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Nucleic acid-based differential diagnostic assays for feline coronavirus

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Feline coronavirus (FCoV) is a pleomorphic, enveloped, positive-sense single-stranded RNA virus. Owing to the differences in its genotype, FCoV belongs to a separate clade along with other viruses, such as transmissible gastroenteritis virus (TGEV) and canine coronavirus (CCoV), which can be isolated from cats. In this study, a PCR assay was developed to differentiate these coronaviruses concurrently. Multiplex differential RT-PCR was performed with primers based on the highly conserved coronavirus membrane protein. Three primer sets were designed: a primer pair (S1 and S2) that can bind to conserved sequences in all target coronaviruses, a CCoV-specific primer (S3), and a TGEV-specific primer (S4). Because of the high sequence homology among FCoV, CCoV, and TGEV, a nucleotide preceding the last pair of dissimilar nucleotides in S3 and S4 was substituted with an inosine to allow primer binding. This assay could detect and differentiate FCoV ($n = 7$), CCoV ($n = 4$), and TGEV ($n = 8$) precisely and did not show any cross-reactivity with other pathogens. These results suggest that this molecular approach provides a rapid and reliable way to detect FCoV, especially in feline clinical specimens.

1. Introduction

Feline coronaviruses (FCoVs; family Coronaviridae) are pleomorphic, enveloped, positive-sense single-stranded RNA viruses (Simons et al., 2005). Viruses in this family have characteristic petal-shaped projections called spike proteins that protrude from the virus particle (Pedersen, 1983), giving it a crown-like appearance, hence the name "coronavirus", when viewed under an electron microscope. These glycoproteins play a key role in attachment of the virus to the surface proteins on the host cell, which act as receptors for the virus (Hartmann, 2005).

FCoV is distributed worldwide in both domestic and wild cats, especially in overcrowded environments (Horzinek and Osterhaus, 1979; Barlough et al., 1983). In cats, the most common route of FCoV infection is oral transmission following contact with virus-containing feces (Pedersen et al., 1995). FCoV are of two subtypes—feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV) (Pedersen et al., 1981). FECV is widespread and often causes asymptomatic or mild enteric infections, while FIPV causes feline infectious peritonitis (FIP), which is a fatal immune-mediated disease (Can-Sahna et al., 2007). Since FIPV arises from mutations in FECV, several reports have suggested that mutation of the avirulent FECV genome alters the cell tropism of the virus, resulting in a virulent variant that induces FIP (Vennema et al., 1992, 1995; Benetka et al., 2004). Recently, it was reported that mutations in the furin cleavage site between the receptor-binding (S1) and fusion (S2) domains of the spike protein could result in altered cell tropism because of the modifications in the spike epitope structure (Licitra et al., 2013).

Since FIP is widespread in cats, a rapid and reliable diagnosis is essential for prescribing a timely and appropriate treatment strategy. Non-specific clinical signs and lack of pathognomonic hematological and biochemical abnormalities hinder definitive diagnosis of FIP in veterinary practices. The tests for detecting the presence of coronavirus antibodies or viral genome, such as enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay (IFA), and polymerase chain reaction (PCR) can suggest a prior exposure to coronavirus or the presence of coronavirus genome (Addie et al., 2003; Hartmann, 2005; Brown et al., 2009). However, CCoV also has a potential to induce enteritis or infectious peritonitis in cats (McArdle et al., 1992; Hartmann, 2005), and TGEV can be carried by asymptomatic cats or cats with mild enteritis (Saif and Sestak, 2006). Thus, a rapid and reliable method that can detect and differentiate FCoV simultaneously from CCoV and TGEV would be very useful for the identification of FCoV-infected cats (i.e., potential spreaders of FIPV) as well as for the isolation of FCoV-infected cats from unaffected cats in order to prevent the spread of the virus in a cattery.

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In this study, a simple and rapid nucleic acid-based test was developed for simultaneous detection and differentiation of FCoV, CCoV, and TGEV. To allow priming to the target sequence in the viral genome, a PCR-based method was developed with a deoxyinosine-containing primer.

