

Detection of feline coronaviruses in cell cultures and in fresh and fixed feline tissues using polymerase chain reaction

Xiantang Li *, Fredric W. Scott

*Cornell Feline Health Center and Department of Microbiology, Immunology and Parasitology,
College of Veterinary Medicine, Cornell University, Ithaca, NY, 14853USA*

Received 10 November 1993; accepted 1 March 1994

Abstract

Feline coronavirus infections in cell cultures and in fresh and fixed feline tissues were detected using a polymerase chain reaction (PCR) test. Cell cultures were inoculated with feline infectious peritonitis virus (FIPV), feline enteric coronavirus (FECV) or sham inoculum. The tissue samples of liver, kidney and spleen were taken from specific-pathogen-free (SPF) cats that were inoculated intranasally with 10^3 TCID₅₀ of FIPV 79-1146 ($n=10$), FIPV UCD1 ($n=3$) or sham inoculum ($n=3$), from clinical cats ($n=43$), and from formalin-fixed archived feline tissues ($n=49$), respectively. Additional tissue samples were taken from the FIPV-inoculated cats ($n=6$) and were kept at 4°C, room temperatures (20–24°C) and 37°C respectively for 0, 6, 12, 24, 48, 72, and 96 hours before frozen (–70°C) for PCR to evaluate the effects of the ambient temperatures and post-mortem intervals on the test. The samples were also fixed in 10% neutrally buffered formalin, 95% ethanol, and Bouin's solution respectively to evaluate the effects of the fixatives on the test.

Positive PCR results were obtained from the cell cultures that were inoculated with FIPV and FECV and from the FIPV-inoculated cats (13/13). Negative PCR results were obtained from the sham-inoculated cell cultures and cats (3/3). Of the 92 clinical cats, 7 of the 8 FIP-suspected cats (87.5%) and 51 of the 84 non-FIP-suspected cats (60.7%) were shown to be virus-positive in at least one of the tissue samples. There was no significant difference in the PCR results between the fresh and the formalin-fixed tissues of the clinical cats ($P>0.05$). Of the FIPV inoculated cats, the virus was detectable equally well in fresh and formalin-, Bouin's solution- or ethanol-fixed tissues. However, the amounts of total RNA extracted from the fixed tissues were significantly less than those from fresh tissues ($P<0.01$). In tissues that were kept at 4°C, the virus was detectable up to 96 h; at room temperatures, up to 48 h; and at 37°C, up to 24 h, respectively.

Keywords Feline coronavirus; Cat; Diagnosis, feline coronavirus; PCR

* Correspondence to: Division of Comparative Medicine, Massachusetts Institute of Technology, 37 Vassar Street, Cambridge, MA 02139-4307, USA Phone (617) 253-9441, Fax: (617) 258-5708

1. Introduction

Feline coronaviruses are ubiquitously present in wild and domestic feline species of all ages worldwide, with seroconversion in up to 45–80% of cats (Addie and Jarrett, 1990, Barlough and Stoddart, 1990, Evermann et al., 1988, Evermann et al., 1991, Horzinek and Osterhaus, 1979, Pedersen, 1987, Pedersen et al., 1981, Pedersen et al., 1984, Scott, 1989, Scott, 1991). Some feline coronaviruses, especially feline infectious peritonitis virus (FIPV) and feline enteric coronavirus (FECV), cause important diseases in feline species, and present considerable challenges to feline practitioners and diagnosticians. The disease associated with coronavirus infection in cats was first described 30 years ago and the virus was observed ultrastructurally 5 years later (Holzworth, 1963, Zook et al., 1968). Since then, many attempts have been made to develop a reliable clinical method and/or laboratory test to diagnose the disease and to detect the virus-carrier cats (Barlough, 1984, Barlough and Stoddart, 1990, Hok, 1989, Hok, 1990, Martinez and Weiss, 1993, Pedersen, 1987, Sparkes et al., 1991).

The difficulties in developing such a method and/or test to detect the coronavirus infection in cats are multiple. First of all, the clinical occurrence (less than 5–10%) of the disease is much lower than that of inapparent viral infection in cats (Evermann et al., 1991, Pedersen, 1987, Scott, 1989, Scott, 1991). Second, the disease spectrum varies greatly and the clinical or pathological manifestations of the virus infection can be similar to and confused by those of many other diseases in cats (August, 1984, Barlough and Stoddart, 1990, McKeirnan et al., 1981, Pedersen, 1987). Third, certain strains of feline coronaviruses do not grow in conventional culture systems and viral isolation is a difficult task (Barlough and Stoddart, 1990, Pedersen, 1987). Fourth, there exist biological and antigenic diversities of feline coronaviruses and host antibody response varies greatly (McKeirnan et al., 1981, Pedersen, 1987, Tupper et al., 1987). Lastly, co-infection of feline coronaviruses with other infectious pathogens often occurs in cats (Pedersen, 1987, Rodgers and Baldwin, 1990, Sparkes et al., 1991).

