



Detection of subgenomic mRNA of feline coronavirus by real-time polymerase chain reaction based on primer-probe energy transfer (P-sg-QPCR)

Ákos Hornyák^{a,b,c}, Ádám Bálint^d, Attila Farsang^e, Gyula Balka^{f,*}, Mikhayil Hakhverdyan^{a,b}, Thomas Bruun Rasmussen^g, Jonas Blomberg^h, Sándor Belák^{a,b}

^a The Joint Research and Development Division, Department of Virology and Parasitology, The Swedish University of Agricultural Sciences, Ulls väg 2B, SE-751 89 Uppsala, Sweden

^b BVF (SLU, BVF) and the National Veterinary Institute, VIP (SVA, VIP), Ulls väg 2 B, SE-751 89 Uppsala, Sweden

^c Department of Microbiology and Infectious Diseases, Faculty of Veterinary Science, Szent Istvan University, Hungária krt. 23-25, H-1143 Budapest, Hungary

^d Department of Virology, Central Agricultural Office Veterinary Diagnostic Directorate, Tábornok u. 2, H 1149 Budapest, Hungary

^e Central Agricultural Office Directorate of Veterinary Medicinal Products, Szállás u. 8, H-1107 Budapest, Hungary

^f Department of Pathology and Forensic Veterinary Medicine, Faculty of Veterinary Science, Szent Istvan University, István u. 2, H-1078 Budapest, Hungary

^g National Veterinary Institute, Technical University of Denmark, Lindholm, DK-4771 Kalvehave, Denmark

^h Section of Clinical Virology, Department of Medical Sciences, Academic Hospital, Uppsala University, Dag Hammarskjölds v 17, SE 751 85 Uppsala, Sweden

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Feline infectious peritonitis is one of the most severe devastating diseases of the *Felidae*. Upon the appearance of clinical signs, a cure for the infected animal is impossible. Therefore rapid and proper diagnosis for both the presence of the causative agent, feline coronavirus (FCoV) and the manifestation of feline infectious peritonitis is of paramount importance. In the present work, a novel real-time RT-PCR method is described which is able to detect FCoV and to determine simultaneously the quantity of the viral RNA. The new assay combines the M gene subgenomic messenger RNA (sg-mRNA) detection and the quantitation of the genome copies of FCoV. In order to detect the broadest spectrum of potential FCoV variants and to achieve the most accurate results in the detection ability the new assay is applying the primer-probe energy transfer (PriProET) principle. This technology was chosen since PriProET is very robust to tolerate the nucleotide substitutions in the target area. Therefore, this technology provides a very broad-range system, which is able to detect simultaneously many variants of the virus(es) even if the target genomic regions show large scale of variations. The detection specificity of the new assay was proven by positive amplification from a set of nine different FCoV strains and negative from the tested non-coronaviral targets. Examination of faecal samples of healthy young cats, organ samples of perished animals, which suffered from feline infectious peritonitis, and cat leukocytes from uncertain clinical cases were also subjected to the assay. The sensitivity of the P-sg-QPCR method was high, since as few as 10 genome copies of FCoV were detected. The quantitative sg-mRNA detection method revealed more than 10–50,000 times increase of the M gene sg-mRNA in organ materials of feline infectious peritonitis cases, compared to those of the enteric FCoV variants present in the faeces of normal, healthy cats. These results indicate the applicability of the new P-sg-QPCR test as a powerful novel tool for the better detection and quantitation of FCoV and for the improved diagnosis of feline infectious peritonitis, this important disease of the *Felidae*, causing serious losses in the cat populations at a global scale.

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1. Introduction

Feline infectious peritonitis is currently the leading cause of infectious death among young domestic and wild *Felidae*. The disease has a worldwide distribution, especially in cats originated from purebred catteries (Pedersen, 1987; Foley et al., 1997). The causative agent of feline infectious peritonitis, feline coronavirus (FCoV), belongs to the *Coronaviridae* family within the order *Nidovirales* (Almeida and Tyrell, 1967; Siddell et al., 1983). FCoV, together with human, porcine, and canine coronaviruses is a member of alpha coronavirus genus (de Groot et al., 2008).

Abbreviations: C_T, threshold cycle; FAM, 6-carboxyfluorescein; FCoV, feline coronavirus; eFCoV, feline enteric coronavirus; FRET, fluorescence resonance energy transfer (or) Förster resonance energy transfer; PCR, polymerase chain reaction; PriProET, primer-probe energy transfer; QPCR, quantitative real-time PCR; RNA, ribonucleic acid; sg-mRNA, sub-genomic messenger RNA; RT-PCR, reverse transcriptase polymerase chain reaction; TxR, Texas Red (sulphorhodamine 101 acid chloride); PP-sg-QPCR, PriProET sg-mRNA QPCR.

* Corresponding author. Tel.: +36 1 478 4181; fax: +36 1 478 4284.

E-mail address: balka.gyula@aotk.szie.hu (G. Balka).

Regarding pathogenicity, FCoV comprises two biotypes: the *feline infectious peritonitis viruses* (FIPV) and the *feline enteric coronaviruses* (eFCoV). The existence of at least two serotypes of FCoV (types I and II) has been shown based on in vitro neutralisation assays using monoclonal antibodies. Each serotype includes viruses of both the FIPV and eFCoV biotypes (Hohdatsu et al., 1991a,b). Type I and II viruses differ mainly in their in vitro growth characteristics, as the members of the serotype I can rarely if ever be propagated in cell-cultures. In the field, the prevalence of FCoV type I appear to be higher, as approximately 70–85% of feline infectious peritonitis cases are due to infection with type I viruses (Hohdatsu et al., 1992; Herrewegh et al., 1998; Addie et al., 2003; Kummrow et al., 2005).

Biotype eFCoV is not a significant cause of morbidity in cats. It may produce mild enteritis, but most experimental or natural infections remain subclinical. It has been observed that the incidence of feline infectious peritonitis in a cat population is strongly correlated with the eFCoV → FIPV mutation rate (Vennema et al., 1998). FIPV causes a fulminant and fatal disease of cats with manifestations of anorexia, chronic fever, and malaise. In addition, ocular and neurological disorders can occasionally occur.

