Chapter 15

Feline Coronavirus RT-PCR Assays for Feline Infectious Peritonitis Diagnosis

Takehisa Soma

Abstract

Feline infectious peritonitis (FIP) is a highly fatal systemic disease in cats, caused by feline coronavirus (FCoV) infection. FCoV usually has little clinical significance; however, a mutation of this avirulent virus (feline enteric coronavirus) to a virulent type (FIP virus) can lead to FIP incidence. It is difficult to diagnose FIP, since the viruses cannot be distinguished using serological or virological methods. Recently, genetic techniques, such as RT-PCR, have been conducted for FIP diagnosis. In this chapter, the reliability of RT-PCR and procedures used to determine FCoV infection as part of antemortem FIP diagnosis is described.

Key words Diagnosis, Feline coronavirus, Feline infectious peritonitis, RT-PCR

1 Introduction

Feline infectious peritonitis (FIP) is an immune-mediated progressive and systemic infectious disease occurring in domestic cats and wild felids, and caused by infection with feline coronavirus (FCoV), a single-stranded RNA virus, which has been classified as *Alphacoronavirus* along with canine coronavirus (CCoV) and transmissible gastroenteritis virus [1, 2]. FCoV is transmitted by the fecal-oral route and usually causes a mild to inapparent enteritis [2]. FIP is considered to be induced by a virulent mutant (FIP virus; FIPV) of this enteric FCoV (feline enteric coronavirus; FECV) [2, 3]. The incidence of FIP is generally as low as 1–3 % in FCoV-infected cats, though it varies depending on age, breed, environment, and superinfection with other viruses [2, 4–6].

It is divided into two basic clinical forms, effusive FIP, in which effusion is observed in the body cavity, and non-effusive FIP, in which multiple pyogranuloma lesions are observed, though differences in lesions are influenced by individual immunity [7]. Furthermore, there are two types (I and II) of FCoV, with FCoV type II considered to arise by a recombination of FCoV type I and

Leyi Wang (ed.), *Animal Coronaviruses*, Springer Protocols Handbooks,
DOI 10.1007/978-1-4939-3414-0_15,

CCoV [8–10]. Based on genetic and serological investigations, FCoV type I is overwhelmingly dominant as compared to type II and mixed infection with both types is not rare [11–14].

Since FIPV and FECV cannot be fully distinguished using serological methods, it is generally difficult to diagnose FIP [1]. Therefore, other laboratory findings such as hematology and serum biochemistry examinations [15, 16] have been referred to FIP diagnosis. Recently, it has been stated that demonstration of FCoV RNA by RT-PCR is one of the most reliable diagnostic indicators of FIP in suspected cases [7, 17]. However, FIPV and FECV are not necessarily distinguished with certainty, and the reliability of RT-PCR for FIP diagnosis depends largely on the test specimens as well as rearing environment of the affected cat.

Test specimens used with FCoV RT-PCR for FIP diagnosis include body cavity fluid (ascitic and pleural effusions), blood, cerebrospinal fluid (CSF), and tissues. As shown in Table 1, effusion is the most suitable, and FCoV RNA detection provides highly sensitive and specific diagnosis [1, 17–19]. When using CSF, RNA detection can also give a highly specific diagnosis. However, the absence of FIP cannot be generally concluded based on negative results, because small amounts of the virus may exist in CSF from FIP cases [1, 20, 21]. Even in non-FIP and healthy carriers, RNA may be detected in blood for several months after FECV infection [22, 23]. Of note, associated RNA is frequently detected in blood from FCoV-endemic multi-cat households. Thus, the reliability of RT-PCR-positive results obtained from a blood specimen is dependent on the rearing environment [23–25]. In contrast, FIP may be excluded when a blood specimen is RT-PCR negative, because the RNA detection sensitivity is relatively high with blood from FIP cases [17, 23, 26, 27]. RNA detection sensitivity varies among tissues, i.e., higher in the liver and spleen, and lower in the kidneys and heart [28–30]. Tissue samples generally contain blood, which compromises the reproducibility of FIP diagnosis with RT-PCR-positive tissues [1, 29].