Polymerase chain reaction (PCR), a test detecting the viral genome itself, has been widely used as a powerful diagnostic tool for various viral infections (Homberger et al., 1991, Yamada and Imanishi, 1992). In combination with reverse transcription of a certain segment(s) of the viral RNA into DNA, the PCR test could detect all and differentiate each of the feline coronavirus infections in cultures and tissues. The present paper describes a PCR test that was able to detect all feline coronaviruses in cell cultures and feline tissues.

2. Materials and methods

2.1. *Viruses, cells and animals*

Confluent Crandell feline kidney (CrFK) cell monolayers were inoculated in triplicates with 10^3 TCID₅₀ each strain of feline coronaviruses, including FECV (1683), type I (UCD1/NW-1, UCD3, UCD4) and type II (1146, UCD2) strains of FIPV, and sham

Table 1

Detection of feline coronavirus (FCoV) infection by polymerase chain reaction (PCR) in clinical cats ($n=92$) that were submitted to pathology service and specific-pathogen-free (SPF) cats that were inoculated with FCoV ($n=13$) or sham inoculum ($n=3$)^a

Clinical and pathologic diagnoses	Case No.	FCoV-Positive No
Feline infectious peritonitis	8	7
Feline leukemia	2	2
Cardiomyopathy	3	2
Trauma/bone fracture	4	2
Thoracic empyema	3	3
Neoplasms (various types)	15	11
Toxoplasmosis	2	1
Megacolon	2	0
Hemorrhagic diathesis	2	1
Myelopathy	3	0
Pulmonary diseases	3	2
Central nervous system diseases	5	3
Hyperthyroidism	2	0
Miscellaneous (one case/diagnosis)	17	11
No definitive diagnosis	9	6
Disposals (diagnosis not performed)	12	7
Total cases.	92	58

^a All 13 FCoV-inoculated SPF cats were shown to be positive and 3 sham-inoculated SPF cats negative for FCoV by PCR.

inoculum, respectively for 48–72 h. The cultures were harvested for viral RNA extraction when cytopathic effects appeared on 50% of the FIPV-1146-infected monolayers. The viruses on the cell monolayers were confirmed by indirect immunofluorescence assay (IFA) (Olsen et al., 1992). The hyper-immune serum was obtained from a specific-pathogen-free (SPF) cat that was inoculated with FIPV-1146, and diluted 1:1000 in phosphate-buffered saline supplemented with 0.5% bovine serum albumin (BSA-PBS) as the primary antibody. The fluorescein-conjugated goat-anti-cat antibody was supplied by Sigma (St. Louis, MO), and diluted 1:50 in 0.5% BSA-PBS as the secondary antibody.

SPF cats (Cornell Feline Health Center, CFHC, Ithaca, NY) were kept in filter-isolation cages, one cat per cage, at 16 weeks of age. After adaptation for one week, the cats were inoculated intranasally with 0.5 ml of 10^3 TCID₅₀ FIPV-1146 ($n=10$), FIPV-UCD1 ($n=3$), or sham inoculum ($n=3$) respectively. When the infected cats developed clinical signs of FIP, they were anesthetized with Ketamine subcutaneously and euthanized with barbiturate fluid intracardially for necropsy. Clinical cats ($n=42$) were randomly selected at necropsy from the Veterinary Pathology Service (VPS) and/or CFHC at Cornell University (Table 1). A pilot study with the FIPV-infected cats and clinical cats ($n=10$) indicated that FIPV was most frequently detected from the liver, kidney and spleen samples among others (e.g., blood, pancreas, heart, brain, cecal apex, mesenteric lymph nodes, conjunctiva, lung, and parotid gland). At necropsy, the tissue samples (1 gram/sample) of liver, kidney and spleen were taken randomly from each cat, quenched in liquid nitrogen, and stored at -70°C for viral RNA extraction. One liver biopsy was taken from a kitten

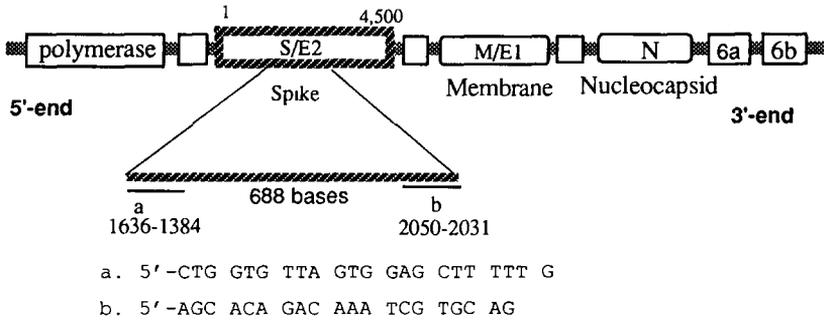


Fig 1 Proposed genomic RNA sequences of feline coronaviruses and the primer sequences used in this study

that was referred to the Veterinary Medical Teaching Hospital at Cornell University because of the hydropericardium and a family history of feline infectious peritonitis (FIP). The liver biopsy sample was processed as above for viral RNA extraction. Additionally, liver, kidney and spleen samples of 49 cats were taken from the VPS archival tissues fixed in 10% neutrally buffered formalin (< 1 year's duration) for viral RNA extraction (Table 1).