There is a vital need to develop reliable and robust laboratory diagnostic method for the detection and identification of FIPV biotype in all doubtful cases, considering the profoundly different biology and clinical consequences caused by the two biotypes of FCoV.

Initially, the determination of antibody titre by indirect immune fluorescence method (Burleson et al., 1992) served for diagnosis followed by the detection of viral RNA from blood (Kennedy et al., 1998). The reliability of these diagnostic approaches were questioned due to the inconsequent correlation of the positive results and the disease outcome. Simons et al. (2005) published a more sophisticated method, targeting subgenomic mRNA (sg-mRNA) production of the virus, detected by conventional RT-PCR. This method was supposed to detect only the intensively replicating viral biotype, since viral sg-mRNAs are served to express the viral structural protein genes. However, the suitability of this test to detect FIPV was questioned by Can-Sahna et al. (2007) on the basis of the high ratio of positive results obtained from the samples of clinically healthy cats. These observations indicate that an assay capable of FCoV sg-mRNA quantitation in the different organs and body fluids would be of very high importance, considering the fact that low-level virus replication occurs even in the case of eFCoV infection (Herrewegh et al., 1997), and hopefully the exact determination of the viral replication rate by measuring the viral sg-mRNA level would be decisive for the establishment of a correct diagnosis (de Groot-Mijnes et al., 2005).

Considering this important diagnostic need, a real-time sg-mRNA detection and measuring system, termed P-sg-QPCR was developed, as a novel tool identification and quantitation of FCoV in order to obtain an improved system for the reliable diagnosis of feline infectious peritonitis. The novel assay is based on the FRET principle (Förster resonance energy transfer; Förster, 1948) and uses the primer-probe energy transfer (PriProET) technology. This robust real-time PCR technology was previously successfully applied to detect a wide variety of viruses comprising vesicular disease viruses, hepatitis E virus, classical swine virus porcine reproductive and respiratory syndrome virus and porcine circovirus (Rasmussen et al., 2005; Hakhverdyan et al., 2006; Bálint et al., 2009). Compared to the most wide-spread real-time PCR system, the TaqMan method, PriProET is providing a higher flexibility in the detection of varying target nucleic acids. PriProET is able to overcome multiple mutations located in the targeted region of the viral genome and to amplify a wide range of nucleic acids despite of the variations in the targeted regions (Rasmussen et al., 2003). This robust feature made the PriProET technique

suitable for the detection of the FCoV, a highly variable RNA virus.

In this study, the robustness and flexibility of PriProET was combined, providing a wide range detection of the virus variants, with the capability to quantitate subgenomic mRNA, allowing the quantitative assessment of virus replication, thus the estimation of the development of feline infectious peritonitis. The quantitative real-time PCR (P-sg-QPCR) enabled us to detect and differentiate a wide range of closely related coronaviruses such as FCoV, canine coronavirus (CCoV) and transmissible gastroenteritis virus (TGEV) and to estimate the tendency of virus replication in various organs, by a single method. Thus, the P-sg-QPCR assay was found as a powerful novel tool for the improved diagnosis of feline infectious peritonitis.

2. Materials and methods

2.1. Viruses

A collection of 24 coronaviruses of human, feline, canine, porcine, bovine, murine and avian origin, summarised in Table 1, was included in the specificity, sensitivity and reproducibility tests of the P-sg-QPCR assay.

2.2. Clinical and pathological samples

Faecal samples from a total of 104 healthy domesticated cats were collected in Sweden in 2006. Small intestine, mesenteric lymph node, spleen, liver, lungs and kidney samples of nine diarrhoeic cats, previously diagnosed as FCoV infected were also included in the tests. Furthermore, a total of 146 organ samples, seven body fluids, and four faecal samples obtained from 27 Swedish and five Hungarian cats perished with feline infectious peritonitis.

A collection of 44 separated leukocyte samples obtained from clinically possible feline infectious peritonitis cases, collected in Hungary between 2007 and 2008, and was also subjected to molecular examination.

Diagnoses were achieved on the results of pathological, histopathological examinations and positive findings of immunohistology and gel based conventional RT-PCR for feline infectious peritonitis (Kiss et al., 2000). The genomic RNA from all the above-mentioned samples was tested by the SYBR Green RT-QPCR described by Escutenaire et al. (2007), while the sg-mRNA levels were examined by the P-sg-QPCR assay.

Approximately 1 g pieces of the faecal samples and organs were homogenised in sterile ceramic mortars using sterile quartz sand. They were diluted in 9 ml sterile phosphate-buffered saline (PBS), and then were centrifuged at 5000 × g for 20 min to remove cell debris and bacterial contamination. The supernatant was collected and used for virus isolation and PCR. After homogenisation, samples were stored at −80 °C. Feline leukocytes were purified from whole blood on Histopaque-1077 (Sigma-Aldrich, Saint Louis, MO, USA) according to the manufacturer's recommendations. The cells were resuspended in PBS to the original volume of the whole blood they originated from.

2.3. Virus propagation and titration in cell culture

One part of the SYBR Green positive specimens, including 28 feline faecal samples collected from healthy cats and 34 organ samples of five recently perished cats diagnosed with feline infectious peritonitis in Sweden were subjected to standard virus isolation procedure in 24-well tissue culture plates (Greiner Bio-One GmbH, Frickenhausen, Germany), incubated for 48 h at 37 °C in a 5% CO₂ atmosphere. Following two blind-passages, cells were scraped off

Table 1

Collection of 24 human and animal coronaviruses included in the specificity, sensitivity and reproducibility tests of sg-QPCR.