2. Materials and methods

2.1. Biological materials

Strains of FIPV (strain 79-1146; ATCC number VR-990) and CCoV (strain 1-71; ATCC number VR-809) purchased from the ATCC (Manassas, VA, USA) were used as reference viruses. Field isolates of FIPV ($n = 3$), FECV ($n = 3$), CCoV ($n = 3$), TGEV ($n = 8$), feline immunodeficiency virus (FIV; $n = 3$), feline leukemia virus (FeLV; $n = 2$), feline parvovirus (FPV; $n = 4$), canine parvovirus (CPV; $n = 2$), *Giardia* spp. ($n = 2$), and *Trichomonas* spp. ($n = 2$) were obtained from the submissions to the College of Veterinary Medicine Teaching Hospital at Chungbuk National University or the Iowa State University Veterinary Diagnostic Laboratory (ISUVDL) or the Veterinary Medical Center (ISUVMC); these isolates were used to assess the analytic specificity of the tests. All field isolates of FIPV were isolated from cats with wet-type FIP, while FECVs were isolated from feces of clinically healthy cats. CCoVs, TGEVs, FIVs, FeLVs, FPVs, CPVs, *Giardia* spp., and *Trichomonas* spp. were isolated from the blood or feces of animals (i.e., dogs, pigs, or cats) presenting with the corresponding disease and positive for each of the mentioned pathogen, as tested by the routine diagnostic procedures such as microscopic examination, ELISA (SNAP® Parvo or Giardia test, IDEXX, Westbrook, ME, USA), and PCR (Pratelli et al., 1999; Kim et al., 2000; Pereira et al., 2000; Gookin et al., 2002; Crawford et al., 2005; Gomes-Keller et al., 2006; Garcia Rde et al., 2011). The identification of all isolates was confirmed by PCR and sequencing analyses.

2.2. Primer design

Membrane protein (M) gene was used to design primers specific for each virus as *M* gene is highly conserved. Sequence alignments of FECV-RM (serotype I; GenBank accession no. FJ943764), FIPV UCD1 (serotype I; GenBank accession no. FJ943771), FECV 79-1683 (serotype II; GenBank accession no. AB086904), FIPV 79-1146 (serotype II; GenBank accession no. DQ010921), CCoV (GenBank accession no. DQ431022), and TGEV (GenBank accession no. FJ755618) were performed by using the CLC SEQUENCE VIEWER, version 4.6.2 (CBS Interactive, San Francisco, CA, USA). Based on nucleotide alignment, four primers were designed, consisting of a common primer pair (S1 and S2, forward and reverse primers) that can bind a common sequence of all the viruses, a CCoV-specific primer (S3, reverse primer), and a TGEV-specific primer (S4, forward primer). While S3 was paired with S1, S4 was paired with S2 and S3 (Fig. 1). Sequences showing minimal possibility of secondary structure with lowest Gibbs free energy (ΔG) were selected as the primer sequences. The sequence specificity of the primer and its priming condition (i.e., ΔG value and hairpin or dimer formation of primers) were verified by BLAST analysis and DNAMAN software (Lynnon, Pointe-Claire, Quebec, Canada) (Table 1).

2.3. Reverse transcriptase polymerase chain reaction (RT-PCR)

Viral RNAs were extracted by using the Viral Gene-Spin™ Viral DNA/RNA Extraction Kit (iNtRON Biotechnology, Sungnam, South Korea), according to the manufacturer's instructions. Viral RNAs were reverse-transcribed into first-strand cDNAs with a random hexamer primer by using the Power cDNA Synthesis Kit (iNtRON).

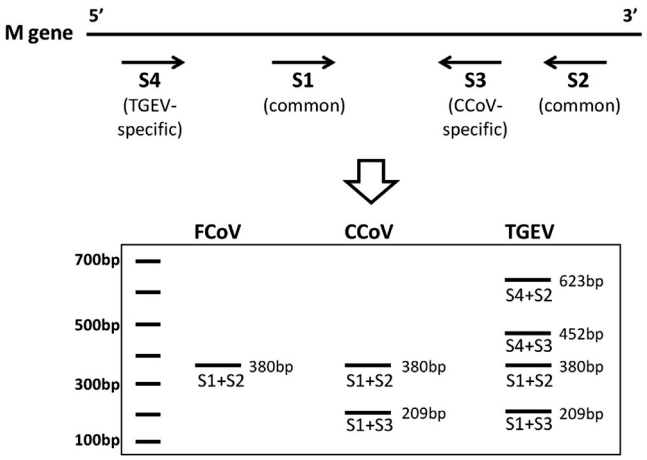

Fig. 1. Diagram of a multiplex PCR assay depicting the position of primer binding and the electrophoretic pattern with S1, S2, S3, and S4 primers. S1 and S4 are forward primers, and S2 and S3 are reverse primers.