In this chapter, three RT-PCR techniques generally employed for FIP diagnosis in Japan are outlined in regard to their usefulness for antemortem diagnosis.

Table 1
Predictive values of FCoV RT-PCR in FIP diagnosis

Predictive value	Effusion	Blood	CSF	Tissue
Positive (specificity)	High	Valuable	High	Valuable
Negative (sensitivity)	High	Moderate to high	Low	Valuable

2 Materials

2.1 Primer Set for RT-PCR

Three FCoV RT-PCR primer sets are recommended for FIP diagnosis, as shown in Table 2. One targets the 3′-untranslated region (3′-UTR) (P205–P211 primer set) [17] for FIP screening. This region is the first choice for RT-PCR, because it is highly conserved among *Alphacoronavirus* and allows sensitive FCoV RNA detection. A second-round (nested) PCR primer set (P276–P204) is also available to check the specificity of the RT-PCR result.

To confirm a positive RT-PCR reaction, a subsequent RT-PCR assay is recommended using a primer set that recognizes subgenomic mRNA of the M gene (212–1179 primer set) [27] (Table 2). Since detection of this gene indicates viral replication, FIPV, which has increased microphage infectivity, is able to be detected with high specificity. This RT-PCR technique is more useful for specimens other than effusion samples and CSF. However, in our experience, mRNA detection tends to be less sensitive than 3′-UTR RT-PCR.

To determine the type of cases shown positive with the above RT-PCR assays, a primer set targeting the S gene should be used for a multiplex RT-PCR (Iffs-Icfs-Iubs primer set) (Table 2) [31]. For negative cases shown by RT-PCR, nested PCR should be conducted using nIffle-nIcfs-nIubs primer set (*see* **Note 1**).

Table 2
Primers for the amplification of FCoV gene

Primer	Sequence (5′–3′)	Orientation	Target	Product size	Reference
P205	GGCAACCCGATGTTTAAAACTGG	Sense	3′-UTR	223 bp	[17]
P211	CACTAGATCCAGACGTTAGCTC	Antisense			
P276	CCGAGGAATTACTGGTCATCGCG	Sense		177 bp	
P204	GCTCTTCCATTGTTGGCTCGTC	Antisense			
212	TAATGCCATACACGAACCAGCT	Sense	M (mRNA)	295 bp	[27]
1179	GTGCTAGATTTGTCTTCGGACACC	Antisense			
Iffs	GTTTCAACCTAGAAAGCCTCAGAT	Sense	S	Type I 376 bp	[31]
Icfs	GCCTAGTATTATACCTGACTA	Sense		Type II 283 bp	
Iubs	CCACACATACCAAGGCC	Antisense			
nIffles	CCTAGAAAGCCTCAGATGAGTG	Sense		Type I 360 bp	
nIcfs	CAGACCAAACTGGACTGTAC	Sense		Type II 218 bp	
nIubs	CCAAGGCCATTTTACATA	Antisense			

Representative positive reaction bands from these three RT-PCR methods and two nested PCR assays are as shown in Figs. 1 and 2.

2.2 Reagent for FCoV RT-PCR

2.2.1 Extraction and Purification of Viral RNA

1. QIAamp Viral RNA Mini Kit (Qiagen).
2. QIAamp Blood RNA Mini Kit (Qiagen).
3. RNeasy Mini Kit (Qiagen).
4. DNase- and RNase-free water (Invitrogen).
5. DNase- and RNase-free ethanol, 99.5 %(V/V) (Wako).