CoV genus	Strain	Reference	Source
Alphacoronavirus			
FCoV type I	FIPV UCD-1	Pedersen and Floyd (1985)	I. Kiss, P. Rottier
FCoV type I	FIPV UCD-2	Pedersen and Floyd (1985)	I. Kiss
FCoV type I	FIPV UCD-3	Herrewegh et al. (1995)	I. Kiss
FCoV type I	FIPV UCD-4	Herrewegh et al. (1995)	I. Kiss
FCoV type I	FIPV Black	Black (1980)	I. Kiss
FCoV type I	Nor15	Herrewegh et al. (1995)	I. Kiss
FCoV type II	FCoV 79-1683	McKeirnan et al. (1981)	I. Kiss
FCoV type II	FCoV 79-1146	McKeirnan et al. (1981)	I. Kiss
FCoV type II	FIPV DF-2	Evermann et al. (1981)	I. Kiss
CcCoV type I	CB/05	Decaro et al. (2007)	C. Buonavoglia
CcCoV type II	144/01	Escutenaire et al. (2007)	S. Escutenaire
TGEV	Purdue	Bohl et al. (1972)	I. Kiss
PRCoV	PRCV	Pensaert et al. (1986)	I. Kiss
PEDV	PEDV 7	Pensaert and Debouck (1978)	I. Kiss
HCoV-229E	229E	Hamre and Procknow (1966)	ATCC
HCoV-NL63	NL63	van der Hoek et al. (2004)	L. van der Hoek
Betacoronavirus			
HCoV-OC43	OC43	McIntosh et al. (1967)	ATCC
MHV	MHV-A59	Manaker et al. (1961)	P. Rottier
BCoV	BCQ-4	Milane et al. (1997)	Kheyar
SARS-CoV	SARSFFM-ic	Drosten et al. (2003)	M. Niedrig
Gammacoronavirus			
IBV	Beaudette	Casais et al. (2001)	I. Kiss
IBV	7/91	Adzhar et al. (1997)	D. Cavanagh
IBV	Arkansas 99	Fields (1973)	D. Cavanagh
PhCoV	PhUK/438/94	Cavanagh et al. (1994)	D. Cavanagh

in 500 µl supernatant, and examined by the genomic and subgenomic PCR assays described above to detect virus replication.

Strain FIPV DF-2 was grown in *Felis catus* whole foetus cells (FCWF-4) in Minimal Essential Medium (EMEM) (Sigma–Aldrich) containing 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, supplemented with 10% foetal calf serum (FCS). Three-day-old cell cultures were split into 1:5 ratio; the remaining cells were adjusted to a concentration of 2×10^5 cells/ml and plated into 12-well plates (Greiner), incubated overnight at 37 °C in a 5% CO₂ atmosphere. On the next day cells were infected with serial ten-fold dilutions of FIPV DF-2, in four wells in parallel, incubated for 1 h at 37 °C allowing the virus to adsorb to the cell receptors and then the inoculates were replaced with EMEM containing 2% FCS. Virus titre was determined on the basis of cytopathic effect (CPE) observed on the third day post inoculation by Reed–Muench method (Reed and Muench, 1938). The titration was repeated three times under the same conditions.

2.4. RNA extraction

Viral RNA was isolated from cat and dog faecal samples, homogenised organ samples of perished cats and feline leukocytes with QIAamp Viral Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. 100 µl of the 10% (w/v) suspension of organ and faeces homogenates, as well as the leukocyte suspension were diluted with 40 µl PBS before RNA extraction. After extraction, the volume of eluted RNA was complemented to 100 µl. The concentration of the extracted samples' total RNA-s was between 100 and 200 ng/µl.

2.5. Primer and probe design

The highly conserved membrane (M) gene regions of 25 feline, canine and one porcine coronaviruses were aligned with CLUSTAL X 1.81 program in order to design the reverse primer and probe capable of detecting both serotypes of FIPV.

The reverse primer was modified with a donor fluorophore (6-carboxyfluorescein, FAM) at the 5' end. The subgenomic forward

primer was designed to bind to the leader sequence of the coronaviral sequences available in the GenBank. The probe was designed to bind upstream of the reverse primer without any nucleotide gap, and was labelled with a reporter fluorophore (Texas Red, TxR) on its 3' end, allowing the real-time detection of the 223 bps long PCR product. GC content, stability, primer-dimer and hairpin formation were analysed by the Oligo Analyzer 2.5 (<http://www.idtdna.com>). Primers and probe were synthesised and HPLC purified by Thermo Electron GmbH (<http://www.thermohyaid.de>). Sequences of the primers and probe are summarised in Table 2.

2.6. P-sg-QPCR

For performing the P-sg-QPCR assay the TITANIUM™ One-Step RT-PCR Kit (Clontech Laboratories, Palo Alto, USA) was applied. The amplification reaction parameters (primer and magnesium concentration, annealing temperature and period, number of cycles, melting temperatures of the reference viruses) were optimised by titration of the different variables in order to achieve low cycle threshold (C_T) values and high fluorescence signal. After optimisation, the reaction mix was as follows: 1× One-Step Buffer, 0.2 mM dNTPs, 100 nM forward primer, 500 nM reverse primer labelled with FAM, 500 nM probe labelled with TxR, 20 U of Recombinant RNase Inhibitor, 0.4 rxn 50× Titanium™ Taq RT Enzyme mix and 2 µl template RNA in a total reaction volume of 20 µl. The ThermoStabilizing Reagent, the GC-Melt™, and the Oligo (dT) primer were not included in the reaction mix to gain more intensive fluorescence signal. All reactions were run in the Corbett Research Rotor-Gene Real Time Amplification system (RG-3000, Corbett Research, Mortlake, NSW, Australia). The thermal profile initiated with reverse transcription at 50 °C for 1 h, followed by initial denaturation at 95 °C for 1 min, and 55 cycles of [94 °C for 15 s, 55 °C for 30 s, 75 °C for 20 s].

Fluorescence signal data were collected for 5 s after the primer annealing, where the wavelength of the source was 470–510 nm and the detector was 610 nm. After cycling, melting point analysis was used to confirm the specificity of amplification at 40–90 °C with 10 s holds at each elevation step of 1 °C.

Table 2
The genome of the 79-1146 FIP strain (GenBank access. no. AY994066) is used as a reference. The T in italic letter is an extra T (spacer arm). Leader = Leader sequence, M gene = Membrane protein encoding gene.