Additional tissue samples of liver, kidney and spleen were taken from the FIPV-1146-inoculated SPF cats (n=6) and kept at 4°C, room temperatures (20-24°C) and 37°C respectively for 0, 6, 12, 24, 48, 72, and 96 h, before quenched in liquid nitrogen and stored at -70°C, to evaluate the effects of the ambient temperatures and post-mortem intervals on the PCR test. The tissue samples were also fixed in 10% neutrally buffered formalin, 95% ethanol, and Bouin's solution respectively to evaluate the effects of the fixatives on the PCR test.

2.2. Viral RNA extraction and slot hybridization

Viral RNA, including cellular RNA, in the cultures and tissues was extracted by a single-step total RNA isolation method (Chomczynski and Sacchi, 1992). The cultures (10⁸ cells/75 cm² cell monolayer) and the tissues (1 gram/sample) were homogenized respectively in 5 ml of denaturing solution containing 4 M guanidinium thiocyanate (Sigma, St. Louis, MO). The viral RNA extracted from the 10 FIPV-1146-infected and 3 sham-inoculated cats was confirmed by slot hybridization to the FIPV spike (S) gene with a [³²P]-labeled S-probe (Sambrook et al., 1989a). The DNA fragment of the FIPV S gene was prepared from a cloned vector (pB1) that was supplied kindly by Dr. Horzinek (De Groot et al., 1987). The fragment was end-labeled with [gamma-³²P]-dATP (DuPont, Boston, MA) and a Multiprime DNA Labeling System (Boehringer Mannheim Co., Indianapolis, IN) as directed by the manufacturer.

2.3. Polymerase chain reaction

The forward (a) and reverse (b) primers were designed to span the FIPV S gene from bases 1363 to 2050, and yielded a PCR product of 688 base pairs as shown in Figure 1 (De Groot et al., 1987). PCR was performed as a two-step process, i.e. reverse transcription of

Table 2

Cycling temperatures and time of polymerase chain reaction to detect feline coronavirus infection in cell cultures and feline tissues

Reverse transcription (with forward primer):			
Soak:	4°C	Prepare reaction mixtures	
Step-cycle:	43°C	20 minutes	1 cycle
	99°C	5 minutes	1 cycle
Soak:	4°C	5 minutes	
Add amplification reagents and spin briefly.			
Amplification (with forward and reverse primers):			
Step-cycle:	94°C	3 min	1 cycle
Thermo-cycle:	94°C	1'15" minute	35 cycles
	53°C	1'15" minute	35 cycles
	72°C	2 min	35 cycles
		(with 5 second ramping between)	
Time-delay:	72°C	5 min	1 cycle
Soak:	4°C	5 min	

the viral RNA into DNA and subsequent amplification of the DNA, in a single vial with the GeneAmp RNA PCR Kit and DNA Thermal Cycler-480 (Perkin-Elmer Cetus, Norwalk, CT) as directed by the manufacturer. The cycling time and temperatures are shown in Table 2. The PCR products were separated on 1% SeaKem agarose gels with 0.5 µg/ml of ethidium bromide and visualized under ultraviolet light.

2.4. Southern hybridization and DNA sequencing of PCR products

Specificity of certain randomly selected PCR products was confirmed by Southern hybridization with the [³²P]-labeled S-probe (Sambrook et al., 1989b). The PCR products from the FIPV-infected and the clinical cats were also selectively cloned into a pCRTM II vector (Invitrogen, San Diego, CA) as directed by the manufacturer. The PCR products within the plasmid were confirmed by colony hybridization with the above probe (Sambrook et al., 1989c) and by partial nucleotide sequencing with a ds-DNA Cycle Sequencing System (GIBCO, Gaithersburg, MD) as directed by the manufacturer (Table 2). Ten pmol of each primer and 10 pmol of plasmid DNA were used respectively. The reaction mixtures were separated on 6% polyacrylamide sequencing gels (Slatko et al., 1992).

3. Results

3.1. Viruses, cells, and animals

The CrFK cell monolayers inoculated with FIPV-1146, FIPV-UCD2 and FECV-1863 all showed moderate to marked, multifocal to diffuse, cytopathic effects (CPE) in 2–4 days after inoculation. Those cultures inoculated with FIPV-UCD1, FIPV-UCD3 and FIPV-UCD4 showed no or very mild CPE, whereas the sham-inoculated monolayers did not show any CPE. With the cat anti-FIPV-1146 serum as the primary antibody, the IFA was able

positively to detect all strains of FIPV and FECV on the CrFK cell monolayers, whereas the results of IFA for the sham-inoculated cell monolayers were all negative.