Singleplex and multiplex PCRs were performed in the same condition. Both PCR amplifications were performed in a total volume of 50 μL containing 50 mM KCl, 10 mM Tris–HCl (pH 8.3; 25 °C), 1.5 mM MgCl, 200 μM of each dNTP, 10 μM of each primer, 5 units of Taq polymerase (iNtRON), and 2 μL of template. PCR was performed by using the TaKaRa Thermal Cycler Dice (TaKaRa Bio, Otsu, Japan) under the following conditions: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 40 s, and a final extension cycle at 72 °C for 5 min. The PCR products were separated by agarose gel electrophoresis for 40 min at 100 V, followed by staining with ethidium bromide for visualization under ultraviolet light. The expected sizes of the amplicon were 380 bp (common), 209 bp (CCoV-specific), and 452 and 623 bp (TGEV-specific).

2.4. Analytic sensitivity and specificity

To assess analytic sensitivity of the assay (i.e., detection limit), each PCR product (common [S1 and S2] 380 bp, the CCoV-specific [S1 and S3] 209 bp and one TGEV-specific [S4 and S2] 623 bp) was cloned into the pDrive vector (Qiagen Inc., Valencia, CA) according to the manufacturer's instruction. Cloning was confirmed by sequencing. Plasmid DNA was purified by using the QIAprep Spin Miniprep Kit (Qiagen) and quantified by spectrophotometry. Each recombinant vector was diluted in diethylpyrocarbonate (DEPC)-treated distilled, deionized water by a 10-fold serial-dilution technique. The analytic sensitivity of the test was assessed in triplicate as singleplex (i.e., individual PCR for each recombinant vector) or multiplex assay on serially diluted recombinant vectors. Each PCR products were separated on 2% agarose gel, stained with ethidium bromide, and scanned under ultraviolet illumination. The band intensity of the image was converted to the corresponding numerical data by using the SigmaScan Pro, version 6.0.0 (IBM, Chicago, IL, USA) and analyzed by using the SigmaPlot version 12.5 (IBM). The reaction fidelity between dilutions and the overall detection efficacy were compared between the singleplex assay and the multiplex assay.

The analytic specificity of the assay was evaluated in triplicate by examining the presence of cross-reactivity between individual PCR including all but the intended target in each assay. All individual PCRs were run on the additional 6 blood or intestinal pathogens described above (i.e., FIV, FeLV, FPV, CPV, giardia, and trichomonas) in order to determine the specificity of recognition.

Table 1
Information of primers used in this study.

Target sequence	Primer	Sequence (5′–3′)	Tm[a]	Δ*G* (kcal/mol)	Size of amplicon
Common	S1 (forward)	CCAGA TATGT AATGT TCGGC	55.3	0	380
	S2 (reverse)	TTTAC ATAGT AAGCC CATCC	53.2	−0.82	
CCoV-specific	S1 (forward)	CCAGA TATGT AATGT TCGGC	55.3	0	209
	S3 (reverse)	AAAGT GACAC CAGTT GICAC	54.5	−4.84	
TGEV-specific	S4 (forward)	TCATG TCGCA ATAGC ACAIC	54.5	−2.18	623
	S2 (reverse)	TTTAC ATAGT AAGCC CATCC	53.2	−0.82	
	S4 (forward)	TCATG TCGCA ATAGC ACAIC	54.5	−2.18	452
	S3 (reverse)	AAAGT GACAC CAGTT GICAC	54.5	−4.84	

Δ*G*, Gibbs free energy of secondary structure.
[a] Melting temperature.