	Sequence 5'–3'	Location on the genome	Position on the genome
Primers of sg-QPCR ^a			
L-66 (sense)	AGATTTGCTCTCGGACACCAACTCGAACT	Leader	66
M-26399 (antisense)	5'-FAM-TCAATATTACAGACCAGCTGAAG	M gene	26,399
Probe (sense)	TGGTGATCTTATTGGCATCTTGCTAACTGGAT-TxR-3'	M gene	26,344
Primers of SYBR Green QPCR ^b			
11-FW (sense)	TGATGATGSNGTTGTNTGYTAYAA	ORF1b	15,647
13-RV (antisense)	GCATWGTGRTGYTGNGARCARAATTC	ORF1b	15,825

The nine reference feline coronavirus strains, the three CCoV strains; 23/03, CB/05, 144/01 and porcine TGE Purdue strain were involved in the specificity tests. The melting point analysis reveals grouping of the viruses into four different genotypes in this very conserved region of the M gene. The four colours denote the four melting points of the genotypes.

^a Positions refer to the genome of 79-1146 FIPV strain (AY994066).

^b According to Escutenaire et al. (2007). Positions refer to the genome of SARS Tor2 (AY274119).

2.7. Determination of sensitivity

The sensitivity of the system was determined by using known amounts of recombinant RNA prepared from FIPV DF-2 as follows: conventional RT-PCR using Qiagen One-Step RT-PCR Kit (Qiagen) was carried out with the unlabelled version of the PriProET primers. A specific T7 promoter sequence was added to the 5' end of the forward primer. The PCR product was gel purified using the QiaQuick Gel Extraction kit (Qiagen, Hilden, Germany). The purified DNA samples were in vitro transcribed using MEGAscript[®] T7 Kit (Ambion, Austin, TX, USA). The RNA concentration was determined with Nanodrop ND 1000 (Nanodrop, Wilmington, DE, USA). The standard curve was generated from a serial ten-fold dilution (10^0 – 10^{10} copy numbers) of the recombinant RNA in RNase free water (Ambion, Huntingdon, UK).

Furthermore, in order to assess the detection limit of subgenomic mRNAs, sensitivity was evaluated from a serial ten-fold dilution from 10^6 to 10^0 of FIPV DF-2 grown in FCWF cell culture. Inoculation of the cells was followed immediately by RNA extraction of the different virus dilutions. Virus titre based on CPE in FCWF was compared to the characteristic parameters of the assay: threshold cycle (C_T), fluorescents signal, and melting point.

2.8. Determination of specificity

The specificity of the assay targeting the M gene subgenomic mRNA was verified by gel electrophoresis, sequencing the amplicons and by melting point analysis. Subsequently, the capability of the PCR assay to detect various FCoV strains was tested. Nine reference feline coronavirus strains representing both serotypes and biotypes were subjected to the specificity tests. On the other hand, non-feline coronaviruses were tested to exclude the cross-reactivity of the assay, including human, canine, porcine, avian, bovine and murine coronavirus strains, representing all the three genus of *Coronavirinae* (Table 1).

2.9. Efficiency and reproducibility of the assay

The efficiency of the assay was calculated using the equation of $E = 10^{(-1/M)} - 1$, where “E” is the amplification efficiency and “M” is the slope of the standard curve. All tests for sensitivity and specificity were performed in triplicate in order to assess the intra-assay variability. Inter-assay variability was calculated in two different ways: (1) comparison of the obtained C_T values of the standard samples included in each experiment; (2) examination the C_T values of the same set of RNA samples in three distinct runs.

3. Results

3.1. Virus propagation and titration

No signs of virus induced CPE were found in the case of the 28 eFCoV positive faecal samples and 34 feline infectious peritonitis positive organ samples after the third passage. Neither the SYBR Green RT-QPCR nor the P-sg-QPCR assay could display fluorescent signals and characteristic melting points during the examination of cell culture supernatants for the detection of their genomic and subgenomic RNAs.

The mean titre of FIPV DF-2 strain reached $10^{6.2}$ TCID₅₀/100 μ l (/ml) based on the typical CPE, such as cell fusion and rounding of cells at the edges of the cythopathic foci.

3.2. Determination of sensitivity

The sensitivity of P-sg-QPCR was determined by generation of standard curves from a serial ten-fold dilution of both the recombinant RNA and FIPV DF-2 grown in FCWF cell culture. The detection of the different dilutions of the recombinant RNA was linear in the range of 7.4×10^6 up to 7.4×10^0 copies per reaction (Fig. 1). This result indicated a sensitivity of 1–10 viral genome copies per PCR assay. The detection limit of the P-sg-QPCR test for subgenomic mRNA detection from the FCWF cell cultured DF-2 virus strain was 1 TCID₅₀/100 μ l.

3.3. Determination of specificity

The specificity of amplification was confirmed by gel electrophoresis of the P-sg-QPCR amplicons, followed by sequencing (data not shown). The P-sg-QPCR was able to detect all nine reference strains of FCoV as well as TGEV and CCoV. The melting point analysis revealed four different genetic variants in this very conserved region of the M gene with the following average temperature values: (1) 69.3 °C (UCD2, UCD3, UCD4, Black, 79-1146, DF-2); (2) 67.5 °C (UCD1); (3) 66.5 °C (NOR15, 79-1683, CCoV CB/05 and 144/01); (4) 63.6 (TGEV Purdue) (Table 3; Fig. 2).