All of the FIPV-1146-infected cats showed typical clinical signs and pathologic changes of FIP in 2–5 weeks after inoculation, the FIPV-UCD1-infected cats showed minimal to mild clinical signs and pathologic changes of FIP, whereas the sham-inoculated cats did not show any clinical or pathologic changes. Of the 92 clinical cats, 8 cats (including the kitten) had history, clinical signs, and/or pathologic changes that were suggestive of FIP, and 84 cats had no clinical signs and/or lesions of FIP (Table 1). Of the non-FIP suspected cats, the disease spectrum varied greatly, including simple traumas, feline leukemia, cardiomyopathy or hydropericardium, thoracic empyema, toxoplasmosis, megacolon, hemorrhagic diathesis, myelopathy, pneumonia, central nervous system diseases, hyperthyroidism, and other miscellaneous diseases (one case per diagnosis) (Table 1). The tumors included lymphoma (5), fibrosarcoma (3), squamous cell carcinoma (2), transitional cell carcinoma (1), colonic adenocarcinoma (1), multiple myelomas (1), pancreatic adenomas (1), and undifferentiated tumor (1). Some of the cats had no definitive diagnoses, and other cats were simply euthanized for disposal.

3.2. *Viral RNA extraction and slot hybridization*

Total RNA, including viral and cellular RNA, extracted from the cell cultures varied from 153.4–229.7 μg , averaging 196.2 $\mu\text{g}/10^8$ cells (75 cm^2 cell monolayer); and from the feline tissues varied from 187.6–242.8 μg , averaging 221.7 $\mu\text{g}/\text{gram}$ tissues. There was no significant difference in the amounts of total RNA extraction between the virus infected and the sham-inoculated cultures or feline tissues ($P > 0.05$). The amounts of total RNA extracted from fresh tissues were significantly higher than those from formalin-, ethanol- or Bouin's solution-fixed tissues ($p < 0.01$) as shown in Figure 2. There is no difference in the amounts of total RNA extraction between or among the formalin-, ethanol- or Bouin's solution-fixed tissues.

The results of slot hybridization of the extracted RNA by the [^{32}P]-labeled S-probe to FIPV S gene indicated that 2 of the 10 FIPV-1146-infected cats were positive for feline coronavirus. Of the 2 FIPV-infected positive cats, the total RNA samples from the liver and kidney of one cat, and from the liver and spleen of the other cat, were shown to be positive for the virus. The RNA samples from the SPF control cat tissues were all shown to be negative for the virus.

3.3. *Polymerase chain reaction*

The RNA samples extracted from the FIPV- and FECV-infected CrFK cell cultures were shown all to be PCR-positive, whereas those from the sham-inoculated cultures were all PCR-negative (Fig. 3). All of the FIPV-1146- and FIPV-UCD1-infected cats were shown to be PCR-positive in at least one of the liver, kidney or spleen samples per cat, whereas all of the sham-inoculated cats were shown to be PCR-negative (Fig. 4).

Of the 92 clinical cats, 7 of the 8 FIP-suspected (87.5%) and 51 of the 84 non-FIP-suspected (60.7%) cats were shown to be virus-positive in at least one of the tissue samples (Table 1, Fig. 4). The FIP-suspected kitten was shown to be feline coronavirus-negative.

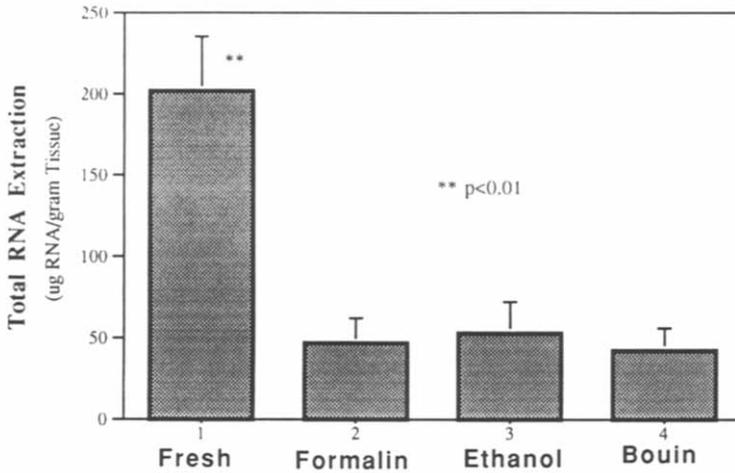


Fig. 2. The results of total RNA extraction from the fresh and formalin-, ethanol- or Bouin's solution-fixed feline tissues. The amounts of total RNA (1 gram/sample) extracted from the fresh tissues were significantly higher than those from formalin-, ethanol- or Bouin's solution-fixed liver tissues ($P < 0.01$). There was no difference in the amounts of total RNA extraction among or between the fixed tissues ($P > 0.05$).

Of the clinical cats, there was no significant difference in the PCR results between the fresh tissues taken at necropsy (61.2%) and those of formalin-fixed archival tissues (58.6%) ($*P > 0.05$). Among the clinical cat tissue samples, the livers (48.6%) appeared to have a higher percentage PCR-positive for coronaviruses than the kidney (21.1%) and spleen (42.3%).

