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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 ($\Delta G$) were selected as the primer sequences. The sequence specificity of the primer and its priming condition (i.e., $\Delta G$ 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).

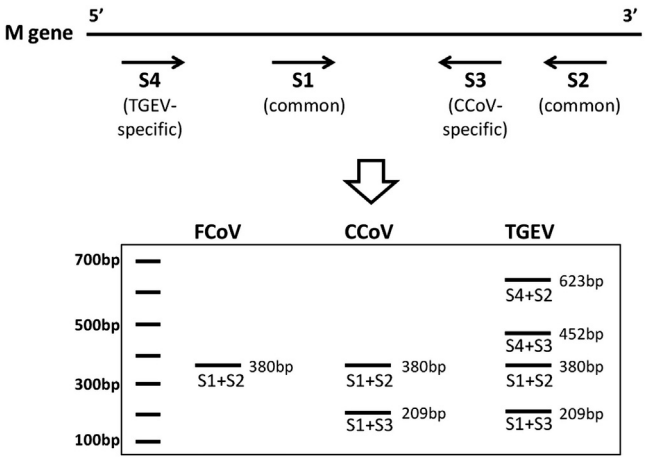


**Fig. 1.** Diagram of a multiplex PCR assay depicting the position of primer binding and the electrophoretic pattern with S1, S2, S3, and S4 primers. S1 and S4 are forward primers, and S2 and S3 are reverse primers.

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.