3.4. Efficiency and reproducibility of the assay

The efficiency of the assay was defined from the standard curve that displayed a linear inversely proportional relationship between the logarithmic amount of copy concentration and the C_T of the original samples. Reaction efficiency was found to be 1 with an R^2 value of 0.988 (Fig. 1). The intra assay variability was assessed using different amounts of RNAs of the nine feline CoV strains. These samples were used in three replicates in the same sg-QPCR run and the intra-assay variability was obtained from two statistical

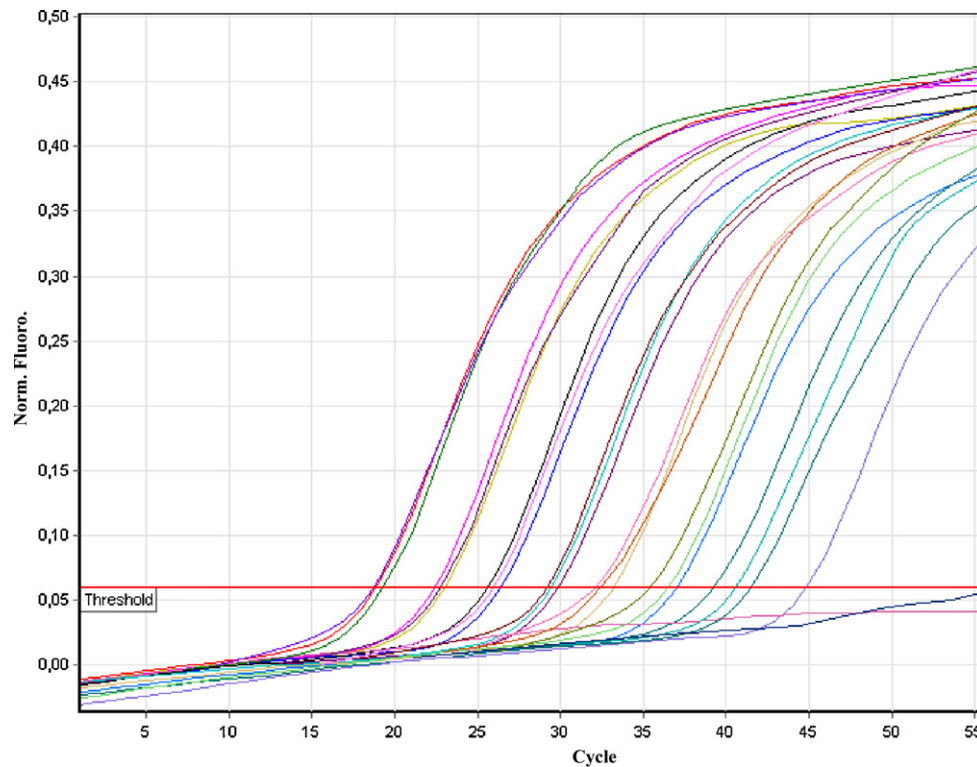


Fig. 1. Amplification plot of the P-sg-QPCR. C_T values of serial triplicate dilutions of the RNA standard ranging from 7.4×10^7 – 10^0 copies/ $2 \mu\text{l}$ samples are represented from left to right.

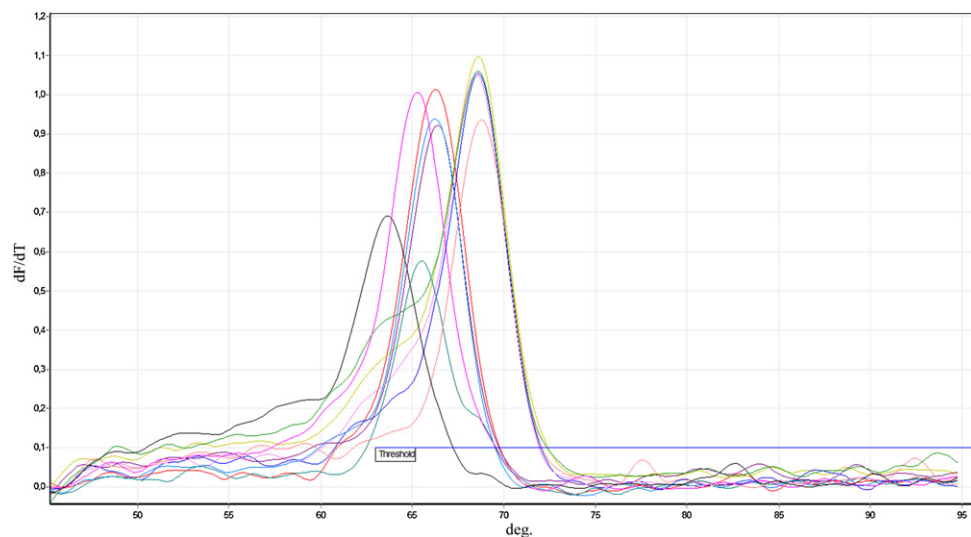


Fig. 2. Melting points of the nine reference coronavirus strains obtained with the P-sg-QPCR. The representatives of the four genotypes can be seen like four groups of melting point peaks as presented in Table 3.

parameters: standard deviation was 0.44 and the standard error of the mean (SEM) was 0.084. The inter assay variability was determined by the same RNA set testing it three times and the standard deviation was obtained as 0.56, the standard error of the mean (SEM) was 0.108 (data not shown).

3.5. Examination of faecal samples

The 104 feline faecal samples from Sweden were analysed for the presence of FIPV-specific nucleic acid by both SYBR-Green QPCR and by the new P-sg-QPCR method, in parallel. Both assays revealed the presence of FCoV in several samples, but to a different extent.

The SYBR-Green method detected 63 positives (60.6%), while the P-sg-QPCR assay revealed only 36 (34.6%). The C_T values of the PriProET positive samples varied between 35.58 and 44.92 (mean: 40.36; SD: 2.61) reflected markedly different sg-mRNA copy number in the intestines of the cats: 1×10^0 – 5×10^2 copies/ $100 \mu\text{l}$ 10% (w/v) faeces suspension.

All the nine diarrhoeic FCoV shedder cats were SYBR-Green positive, but only five of them proved to be P-sg-QPCR. The C_T value of the FCoV positive samples varied between 43.18 and 44.91 (mean: 44.10; SD: 0.74), which is equivalent with approximate 1×10^0 – 1×10^1 copies/ $100 \mu\text{l}$ 10% (w/v) faeces suspension (Table 5).

Table 3

The C_T values and the melting points of the nine reference coronavirus strains obtained with the P-sg-mRNA QPCR. The representatives of the four genotypes can be seen in the alignment with violet, yellow, green and blue backgrounds.