Among the coronavirus-positive clinical cats, there was no predilection of animal age, sex, breed, and health status. The ages varied from 3 months to 18 years. Female and male cats were approximately equally distributed. Domestic short hair cats were most common,

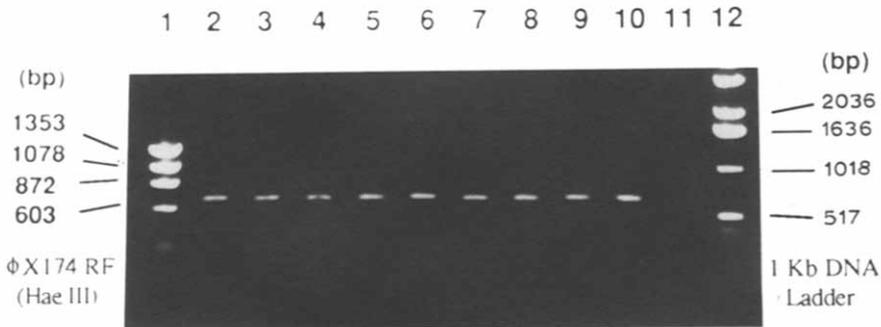


Fig. 3. Detection of different strains of feline infectious peritonitis virus (FIPV) and feline enteric coronavirus (FECV) in cell cultures by polymerase chain reaction. Lanes 1 (ϕ X174 RF DNA/Hae III fragments, GIBCO) and 12 (1 kb DNA Ladder, GIBCO) are molecular markers respectively. Lanes 2–4 (1146), 5 (UCD1), 6 (UCD2), 7 (UCD3), 8 (UCD4) are different FIPV strains. Lanes 9–10 are FECV (strain 1683). Lane 11 is uninfected control cell culture.

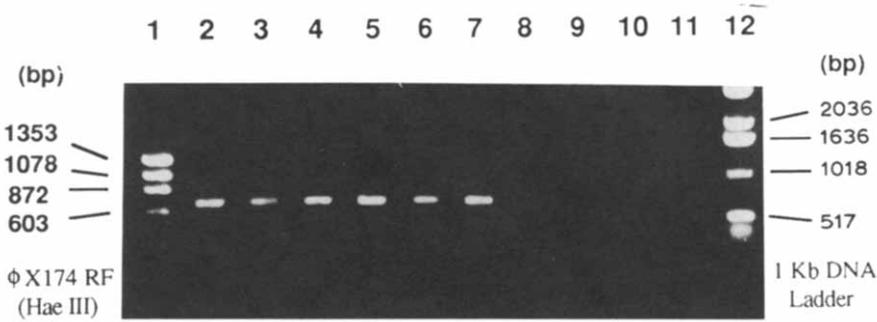


Fig. 4 Detection of feline coronavirus (FCoV) infection by polymerase chain reaction from liver, kidney and spleen tissues respectively of a specific-pathogen-free (SPF) cat (lanes 2–4) inoculated with feline infectious peritonitis virus (FIPV-1146), a clinical cat (lanes 5–7) referred to pathology service, and a sham-inoculated SPF cat (lanes 8–10). Lanes 1 (ϕ X174 RF DNA/Hae III fragments, GIBCO) and 12 (1 kb DNA Ladder, GIBCO) are molecular markers respectively.

due to the geographical distribution of cat populations. No particular diseases or etiologies could be linked or correlated to the PCR results.

3.4. Southern hybridization and DNA sequencing of PCR products

Southern blotting and hybridization with the [32 P]-labeled S probe indicated that the PCR products that were randomly selected for analysis were all positive for the FIPV S gene.

With the above DNA sequencing method, 50 to 150 base sequences adjacent to either the forward or reverse primer were often clearly readable on the gels. Of the clearly readable base sequences, they were either identical (100% homology) or quite similar (>97% homology) to the corresponding DNA sequences (bases 1363 to 2050) of the FIPV-1146 S gene.

3.5. Effects of post-mortem intervals and environmental temperatures on the PCR test

The post-mortem intervals and environmental temperatures appeared to affect the PCR results. In the FIPV-1146 infected feline tissues that were kept at 4°C, the virus was detectable up to 96 h by PCR in all of the liver, kidney and spleen samples that were PCR-positive at the 0 time interval. In the tissues that were kept at room temperatures, the virus was detectable up to 48 h in all samples that were PCR-positive at the 0 time interval, and partially up to 72 h. In the tissues that were kept at 37°C, the virus was detectable up to 24 h in all samples that were PCR-positive at the 0 time interval, and partially up to 48 h respectively. The tissues became severely autolysed in 96 h at room temperatures and in 72 h at 37°C, and the subsequent PCR test was not performed on these samples.