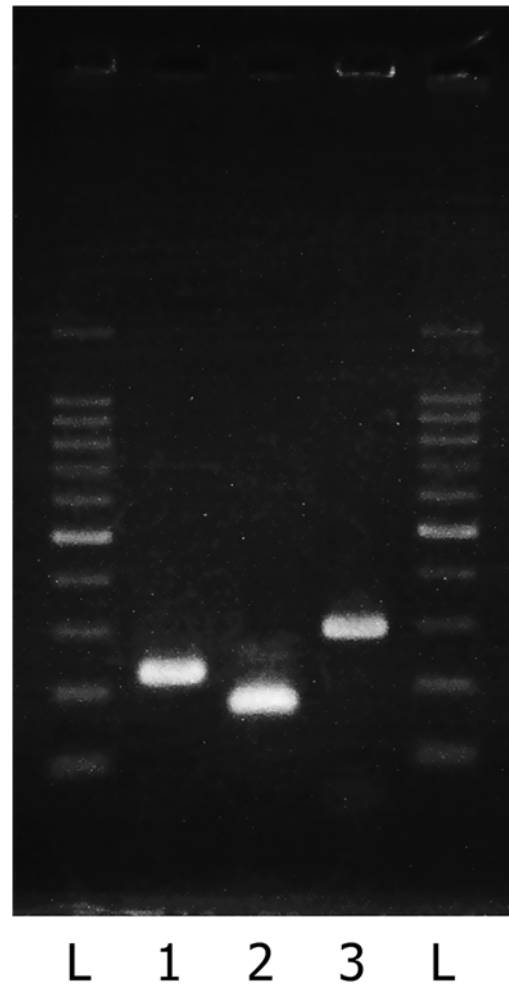

Fig. 1 Agarose gel electrophoresis of products obtained by FCoV RT-PCR targeting 3′-UTR and M (mRNA) genes. Lane 1: 3′-UTR RT-PCR (first-round PCR) (223 bp), lane 2: 3′-UTR nested PCR (177 bp), lane 3: M (mRNA) RT-PCR (295 bp), L: 100 bp DNA ladder marker

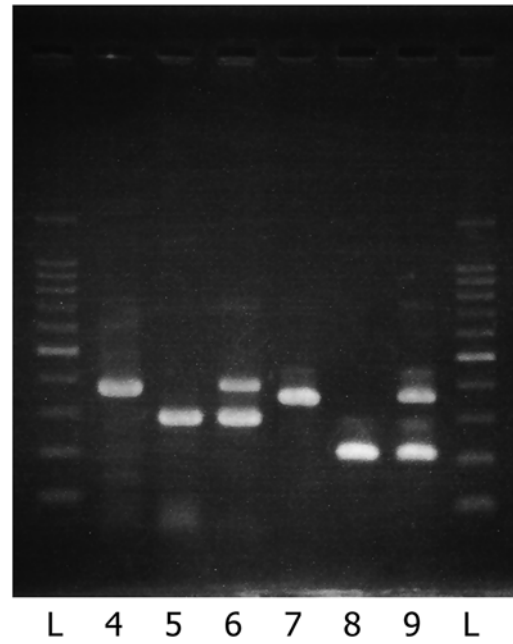

Fig. 2 Agarose gel electrophoresis of products obtained by FCoV multiplex RT-PCR targeting S gene. Lanes 4–6: RT-PCR (first-round PCR), Lanes 7–9: nested PCR, Lanes 4 and 7: Type I (376 bp and 360 bp, respectively), Lanes 5 and 8: Type II (283 bp and 218 bp, respectively), Lanes 6 and 9: Both type infections,, L: 100 bp DNA ladder marker

2.2.2 RT-PCR

1. Qiagen One-Step RT-PCR kit, containing 5× RT-PCR buffer, enzyme mix, and dNTP mix (10 mM each) (Qiagen).
2. RNase inhibitor, 40 U/mL (Promega).
3. Primers, 10 μM (shown in Table 2).

2.2.3 Second-Round (Nested) PCR

1. DNase- and RNase-free water (invitrogen).
2. AmpliTaq Gold DNA polymerase, 5 U/mL, with 10× PCR buffer, $MgCl_2$ solution (25 mM), and dNTP mix (2 mM each) (Applied Biosystems).
3. Primers, 10 μM (shown in Table 2).

2.2.4 Agarose Gel Electrophoresis

1. Tris-borate-EDTA (TBE) buffer, pH 8.3 (TaKaRa).
2. Agarose-LE powder (Ambion).
3. 6× Gel loading dye, containing bromophenol blue and orange G (Toyobo).
4. 100 bp DNA ladder marker, with loading dye (Toyobo).

2.2.5 EtBr Staining

1. Ethidium bromide (EtBr), 10 mg/mL (invitrogen).
2. Distilled water (for diluting EtBr stock solution), not necessarily DNase- and RNase-free water.