No.	Name	Type	C_T	Peak
1	UCD1_subgen_cDNA	FCoV I	25.20	67.5
2	UCD2_subgen_cDNA	FCoV I	38.36	69.1
3	UCD3_subgen_cDNA	FCoV I	46.89	69.5
4	UCD4_subgen_cDNA	FCoV I	45.62	69.4
5	Black_subgen_cDNA	FCoV I	40.34	69
6	NOR15_subgen_cDNA	FCoV I	39.08	66.9
7	79-1683_subgen_cDNA	FCoV II	40.98	66.3
8	79-1146_subgen_cDNA	FCoV II	43.83	69.5
9	DF-2_subgen_cDNA	FCoV II	25.72	69.2
10	TGEV_subgen_cDNA	PCoV	24.42	63.6
11	CanineCoV_U1/04_cDNA	CCoV	43.66	66.3
12	CanineCoV_U1/04_cDNA	CCoV	43.66	66.3
13	NTC	NTC		48.5

The melting point analysis resulted in 4 different groups of the amplicons: (1) 69.3 °C (eight cases, 22.2%); (2) 68.3 °C (16 cases, 44.4%); (3) 66.1 °C (11 cases, 30.6%); (4) 63.1 °C (one case, 2.8%).

3.6. Examination of organ samples of eFCoV positive and cats with feline infectious peritonitis

None of spleen, liver, lungs and kidney samples of the nine FCoV positive cats showed positive results when tested with the two real-time PCR methods.

The 146 feline organ samples representing cats succumbed to feline infectious peritonitis in Sweden and Hungary were analysed by SYBR-Green QPCR, while only 66 organ samples by the P-sg-QPCR.

A total of 58 (87.9%) of 66 samples analysed by both methods were positive by SYBR-Green QPCR and 47 (71.2%) by the P-sg-QPCR. The P-sg-QPCR C_T values ranged between 23.93 and 44.91 (mean: 36.28; SD: 4.30), which is equivalent with 1×10^0 – 6×10^5 copies/100 µl 10% (w/v) tissue suspension.

The detailed analysis of the feline infectious peritonitis positive cat samples by SYBR-Green QPCR method revealed that the following organs harbour FCoV most frequently: lungs 6/6 (100%), liver 6/6 (100%), kidney 10/11 (90.9%), mesenteric lymph node 18/20 (90%), spleen 16/20 (80%) and gut 15/10 (66.7%).

Based on the results obtained from the newly developed P-sg-QPCR, the list of organs most frequently harbouring sg-mRNA in quantitative order is the following: mesenteric lymph node 14/14 (100%), spleen 7/8 (87.5%), lung 6/8 (85.7%), liver 6/8 (75%), bronchiolar lymph node 2/3 (66.6%), gut 10/6 (60.0%), kidney 5/8 (62.5%).

Analysis of body fluids and excreta collected from the carcasses gave the following results: heart blood 2/2 (100%), ascitic fluid 4/5 (80%) and faeces 3/4 (75%). Interestingly, the three tonsils involved in the examinations consequently remained FCoV negative with both two QPCR methods; 0/3 (0%).

The P-sg-QPCR C_T values in the gut, the primary replication site of FCoV reflected significantly lower viral replication: the values ranged between 35.26 and 38.29 (mean: 37.52; SD: 1.32), which is equivalent with 6×10^1 – 4×10^2 copies/100 µl 10% (w/v) faeces suspension (Table 5).

The melting point analysis revealed only 0.5 °C difference regarding the organs of the same carcass, but resulted in four different groups regarding the different animals: 68.1 °C average

temperature (one cat of eight, 12.5%), 66.3 °C (2/8, 25%), 64.6 °C (4/8, 50%) and 63.8 °C (1/8, 12.5%).

The obtained P-sg-QPCR C_T values revealed higher subgenomic M gene mRNA transcription levels in the different organs compared to those of the faecal samples. This phenomenon can be explained by the lower cell density of the faecal samples, or the lower replication rate of the virus in the intestinal epithelial cells. Furthermore, the C_T values of the sg-mRNA positive gut samples demonstrated a much smaller deviation compared with those of the organ samples.

3.7. Examination of leukocytes in cases of possible feline infectious peritonitis

The 44 leukocyte specimens of clinically sick cats displaying fever, lymphocytopenia, hypergammaglobulinaemia, in a few cases enlargement of the abdomen were subjected initially to the SYBR-Green QPCR method revealing 5 positive cases (11.4%). In contrast to the previous findings, the P-sg-QPCR led to the same result. The obtained C_T values (24.5, 25.3, 26.7 and 30.3 respectively) correspond to approximately 1×10^4 – 5×10^5 copies/100 µl leukocyte suspension (Table 5). One sample showed C_T value of 48.0. As the C_T 48 can be excluded from this series due to its abnormal high value, the mean value of 26.7 corresponds to 10^5 copies/100 µl leukocyte suspension of sg-mRNA in the leukocytes of sick cats suffering with feline infectious peritonitis (confirmed earlier by pathological, histopathological, immunohistological and RT-PCR examinations). The melting points of the five amplicons were 68.1 °C, 68.1 °C, 68.2 °C, 68.3 °C and 68.3 °C. The follow-up investigations revealed that all five cats perished with FIP in 40 days after the P-sg-QPCR examinations (data not shown).

4. Discussion

The diagnosis of feline infectious peritonitis, this major viral disease of the *Felidae* is extremely difficult, due to several factors in the complicated infection biology of FCoV. First, no exact genetic marker(s) has been identified yet in the viral genomes that would allow the differentiation of the two FCoV biotypes: FIPV and eFCoV. Second, during the viral replication genomic alterations are generated continuously by this virus, leading to the rapid appearance of a variety of viruses in the body of the host. Since reliable means for the molecular determination of the differences between the avirulent and virulent variants of FCoV are not available, the diagnosis of feline infectious peritonitis should address other biological characteristics of the virus ways today.