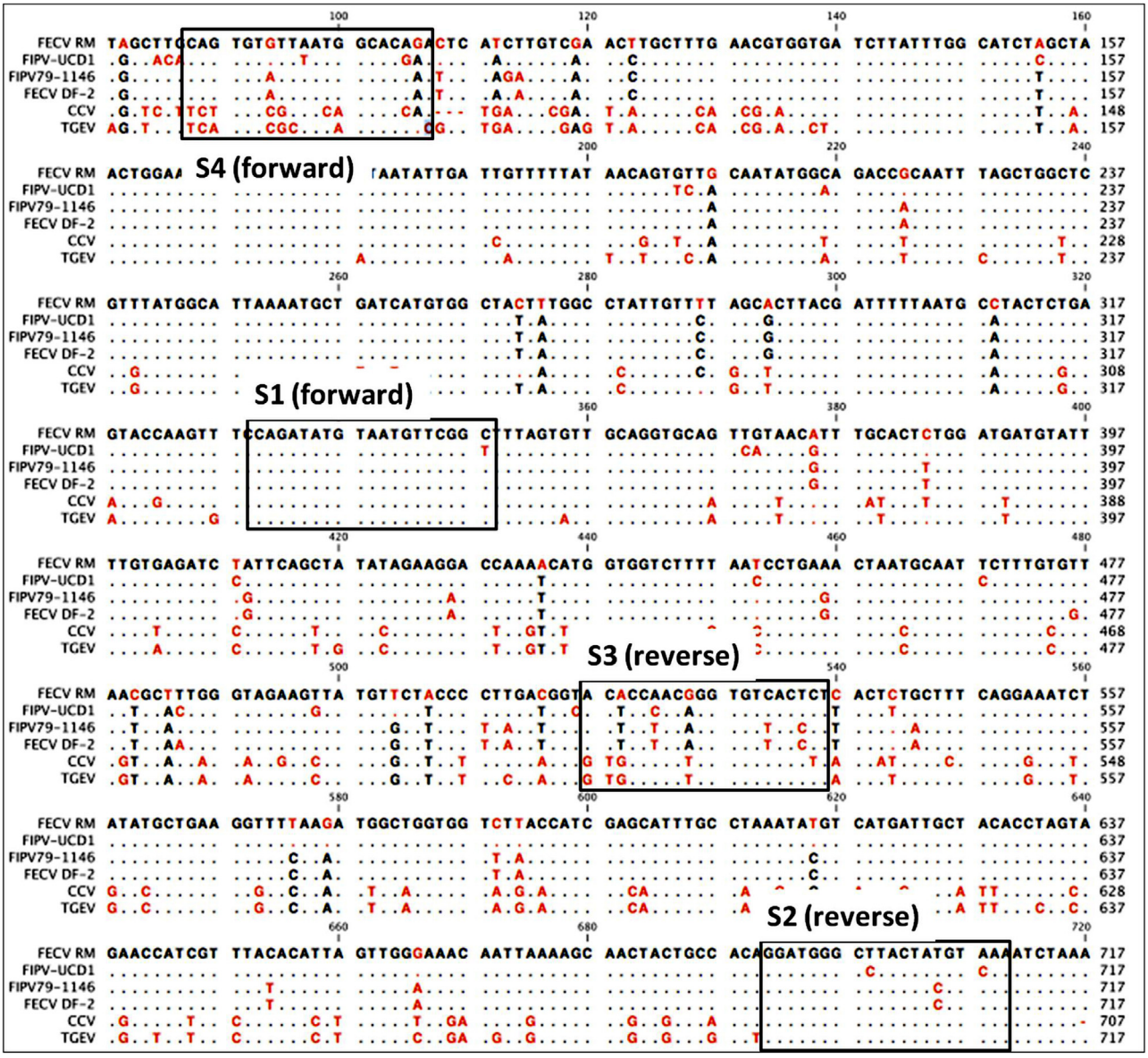

Fig. 2. Alignment of the nucleotide sequences of the *M* gene coding sequences from the FECV RM (GenBank accession no. FJ938051), FIPV UCD1 (GenBank accession no. AB086902), FIPV 79-1146 (GenBank accession no. DQ010921), FECV 79-1683 (GenBank accession no. JN634064), CCoV (GenBank accession no. DQ431022), and TGEV (GenBank accession no. FJ755618). The positions corresponding to the primers S1, S2, S3, and S4 are indicated. Dots indicate nucleotides with complete identical homology to the FECV RM sequence. S1 and S2 are common primers that can bind to all target coronaviruses. S3 is a reverse primer that can amplify CCoV-specific sequence with S1. S4 is a forward primer that can amplify TGEV-specific sequence with S2 or S3.