3 Methods

3.1 RNA Extraction and Purification

Viral RNA is extracted from effusion, serum, plasma, whole blood, cerebrospinal fluid (CSF), and tissue (biopsy) specimens using a QIAamp Viral RNA Mini Kit, QIAamp Blood RNA Mini Kit, or RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions (*see* **Notes 2–6**).

3.2 RT-PCR

Next, reaction mixtures for RT-PCR are prepared, as shown in Table 3. Five microliters of the template (purified RNA) is added to the reaction mixture and subjected to amplification in a thermal cycler (Table 4) (*see* **Notes 7–9**).

Table 3
Reaction mixtures for FCoV RT-PCR

Component	Primer set	
	P205–P211, 212–1179	Iffs-Icfs-Iubs
DNase-free, RNase-free water	27.8 μL	26.3 μL
5× QIAGEN OneStep RT-PCR Buffer	10.0 μL	10.0 μL
dNTP mix (containing 10 mM of each dNTP)	2.0 μL	2.0 μL
10 μM Primers	1.5 μL each	1.5 μL each
QIAGEN OneStep RT-PCR enzyme mix	2.0 μL	2.0 μL
RNase inhibitor (10 U/μL)	0.2 μL	0.2 μL
Total volume	45.0 μL	45.0 μL

Table 4
Reaction conditions for FCoV RT-PCR

	Primer set		
	P205-P211	212–1179	Iffs-Icfs-Iubs
Reverse transcription	50 °C for 30 min	50 °C for 30 min	50 °C for 30 min
Inactivation of reverse transcriptase and denaturation of cDNA template	95 °C for 15 min	95 °C for 15 min	95 °C for 15 min
(Sequential cycle)	(40 cycles)	(30 cycles)	(35 cycles)
Denaturation	94 °C for 50 s	94 °C for 1 min	94 °C for 1 min
Annealing	55 °C for 1 min	62 °C for 1 min	50 °C for 1 min
Extension	72 °C for 1 min	72 °C for 1 min	72 °C for 1 min
Final extension	72 °C for 7 min	72 °C for 7 min	72 °C for 7 min

3.3 Second-Round (Nested) PCR

Reaction mixtures for the nested PCR assay are then prepared, as shown in Table 5. Five microliters of the RT-PCR product diluted 100 times with DNase- and RNase-free water is added to the reaction mixtures, and then subjected to amplification (Table 6) (*see* **Notes 7–9**).

3.4 Agarose Gel Electrophoresis

Five microliters of the PCR product is then added to 6× gel loading dye at a 1/6 volume ratio and electrophoresed with TBE buffer at 100 V for 35 min on a 2 % agarose gel at room temperature.

3.5 EtBr Staining

Following electrophoresis, the gel is immersed into 10 mg/mL of EtBr solution. After staining for 30–40 min, the gel is photographed under UV illumination (*see* **Notes 10–12**).

Table 5
Reaction mixtures for FCoV nested PCR

Component	Primer set	
	P276–P204	nlffles-nlcfs-nlubs
DNase- and RNase-free water	29.8 μL	27.75 μL
10× PCR buffer (containing no $MgCl_2$)	5.0 μL	5.0 μL
25 mM $MgCl_2$	3.0 μL	4.0 μL
dNTP mix (containing 2 mM of each dNTP)	5.0 μL	5.0 μL
10 μM Primers	1.0 μL each	1.0 μL each
Taq polymerase (5 U/μL)	0.2 μL	0.25 μL
Total volume	45.0 μL	45.0 μL

Table 6
Reaction conditions for FCoV nested PCR

	Primer set	
	P276–P204	nlffles-nlcfs-nlubs
Initial denaturation		90 °C for 5 min
(Sequential cycle)	(35 cycles)	(35 cycles)
Denaturation	94 °C for 50 sec	94 °C for 1 min
Annealing	55 °C for 1 min	47 °C for 1 min
Extension	72 °C for 1 min	72 °C for 1 min
Final extension	72 °C for 7 min	72 °C for 7 min

4 Notes

For FCoV RT-PCR implementation and FIP diagnosis, the following points should be noted.