In the present work, two peculiar features of the infection biology of feline coronaviruses were utilised. On the one hand, the enhanced capability of the FIPV biotype to replicate intensively in various organs and tissues of the host animal was exploited (Stoddart and Scott, 1988; Kipar et al., 2005; Rottier et al., 2005). On the other hand, the ability of *Nidovirales* to initiate sg-mRNA synthesis during replication phase was addressed. In the past few years, by the use of sg-mRNA PCR techniques (Gillim-Ross et al., 2004; Simmons et al., 2005), replicating CoVs have been detected successfully. For this reason, to facilitate correct feline infectious peritonitis diagnosis and prognosis, the present study aimed at developing a P-based real-time PCR assay to detect the virus and to determine the level of virus replication, in a single diagnostic platform.

The flexibility of the P-sg-QPCR technique is indicated by the fact that CoV strains even with three mismatches in their genomes at the primer and probe binding sites could simultaneously be detected by this novel method (Table 4), without decrease of the fluorescent signal. Therefore, the P-sg-QPCR method may be regarded as a real-time amplification system with unique capability for the identification of all previously published FCoV M gene sg-mRNAs.

Table 4

Alignment of feline, canine and porcine CoV strains; viruses even with 3 substitutions could be demonstrated by the sg-mRNA detecting QPCR system. Sense, unlabeled primer region with grey, M probe (labelled with TxR) region with violet, M antisense primer (labelled with 6-FAM) region with green background are depicted. UCD-4 sequence data on the M gene has not been available in the GenBank yet. The representatives of the three other genotypes can be seen in the alignment with yellow, green and blue backgrounds (see Table 3).

Leader sequence			
AY994055	50	TAAC TAGCCTTGTGCTAGATTT-GTCTTCGGACACCAACTCGAACTAAAC	
DF2	50-.....	
79_1146FIP(D	50-.....	
WSU_79_1146	50-.....	
DQ848678C1Je	50-.....	
NC_002306	50T.....	
M gene sequence			degree C
AY994055	79-1146	69,5	TGAACGTGGTGATCTTATTGGCATCTTGCTAACTGGAACTTCAGCTGGTCTGTAATATTGATTG
NC007025	79-1146	69,5
DQ286389	DF2	69,2
EU186072	FCoV_Black	69,0
AY452033	FIPV_vaccine_PRC	
DQ160294	FCoV_NTU2		C.....C.....A.....
DQ848678	FcoV_C1Je		C.....C.....G.....C.....
Y13921.1	79-1683		CA.TG.A.....A.....A.....C.....
AB086904	79-1683	66,3	-----A.....A.....A.....C.....
AB086903	Black	69,0
AB086902	UCD1	67,5C.....
FJ943762	UCD3a	69,5	C.....C.T.....
FJ943762	UCD3b	69,5	C.....C.T.....
FJ943763	UCD4	69,4C.T.....
AY548235	23_03_CCoV		-----A.....A.....A.....
DQ112226	CB/05_CCoV	66,3	C...ACA.....A.....CA
-----	144/01_CCoV	66,0	CA.CG.A.....A.....A.....C.....
AF502583	259/01_CCoV	AG.....A.....CA
NC_002306	TGEV	63,6	CA.CG.A.C.....A.....A.....C.....
AY335549	TGEV_TS		CA.CG.A.C.....A.....A.....A.....
AY587883	TGEV_HN2002		-----A.C.....A.....A.....A.....
AF104420	TGEV_96-1933		CA.TG.A.C.....A.....A.....A.....CA

The assay enabled the detection of nine reference FCoV and three closely related non-feline CoV strains: CB/05, 144/01 strain of canine, and TGEV Purdue strain of porcine origin with the ability of differentiation based on melting curve analysis. The other 14 CoV strains belonging to all three genus of *Coronavirinae* did not give specific fluorescent signals, excluding the cross reactivity of the test.

The sensitivity of the assay was determined in two different ways, and the detection limit proved to be as low as 10 copies in each case. The efficiency of The P-sg-QPCR test was calculated by the establishment of two separate calibration curves: one from serial 10-fold dilution of the recombinant RNA, while the other from the serial 10-fold dilution of FIPV DF-2 virus grown on FCWF cell line. Both standard curves displayed a linear detection of sg-mRNA in the range of 10^0 – 10^7 range with a correlation coefficient (R^2) of 0.99 and efficiency of 100%, and R^2 of 0.99 and efficiency of 95%,

respectively. Difference between the two efficiency values may be attributed to the superior purity of the recombinant RNA standard.

These results revealed a significant difference between rate of positive results on feline faecal samples using the genomic SYBR Green and the P-sg-QPCR assays. eFCoV can be detected in a high proportion of intestinal samples of cats by PCR (Herrewegh et al., 1995; Foley et al., 1997). In contrast, the limited number of P-sg-QPCR positive results may indicate a relatively low percentage of animals with extensive virus replication level in the intestinal tract. Since the P-sg-QPCR assay is demonstrating intensive virus replication, it is tempting to speculate that this test is capable of identifying the persistent virus-shedder cats, which contribute to the maintenance CoV infection in the catteries, and their ratio can reach even 13% (Addie et al., 2003), and this group includes the cats with the risk of the fatal FIPV variant emerging. However, the majority of cats shed eFCoV intermittently for weeks or months, in

Table 5

Frequency of genomic FCoV positive samples by SYBR Green method and frequency of FCoV sg-mRNA positive samples by PriProET method completed by sg-mRNA copy number. The copy number data refer to 100 µl of 10% (w/v) tissue and faeces suspension or 100 µl of leukocyte suspension.