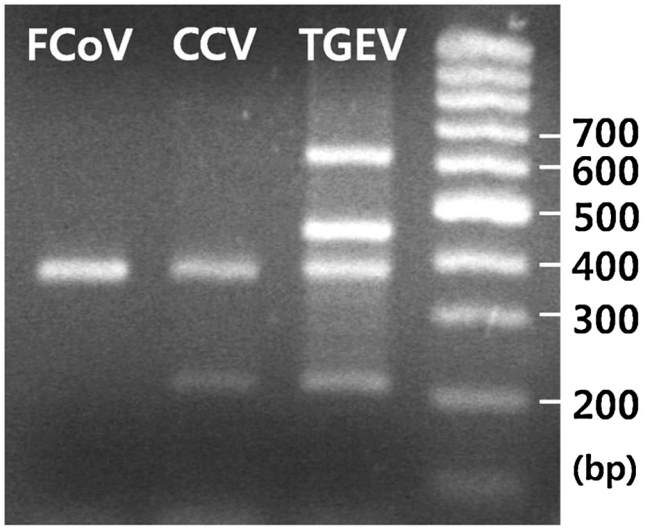

Fig. 3. The electrophoretic pattern of a multiplex PCR assay with S1, S2, S3, and S4. The multiplex PCR assay revealed a common band (380 bp) for all viruses, a CCoV-specific band for CCoV (209 bp), and a TGEV-specific band for TGEV (623 and 452 bp), suggesting efficient discriminating capacity of the PCR.

3. Results

To detect FCoV, CCoV, and TGEV in a differential manner, primers targeting the highly conserved region in the *M* gene were sought. Because of the high sequence diversity among FCoV, CCoV, and TGEV, no consistent difference of more than three continuous nucleotides in the alignments of CCoV and TGEV with FCoV sequences were noted (Fig. 2). Therefore, a primer pair (S1 and S2) specific for common sequences in all the viruses, CCoV-specific primer (S3), and TGEV-specific primer (S4) were selected for the multiplex RT-PCR (Fig. 2). S1 and S4 were used as the forward primers, whereas S2 and S3 were used as the reverse primers. The values of ΔG of each primer or among all primer pairs ranged from −5.40 to 1.20 kcal/mol. In addition, the nucleotide at the 3′-terminal immediate to the last unique nucleotides in S3 and S4 was substituted with an inosine.

The multiplex RT-PCR with S1, S2, S3, and S4 primers permitted successful detection and differentiation of each virus (Fig. 3). PCR products from each of the three viruses showed a common band amplified by S1 and S2 (380 bp) as a control. CCoV revealed its genome-specific band amplified by S1 and S3 (209 bp) in addition to the common band. TGEV revealed four different-sized bands: the common band (380 bp), a band amplified by S3 and S4 (452 bp), a band amplified by S2 and S4 (623 bp), and a band amplified by S1 and S3 (209 bp). Sequencing analyses demonstrated that each of the amplifications were specific for the intended target sequence. In addition, none of the RT-PCR assays recognized any other pathogens in the repeated experiments.

The analytic sensitivity of singleplex assay and multiplex assay was estimated by using serially diluted recombinant vectors with known copy numbers (molecules/μL). The estimated detection limit of the multiplex PCR was 780, 2800, and 550 copies for FCoV, CCoV, and TGEV, respectively (Table 2). For the materials with the same concentrations of the intended targets, the multiplex assays for CCoV and TGEV yielded positive results with 10-fold lower concentration of each virus compared to that used in the singleplex assay. The assay for FCoV yielded the same detection limit between the singleplex and multiplex formats. Standard curves generated by the multiplex test using 10-fold serial dilutions of each recombinant vector showed correlation coefficients (R^2) of 0.950–0.968 and slopes of 27.57–47.95 (Fig. 4), indicating good linearity of the PCR reaction.

Table 2
Detection limits of the singleplex and multiplex reverse transcriptase-polymerase chain reaction assays.

Target product	Detection limit		
	Singleplex	Multiplex	
Feline coronavirus	7.8×10^2	7.8×10^2	molecules/ml
Canine coronavirus	2.8×10^2	2.8×10^3	
Transmissible gastroenteritis virus	5.5×10^1	5.5×10^2	

4. Discussion

In this study, a multiplex RT-PCR assay was developed and evaluated for the detection and differentiation of three coronaviruses (FCoV, CCoV, and TGEV) with similar genetic properties. In this study, the sequences of *M* gene targeted by CCoV-specific and TGEV-specific primers showed minimal consistent differences (three bases and one base, respectively). Thus, inosine was used to allow primer binding. Inosine is a synthetic universal base that can bind any of the four natural nucleotide bases in DNA (Hill et al., 1998). The primers containing inosine thus compensate for the high rate of degeneracy of the target sequence and can substantially reduce the overall primer degeneracy and false priming by overcoming individual nucleotide mismatch (Knoth et al., 1988). In this study, CCoV- and TGEV-specific primer contained inosine in the 3′-terminal sequence, and the analytic performance showed good discriminating capacity among FCoV, CCoV, and TGEV simultaneously.

