene. J Vet Med Sci. 81: 1455-1460.

Pedersen NC, Boyle JF, Floyd K, Fudge A and Barker J 1981. An enteric coronavirus infection of cats and its relationship to feline infectious peritonitis. Am J Vet Res. 42: 368-377.

Pesteanu-Somogyi LD, Radzai C and Pressler BM 2006. Prevalence of feline infectious peritonitis in specific cat breeds. J Feline Med Surg. 8: 1-5.

Riemer F, Kuehner KA, Ritz S, Sauter-Louis C and Hartmann K 2016. Clinical and laboratory features of cats with feline infectious peritonitis--a retrospective study of 231 confirmed cases (2000-2010). J Feline Med Surg. 18: 348-356.

Rohrbach BW, Legendre AM, Baldwin CA, Lein DH, Reed WM and Wilson RB 2001. Epidemiology of feline infectious peritonitis among cats examined at veterinary medical teaching hospitals. J Am Vet Med Assoc. 218: 1111-1115.

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 30: 2725-2729.

Vennema H, Poland A, Foley J and Pedersen NC 1998. Feline infectious peritonitis viruses arise by mutation from endemic feline enteric coronaviruses. Virology. 243: 150-157.

Vennema H, Rossen JW, Wesseling J, Horzinek MC and Rottier PJ 1992. Genomic organization and expression of the 3' end of the canine and feline enteric coronaviruses. Virology. 191: 134-140.

Vogel L, Van der Lubben M, te Lintelo EG, Bekker CP, Geerts T, Schuijff LS, Grinwis GC, Egberink HF and Rottier PJ 2010. Pathogenic characteristics of persistent feline enteric coronavirus infection in cats. Vet Res. 41: 23.

Whittaker GR, André NM and Millet JK 2018. Improving Virus Taxonomy by Recontextualizing Sequence-Based Classification with Biologically Relevant Data: the Case of the Alphacoronavirus 1 Species. mSphere. 3: 00463-00417.