Sample	SybrGreen:	PriProET	sg-mRNA load (copies/100 µl sample)
Faecal samples from healthy cats	63/104 (60.6%)	36/104 (34.6%)	1×10^0 – 5×10^2
Faecal samples from feline enteric coronavirus positive diarrhoeic cats	9/9 (100%)	5/9 (55.6%)	1×10^0 – 1×10^1
Faecal samples from cats succumbed with feline infectious peritonitis	4/4 (100%)	3/4 (75%)	6×10^1 – 4×10^2
Organ samples from feline enteric coronavirus positive diarrhoeic cats	0/36 (0%)	0/36 (0%)	0
Organ samples from cats succumbed with feline infectious peritonitis	58/66 (87.9%)	47/66 (71.2%)	1×10^0 – 6×10^5
Leukocytes (heart blood) from cats succumbed with feline infectious peritonitis	2/2 (100%)	2/2 (100%)	2×10^2 – 5×10^2
Leukocytes from possible feline infectious peritonitiscases	5/44 (11.4%)	5/44 (11.4%)	1×10^4 – 5×10^5

some cases at very low level (Addie and Jarrett, 2001; Lutz et al., 2002). Hence, to avoid false negative diagnosis, negative results of the P-sg-QPCR method should be interpreted with caution, and in these cases this assay is a method of choice complementing the widely used genomic real-time QPCR assays in the determination of FCoV status. Another explanation for the surprisingly low copy number of the FCoV sg-mRNA in the gut may be explained with the balanced immune response of the host animals that keeps FCoV replication at low level, and as a consequence, the subgenomic RNA levels may reach much lower rates than their genomic counterparts. Whichever explanation is true, evaluation of quantitative PCR results has to take into consideration the inhibitory compounds of the faecal samples (Dye et al., 2008).

The different melting point values of the faecal samples indicating variable number of nucleotide substitutions at the probe-binding site (Rasmussen et al., 2003; Hakhverdyan et al., 2006) revealed at least four different FCoV virus genetic variants in the targeted region of the M gene. Comparing these melting points with those of the reference viruses it can be concluded that the most frequent virus types in the Swedish collection may be the UCD1-like, the 79-1683-like FCoV, the NOR15 and 144/01 CCoV strains. Although recombination cannot be excluded in some of this FCoVs the majority of the viruses detected must correspond with these strains, because the M gene is the most conserved structural protein gene.

The spleen, liver, lungs and kidney samples of the nine FCoV positive cats were all negative with the applied two different real-time PCR methods, reflecting an FCoV negative status of these organs.

Analysis of the organ samples obtained from feline infectious peritonitis cases revealed a high copy number of FCoV, which is not surprising, since several earlier publications reported enhanced virus replication in the cats diseased with feline infectious peritonitis. The negative results in the case of a few samples are supposed to be a consequence of the different distribution of FIPV in various organs. The most frequently affected organs are the liver, the lungs, the kidneys and mesenteric lymph nodes with 90–100% positive result rate. This is a reflection of their dense vascularisation, rendering them particularly susceptible to the virus that is transported by the infected monocytes and macrophages. A slight difference was observed in the same organs regarding their sg-mRNA contents, where the order of frequency was: mesenteric lymph nodes, spleen, lungs and liver ranging from 100% to 75% positive result. Concluded by their enhanced sg-mRNA content these are the main susceptible organs, which the FCoV possessing its altered genome can first colonise and then intensively multiply.

The high rate sg-mRNA positive results observed in the body fluids demonstrate the value of the clinical and pathological samples allowing readily diagnosing feline infectious peritonitis. The P-sg-QPCR C_T values revealed an average of 100 times more coronaviral sg-mRNA in the feline infectious peritonitis positive samples than in the faecal samples of the clinically healthy FCoV young shedder animals, in some organs (liver, spleen) even 1000–10,000 times more, proving the very high virus multiplication rate in the diseased cats. Interestingly, the basic organ of FCoV replication the gut, displays virus depletion in all examined feline infectious peritonitis cases, the maximal virus replication occurred at a remarkably low level, never even exceeding 1000 copy numbers. These data suggest that the mutated FCoV variants are prone to abandon this organ after acquiring successful access to other organs via monocytes and macrophages. The interesting phenomenon that the same melting point detected in the different organs within the body may be explained by the absence of concomitant CoV infection.

Compiling these findings, it can be hypothesised that once an FCoV variant had colonised the enteric system of a cat, the

virus could either be eliminated, or kept under control by the specific immune response of the host animal, or the continuous/intermittent high level virus replication in the worst case lead to the generation of the highly virulent fatal FIPV variant by mutation.

The different melting points of the FIPV amplicons obtained from different carcasses suggest the same phenomenon that was observed in the case of the FCoV faecal sample collection, i.e. at least four different FIPV variants existed among cats that have suffered from and succumbed to FIP. The 27 Swedish FIPV cases revealed a melting point between NOR15, 144/01, 79-1683 and the TGEV Purdue strains, and may represent a still non-characterised FIPV variant regarding the targeted stretch of the M gene. On the contrary, the five Hungarian FIPVs can be classified into four different groups based on their melting point values: (i) resembling to the UCD1 (20%); (ii) two comparable to the NOR15, 144/01 and 79-1683 (40%); (iii) similar to the Swedish strains (20%); and (iv) having a melting point similar to the TGEV (20%). This difference is in accordance with the previously described existence of different genetic patterns of FCoV isolates in geographically diverse areas.

The P-sg-QPCR analysis of the leukocytes revealed a relatively low prevalence of feline infectious peritonitis among the uncertain cases, underlines the limited capability of the exclusive use of clinical and conventional laboratory examinations for the establishment of a correct diagnosis. The consequently high FCoV copy numbers of the leukocyte samples demonstrate (with one exception) the feasibility of this diagnostic tool to obtain a correct prognosis from the blood samples of live animals. In contrast to the faecal and organ samples, the same melting point was obtained from the leukocytes, which raised several questions that can be answered only by the examination of a sufficient amount of blood samples.

Taken together, these results indicate the applicability of the new P-sg-QPCR test as a novel assay for the better detection and quantitation of FCoV replication in various organs, and/or in the separated monocyte-macrophage fractions of the diseased cats. In combination with genomic quantitative PCR methods, the assay also provides a practical tool for the identification of persistently infected strong shedder individuals in a closed cat populations e.g., in catteries. Furthermore, this method facilitates the early diagnosis of FIP, allowing a prompt treatment of the infected cats. Considering these practical strengths, this assay provides a practical novel tool for the better understanding of the infection biology as well as for improved control of feline infectious peritonitis, this mysterious viral disease of the *Felidae*.

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