3.6. Effects of fixatives on the PCR test

Different fixatives for the tissue samples did not appear to affect the PCR results. Of the SPF cats that were inoculated with FIPV-1146, the virus was detectable in fresh and

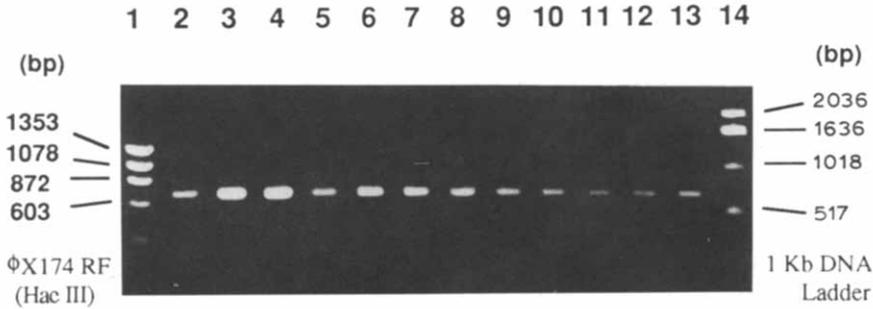


Fig. 5 Detection of viral RNA by polymerase chain reaction from the fresh (lanes 2–4) and formalin- (lanes 5–7), ethanol- (lanes 8–10) or Bouin's solution- (lanes 11–13) fixed liver, kidney and spleen tissues respectively of a specific-pathogen free cat that were inoculated with feline infectious peritonitis virus. Lanes 1 (ϕ X174 RF DNA/HaeIII fragments, GIBCO) and 14 (1 kb DNA Ladder, GIBCO) are molecular markers respectively.

formalin-, ethanol- or Bouin's solution-fixed tissues equally well (Figure 5), although the amounts of total RNA extracted from the fixed tissues were significantly less than those from fresh tissues.

4. Discussion

The present study described a laboratory test to detect feline coronaviruses in cell cultures and feline tissues using PCR. The results of slot hybridization, Southern hybridization, IFA, and the sizes and nucleotide sequences of the PCR products, all indicated that the PCR test was specific for the FIPV-1146 S gene. The S gene is highly conserved among feline and other coronaviruses, and therefore we designed the primers to span a region of this gene so that most, if not all, of the feline coronaviruses in cells and tissues could be detected. On the other hand, the 6b gene of the feline and other coronaviruses is the least conserved (De Groot et al., 1988, Vennema et al., 1992). Our next study is to differentiate each strain (at least major strain) of feline coronavirus infections in cats by PCR with primers that span the 6b gene (Fig. 1). By combination of the two different sets of primers, feline coronaviruses in cells or tissues could be both detected and differentiated simultaneously by a single PCR test.

The results that 87.5% of the FIP-suspected clinical cats were positive for feline coronaviruses suggested that the PCR test could potentially aid the diagnosis of coronavirus disease in cats by providing direct evidence of the virus infection. The currently used clinical methods and/or laboratory tests can not be linked to feline coronaviruses themselves in cats and are often not useful in subclinical viral infections. The history, clinical signs, clinical chemistry, and hematological findings often are not specific, and the manifestations of many diseases in cats can be similar in these aspects (August, 1984, Barlough and Stoddart, 1990, Pedersen, 1987, Scott, 1989). A diagnosis based on the commonly used histopathologic examination often results in a wide range of differential diagnoses, especially for the "dry form" of FIP (Barlough and Stoddart, 1990, Pedersen, 1987, Rodgers and Baldwin, 1990, Scott, 1989, Sparkes et al., 1991). Serum antibody response in cats does not correlate

directly to the coronavirus infections. Some cats may not develop antibody response to coronavirus infection and serum antibody disappears in some terminally ill cats (Barlough and Stoddart, 1990, Sparkes et al., 1991). In kittens, it is often uncertain whether the presence of serum antibody is due to maternal immunity or acquired infection (Addie and Jarrett, 1990, Addie and Garde, 1992). On the other hand, the PCR test does not correlate to the severity, progression and prognosis of the virus infections at the time of testing.

The PCR test appeared to be more sensitive than many other laboratory tests in detecting the coronaviral infection in cats. The PCR results were positive for all 13 FIPV-infected cats, whereas the results of slot hybridization were positive for only 2 of the 10 FIPV-infected cats in this study. A dot blot hybridization assay has been described recently to detect feline coronaviruses in cell cultures and feline peripheral blood mononuclear leukocytes (Martinez and Weiss, 1993). The assay can detect the viruses themselves during the acute viremic infection, but not during chronic infection. It is very difficult to isolate the virus from cats with clinical FIP, since some feline coronaviruses do not grow well in the conventional culture systems (Barlough and Stoddart, 1990, Pedersen, 1987). It is thus practically impossible to use the viral isolation method in routine diagnoses or surveys of coronavirus infections in cats. Since the PCR test detects only a specific segment of viral genome, e.g. 688 nucleotides out of the whole coronavirus genome in this study, PCR results could be positive as long as this specific segment is intact, even if the whole viral particle and/or genome had been disintegrated.