1. False-negative results may be obtained when no viral RNA is detected with the indicated primers because of viral mutations. This is more likely to occur with primers targeting the S gene.
2. Care should be exercised to prevent coagulation of whole blood samples. EDTA is suitable as an anticoagulant, while heparin is not recommended, because it may cause coagulation during transportation.
3. Care should be exercised to prevent blood contamination during CSF sampling, as viral RNA may be contained in blood even in non-FIP cases.
4. Care should be exercised during sampling and transportation, because RNA is fragile, and disposable DNase- and RNase-free sampling containers should be used. Collected samples should be immediately transported to a laboratory in a refrigerated state.
5. DNase- and RNase-free phosphate buffer saline (PBS) should be used to increase sample volume before testing as needed.
6. Effusion, serum, and plasma specimens should be centrifuged with a refrigerated centrifuge prior to purification with the QIAamp Viral RNA Mini Kit, and the resulting supernatants should then be purified.
7. Reaction mixtures should be prepared and dispensed on ice.
8. PCR is highly sensitive and may yield false-positive results when contaminated by even a small amount of nucleic acid. Thus, reaction mixtures should be prepared and dispensed in clean environments, such as a clean bench, and only test results obtained by skilled experimenters are considered to be reliable.
9. Only DNase- and RNase-free instruments, such as test tubes and pipette chips, should be used.
10. Since EtBr is deactivated by light, its solution should be stored in a light-shielded condition.
11. Care should be exercised in handling EtBr for gel staining, because EtBr is toxic to humans. It should be also detoxified in appropriate manners, such as activated carbon adsorption, reductive decomposition, and oxidative decomposition, before disposal. A detoxifying reagent is commercially available (EtBr destroyer, Wako).
12. Care should be exercised in regard to UV irradiation during gel observation, as UV may damage eyes and skin.