The efficiency of multiplex PCR can be affected by suboptimal annealing temperature, poorly designed primers, and poor-enzyme qualities (Elnifro et al., 2000; Xie et al., 2009). The multiplex PCR includes more than one primer pair that increases the possibility of mis-pairing or dimer formation (i.e., hairpin, self, or cross-dimer), resulting in a decrease in the reaction efficiency. Considering these factors, the primer sequences were selected based on the lowest possibility of mis-paring or dimer formation, as estimated by the secondary structure or ΔG value for the primer itself or among all primers. For the enzyme, an enzyme mixture with hot-start function was evaluated to confirm that the reaction efficiency between

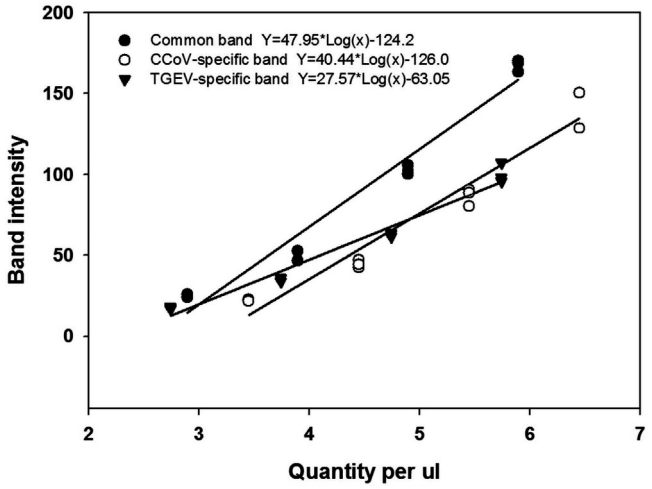

Fig. 4. Multiple detection of a common sequence, a CCoV-specific sequence, and a TGEV-specific sequence. A recombinant plasmid vector containing the target sequence with a known quantity (i.e., copy number/μL) was prepared and used. The *Y*-axis indicates the band intensities of PCR products using each dilution of the vector. Each regression line was constructed based on triplicate measurements.

the enzyme mixtures was not different. Evaluation of the annealing temperature of the multiplex assay ranging from 46 °C to 60 °C also revealed that the highest reaction efficiency was obtained with 50 °C temperature, suggesting that the reaction condition of singleplex assay was suitable for multiplex assay.

FECV replicates in enterocytes; therefore, infected cats are asymptomatic carriers or have mild diarrhea, whereas FIPV is distributed by macrophages throughout the body (Stoddart and Scott, 1989). Phagocytosis by macrophages is a key event in the pathogenesis of FIP. However, FECV can also induce viremia without phagocytosis, suggesting that FECV and FIPV cannot be differentiated based on the evaluations of peripheral blood samples (Gunn-Moore et al., 1998). In addition, mutations in the FECV genome can change the surface structures of the virus to enable phagocytosis by macrophages (Vennema et al., 1992, 1995; Benetka et al., 2004). As described above, CCoV and TGEV can also be isolated from cats (McArdle et al., 1992; Hartmann, 2005; Saif and Sestak, 2006). Thus, the rapid detection and isolation of FCoV-infected cats is the only effective preventive strategy to avoid spread of coronaviruses. For this purpose, a PCR test was developed for detecting and differentiating FCoV, CCoV, and TGEV. It is expected that the proposed multiplex RT-PCR assay will enable detection and discrimination of FCoV, CCoV, and TGEV at a lower cost and in less time than individual tests and thus prevent unnecessary isolation or euthanasia of FCoV-uninfected cats due to misdiagnosis.

In summary, the multiplex PCR method with S1, S2, S3, and S4 primers successfully detected and differentiated FCoV, CCoV, and TGEV. This simple method does not require additional tests or equipment and provides an effective way to detect and differentiate FCoV infection in cats. Until date, the prevalence of FCoV infection and the distribution of its serotype have not been reported in South Korea. Therefore, in future, the prevalence and genetic features of FCoV, including serotype specificity, of coronavirus-infected cats in South Korea will be investigated.

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