The fact that 60.7% of the non-FIP-suspected clinical cats were positive for coronaviruses indicated that the persistent infection of coronaviruses in cats did exist in nature. The PCR test could be used potentially as a valuable tool to identify the virus-carrier cats. The results of this study were also in agreement with those of serologic tests in that up to 45–80% of cats were coronavirus-positive, and further confirmed that feline coronavirus infection is common in cats (Barlough and Stoddart, 1990, Evermann et al., 1991, Scott, 1991). On the other hand, the result of this study might not represent the real prevalence of coronavirus infection in cats in general, since the cats used in this study were mostly ill or dead animals. Although the cats did come from a random resource, one should be cautious when interpreting the results of this study.

The results of this study were also in agreement with other studies in that co-infection of feline coronaviruses and other infectious agents or co-existence of coronaviral diseases and other disease conditions occurred in cats (Barlough and Stoddart, 1990, Pedersen, 1987, Rodgers and Baldwin, 1990, Sparkes et al., 1991). A high percentage (60.7%) of the non-FIP suspected cats with various other disease conditions were shown also to be coronavirus-positive by PCR in this study (Table 1). The relationship between the subclinical coronavirus infection and the miscellaneous clinical disease conditions in these cats is unknown. No specific disease patterns or certain infectious agents could be linked to coronavirus infection in cats in this study.

Our pilot study indicated that the coronaviruses could be detected by the PCR test in many types of tissues of the FIPV-infected cats, with variable frequencies (unpublished observation). In addition to liver, kidney and spleen, the virus was also detected in the peripheral blood mononuclear leukocytes, pancreas, heart, brain, cecal apex, lymph nodes (mesentery), conjunctiva, lung, and parotid gland. The peripheral blood mononuclear leukocytes and conjunctival swabs could be considered as the tissue sample of choice for a

non-invasive PCR diagnostic test or viral survey in cats. However, the virus may not be present simultaneously in every tissues or homogenously within a given tissue. Another advantage of the PCR test is the flexibility of sample handling, i.e. the preservation and shipping of the tissue samples. The tissue samples appeared to be good for the PCR test if, after death or biopsy, the cat tissues were kept at 4°C for up to 96 h, at room temperatures for up to 48 h, and at 37°C for up to 24 h. Alternatively, the tissue samples could be fixed in 10% formalin, ethanol, or Bouin's solution, although tissue fixation would decrease the amount of viral RNA extraction from the tissues. This information could be used as a guideline when submitting tissue samples from cats for the PCR test in various environmental or laboratory conditions.

5. Conclusions

The PCR test detected feline coronaviruses themselves and thus provided direct evidence of coronavirus infection in cultures and feline tissues. This study indicated that the prevalence of coronavirus infection in cats was high and that persistent infection of coronaviruses in cats did exist in nature. The PCR test could aid the diagnosis of coronavirus infections and detection of the virus-carriers in cats.

Acknowledgements

This work was supported by private donations to the Cornell Feline Health Center. The authors appreciate the excellent technical help from Wendy A. Hoose, and thank Dr. M.C. Horzinek (University of Utrecht, Utrecht, the Netherlands) for supplying the FIPV S gene cloned in a vector and Drs. Peter H. Rowland and Kurt J. Williams for providing the clinical case materials.

References

- Addie, D.D. and Jarrett, O., 1990 Control of feline coronavirus infection in kittens. *Vet. Rec.*, 126: 164
- Addie, D.D. and Garde, O., 1992 A study of naturally occurring feline coronavirus infections in kittens. *Vet. Rec.*, 130: 133-137
- August, R.A., 1984. Feline infectious peritonitis: An immune-mediated coronaviral vasculitis. *Vet. Clin. North Am.*, 14: 971–984.
- Barlough, J.E., 1984 Serodiagnostic aids and management practice for feline retrovirus and coronavirus infection. *Vet. Clin. North Am.*, 14: 955–969
- Barlough, J.E. and Stoddart, C.A., 1990 Feline coronaviral infections. In: C.E. Greene (Editor), *Infectious diseases of the dog and cat*. W.B. Saunders Company, Philadelphia, pp. 300–312.
- Chomczynski, P. and Sacchi, N., 1992. Single-step RNA isolation from cultured cells or tissues. In: F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl (eds), *Current protocols in molecular biology*, vol. 1. John Wiley and Sons, New York, pp. 4.2.4–8
- De Groot, R.J., Maduro, J., Lenstra, J.A., Horzinek, M.C., Van Der Zeijst, B.A.M. and Spaan, W.J.M., 1987 cDNA cloning and sequence analysis of the gene encoding the peplomer protein of feline infectious peritonitis virus. *J. Gen. Virol.*, 68: 2639–2646