References

1. Addie DD (2012) Feline coronavirus infections. In: Greene CE (ed) Infectious disease of the dog and cat, 4th edn. Saunders Elsevier, St. Louis, pp 92–108
2. Hartmann K (2005) Feline infectious peritonitis. Vet Clin North Am Small Anim Pract 35:39–79
3. Vennema H, Poland A, Foley J, Pedersen NC (1998) Feline infectious peritonitis viruses arise by mutation from endemic feline enteric coronaviruses. Virology 30:150–157
4. Foley JE, Pedersen NC (1996) The inheritance of susceptibility to feline infectious peritonitis in purebred catteries. Feline Pract 24:14–22
5. Pedersen NC (1976) Feline infectious peritonitis: something old, something new. Feline Pract 6:42–51
6. Sparkes AH, Gruffydd-Jones TJ, Howard PE, Harbour DA (1992) Coronavirus serology in healthy pedigree cats. Vet Rec 131:35–36
7. Addie D, Belák S, Boucraut-Baralon C et al (2009) Feline infectious peritonitis. ABCD guidelines on prevention and management. J Feline Med Surg 11:594–604
8. Herrewegh AAPM, Smeenk I, Horzinek MC, Rottier PJ, de Groot RJ (1998) Feline coronavirus type II strains 79-1683 and 79-1146 originate from a double recombination between feline coronavirus type I and canine coronavirus. J Virol 72:4508–4514
9. Vennema H, Poland A, Hawkins KF, Pedersen NC (1995) A comparison of the genomes of FECVs and FIPVs and what they tell us about the relationships between feline coronaviruses and their evolution. Feline Pract 23:40–44
10. Terada Y, Matsui N, Noguchi K et al (2014) Emergence of pathogenic coronaviruses in cats by homologous recombination between feline and canine coronaviruses. PLoS One 9:e106534
11. Benetka V, Kubber-Heiss A, Kolodziejek J et al (2004) Prevalence of feline coronavirus types I and II in cats with histopathologically verified feline infectious peritonitis. Vet Microbiol 99: 31–42
12. Kummrow M, Meli ML, Haessig M et al (2005) Feline coronavirus serotypes 1 and 2: seroprevalence and association with disease in Switzerland. Clin Diagn Lab Immunol 12: 1209–1215
13. Lin CN, Su BL, Wang CH et al (2009) Genetic diversity and correlation with feline infectious peritonitis of feline coronavirus type I and II: 5-year study in Taiwan. Vet Microbiol 136:233–239
14. Soma T, Wada M, Taharaguchi S, Tajima T (2013) Detection of ascitic feline coronavirus RNA from cats with clinically suspected feline infectious peritonitis. J Vet Med Sci 75: 1389–1392
15. Sparkes AH, Gruffydd-Jones TJ, Harbour DA (1994) An appraisal of the value of laboratory tests in the diagnosis of feline infectious peritonitis. J Am Anim Hosp Assoc 30:345–350
16. Hartmann K, Binder C, Hirschberger J et al (2003) Comparison of different tests to diagnose feline infectious peritonitis. J Vet Intern Med 17:781–790
17. Herrewegh AAPM, de Groot RJ, Cepica A et al (1995) Detection of feline coronavirus RNA in feces, tissues, and body fluids of naturally infected cats by reverse transcriptase PCR. J Clin Microbiol 33:684–689
18. Soma T, Kawashima S, Osada H, Ishii H (2010) Diagnostic value of feline coronavirus PCR testing in clinical cases. J Environ Dis 19:1–7
19. Kennedy MA, Millsaps BRK, Potgieter BLND (1998) Correlation of genomic detection of feline coronavirus with various diagnostic assays for feline infectious peritonitis. J Vet Diagn Invest 10:93–97
20. Foley JE, Lapointe JM, Koblik P, Poland A, Pedersen NC (1998) Diagnostic features of clinical neurologic feline infectious peritonitis. J Vet Intern Med 12:415–423
21. Doenges SJ, Weber K, Dorsch R, et al Detection of feline coronavirus in cerebrospinal fluid for diagnosis of feline infectious peritonitis in cats with and without neurological signs. J Feline Med Surg Mar 3. pii: 1098612X15574757. [Epub ahead of print]
22. Fehr D, Bolla S, Herrewegh AA, Horzinek MC, Lutz H (1996) Detection of feline coronavirus using RT-PCR: basis for the study of the pathogenesis of feline infectious peritonitis (FIP). Schweiz Arch Tierheilkd 138:74–79
23. Gunn-Moore DA, Gruffydd-Jones TJ, Harbour DA (1998) Detection of feline coronaviruses by culture and reverse transcriptase-polymerase chain reaction of blood samples from healthy cats and cats with clinical feline infectious peritonitis. Vet Microbiol 62:193–205
24. Can-Sahna K, Soydal Ataseven V, Pinar D, Oğuzoğlu TC (2007) The detection of feline coronaviruses in blood samples from cats by mRNA RT-PCR. J Feline Med Surg 9:369–372
25. Herrewegh AA, Mähler M, Hedrich HJ et al (1997) Persistence and evolution of feline coronavirus in a closed cat-breeding colony. Virology 234:349–363
26. Sharif S, Arshad SS, Hair-Bejo M et al (2010) Diagnostic methods for feline coronavirus: a review. Vet Med Int 2010, 809480

27. Simons FA, Vennema H, Rofina JE et al (2005) A mRNA PCR for the diagnosis of feline infectious peritonitis. J Virol Methods 124:111–116
28. Pedersen NC, Eckstrand C, Liu H, Leutenegger C, Murphy B (2015) Levels of feline infectious peritonitis virus in blood, effusions, and various tissues and the role of lymphopenia in disease outcome following experimental infection. Vet Microbiol 175:157–166
29. Li X, Scott FW (1994) Detection of feline coronaviruses in cell cultures and in fresh and fixed feline tissues using polymerase chain reaction. Vet Microbiol 42(65–77):1994
30. Sharif S, Arshad SS, Hair-Bejo M et al (2011) Evaluation of feline coronavirus viraemia in clinically healthy and ill cats with feline infectious peritonitis. J Anim Vet Adv 10:18–22
31. Addie DD, Schaap IAT, Nicolson L, Jarrett O (2003) Persistence and transmission of natural type I feline coronavirus infection. J Gen Virol 84:2735–2744