- De Groot, R.J., Andeweg, A.C., Horzinek, M.C. and Spaan, W.J.M., 1988. Sequence analysis of the 3' end of the feline coronavirus FIPV 79-1146 genome: Comparison with the genome of porcine coronavirus TGEV reveals large insertions. *Virology*, 167: 370–376
- Evermann, J.F., McKeirnan, A.J. and Ott, R.L., 1991. Perspectives on the epizootiology of feline enteric coronavirus and pathogenesis of feline infectious peritonitis. *Vet Microbiol*, 28: 243–255.
- Evermann, J.F., Heeney, J.L., Roelke, M.E., McKeirnan, A.J. and O'Brien S.J., 1988. Biological and pathological consequences of feline infectious peritonitis virus infection in the cheetah. *Arch Virology*, 102: 155–171
- Hok, K., 1989. Demonstration of feline infectious peritonitis virus in conjunctival epithelial cells from cats. A simple and reliable method for clinical veterinary virology screening. *APMIS*, 97: 820–824
- Hok, K., 1990. Demonstration of feline corona virus (FCV) antigen in organs of cats suspected of feline infectious peritonitis (FIP) disease. *APMIS*, 98: 659–664
- Holzworth, J., 1963. Some important disorders of cats. *Cornell Vet*, 53: 157–160.
- Homberger, F.R., Smith, A.L. and Barthold, S.W., 1991. Detection of rodent coronaviruses in tissues and cell cultures by using polymerase chain reaction. *J. Clin. Microbiol*, 29: 2789–2793
- Horzinek, M.C. and Osterhaus A.D.M.E., 1979. Feline infectious peritonitis. A worldwide serosurvey. *Am J. Vet Res*, 40: 1487–1492
- Martinez, M.L. and Weiss, R.C., 1993. Detection of feline infectious peritonitis virus infection in cell cultures and peripheral blood mononuclear leukocytes of experimentally infected cats using a biotinylated cDNA probe. *Vet Microbiol.*, 34: 259–271.
- McKeirnan, A.J., Evermann, J.F., Hargis, A., Miller, L.M. and Ott, R.L., 1981. Isolation of feline coronaviruses from two cats with diverse disease manifestations. *Fel Pract*, 11: 16–20
- Olsen, C.W., Corapi, W.V., Ngichabe, C.K., Baines, J.D. and Scott, F.W., 1992. Monoclonal antibodies to the spike protein of feline infectious peritonitis virus mediate antibody-dependent enhancement of infection of feline macrophages. *J. Virology*, 66: 956–965
- Pedersen, N.C., 1987. Coronavirus diseases (coronavirus enteritis, feline infectious peritonitis). In J. Holzworth (Editor), *Diseases of the cats*. W.B. Saunders, Philadelphia, p. 193–214
- Pedersen, N.C., Boyle, J.F., Floyd, K., Fudge, A. and Barker, J., 1981. An enteric coronavirus infection of cats and its relationship to feline infectious peritonitis. *Am J. Vet Res*, 42: 368–377.
- Pedersen, N.C., Evermann, J.F., McKeirnan, A.J. and Ott, R.L., 1984. Pathogenicity studies of feline coronavirus isolates 79-1146 and 79-1683. *Am J. Vet Res*, 45: 2580–2585
- Rodgers, S.J. and Baldwin, C.A., 1990. A serologic survey of Oklahoma cats for antibodies to feline immunodeficiency virus, coronavirus, and *Toxoplasma gondii* and for antigen to feline leukemia virus. *J. Vet. Diagn. Invest*, 2: 180–183.
- Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989a. Slot hybridization of RNA. In *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 2. Cold Spring Harbor Laboratory Press, New York, pp. 754–755
- Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989b. Analysis and cloning of eukaryotic genomic DNA. In *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 2. Cold Spring Harbor Laboratory Press, New York, pp. 9.31–59
- Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989c. Colony hybridization. In *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 2. Cold Spring Harbor Laboratory Press, New York, pp. 10.18–66
- Scott, F.W., 1989. Update on FIP. *Proc. 12th KalKan Symp*, 12: 43–47
- Scott, F.W., 1991. Transmission and epidemiology. In: *New perspectives on prevention of feline infectious peritonitis*, Proc. symp. Orlando, Florida, p. 8–13.
- Slatko, B.E., Albright, L.M. and Tabor, S., 1992. DNA sequencing by the dideoxyl method. In: F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (Editors), *Current protocols in molecular biology*, vol. 1. John Wiley and Sons, New York, pp. 7.4.1–27.
- Sparkes, A.H., Gruffydd, T.J. and Harbour, D.A., 1991. Feline infectious peritonitis, a review of clinicopathological changes in 65 cases, and a critical assessment of their diagnostic value. *Vet. Rec.*, 129: 209–212.
- Tupper, G.T., Evermann, J.F., Russell, R.G. and Thouless, M.E., 1987. Antigenic and biological diversity of feline coronaviruses: feline infectious peritonitis and feline enteritis virus. *Arch. Virology*, 96: 29–38
- Vennema, H., Rossen, J.W.A., Wesseling, J., Horzinek, M.C. and Rottier, P.J.M., 1992. Genomic organization and expression of the 3' end of the canine and feline enteric coronaviruses. *Virology*, 191: 134–140.

- Yamada, A. and Imanishi, J., 1992. Detection of influenza B virus in throat swabs using the polymerase chain reaction. *Acta Virol.*, 36: 320–325
- Zook, B.C., King, N W., Robinson, R.L. and McCombs, H.L., 1968. Ultrastructural evidence for the viral etiology of feline infectious peritonitis. *Pathol Vet.*, 5: 91–95