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Correlation of genomic detection of feline coronavirus with various diagnostic assays for feline infectious peritonitis

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Feline infectious peritonitis (FIP) is a fatal disease of domestic and nondomestic felids caused by a feline coronavirus (FCV). It is a significant problem in catteries, multiple cat households, and shelters.[14,18] FIP can manifest as an effusive peritonitis and/or pleuritis, with a relatively short disease course ending in death. A protracted course with granulomatous lesions affecting multiple organs may occur, which also invariably progresses to death.[8,11,12]

The FCVs are closely related and include two biotypes: those that are virulent and cause FIP and those that are avirulent.[7] The avirulent group, known as feline enteric coronaviruses (FECV), may be associated with mild enteric disease or subclinical infection in cats.[11,12] FCVs are also classified into serotypes 1 and 2 based on antigenicity.[7,12] Both serotypes contain virulent and avirulent biotypes, and FCVs within a serotype are indistinguishable in the laboratory. FCVs are ubiquitous, especially in environments such as catteries, where large numbers of cats are housed together and where the majority of cats are seropositive.[12] As a result, antemortem diagnosis for FIP is difficult because no detection assay sensitive enough and specific for the virus causing FIP is routinely available.[12] This problem is further compounded because consistent genetic differences between the virulent and avirulent biotypes of FCV have not been identified.[16]

An assay has been developed using reverse transcription and a nested polymerase chain reaction (PCR) to detect FCV genomic material in a variety of biologic samples from cats. The genomic region targeted encompasses the 7a and 7b open reading frames (ORFs). This region, specifically that encoding the 7b ORF, may correlate with virulence.[6] In addition, the 7b region is among the least conserved regions of the FCV genomes.[10] The goal of this investigation was to optimize the PCR technique for FCV detection in biologic samples, compare this assay to those routinely used in diagnostic laboratories, and determine whether PCR amplification of the 7a/7b ORFs could be used to distinguish virulent from avirulent FCV biotypes.

Primers for reverse transcription (RT) and PCR were designed to encompass the 7a and 7b ORFs of the FCV genome (Fig. 1).[17] Virus propagated in vitro was amplified using type 1 (UCD1)[a] and type 2 (WSU1143)[a] strains of FIP virus (FIPV) and the external primer pair (primers 1 and 2). Viruses were propagated by standard methods.[2] Virus-infected cells were collected when cytopathic effects were observed, and the virus suspension was centrifuged at 600 × *g* for 5 minutes to pellet the cells. The cell pellet was used for RNA extraction. The procedures for RNA extraction were done as described.[b,3] Approximately 5 μg of RNA was used for RT with Moloney murine leukemia virus reverse transcriptase[b] by standard methods.[13] PCR was performed with *Taq* polymerase[c] also by standard methods.[13] The concentration of primer for RT and PCR was 0.5 μM. The amplification product was predicted to be approximately 1 kilobase (kb) long. The amplification product from the virus propagated in vitro was the appropriate size.

To verify the identity of the amplification product and confirm the specificity of these primers, the DNA product from amplification of strain UCD1 was cloned into the pCRII vector using the TA cloning method[d] according to the manufacturer's directions. The base sequence of cloned cDNA was determined by the dideoxy chain termination method using ^{35}S-ATP[e] with standard methods.[13] The product had 78% homology to the published sequence for the 7a/7b ORF of FECV strain 79-1683 (data not shown).[17] Primers 3, 4, 5, and 6 were designed from the cDNA sequence data (Fig. 1). Primers 3 and 4 were used in the second round of PCR on 5 μl of the product from the first round of amplification using the same parameters as for the first round of PCR. Primers 5 and 6 were used for nucleotide sequencing of amplification products.

The nested PCR procedure was done also with materials containing other feline viruses that could be present in biologic samples and with feline leukocyte RNA to confirm the specificity of the primers for FCV. These viruses included feline calicivirus,[f] feline herpesvirus-1,[f] feline immunodeficiency virus,[a] feline leukemia virus,[a] and feline syncytial virus.[a] Uninfected leukocytes used in primer specificity testing were from a healthy cat seronegative for FIPV.

Primer specificity was high because no product was produced with uninfected leukocyte RNA or RNA from cell cultures infected with the heterologous viruses (data not shown).

To evaluate the sensitivity of the amplification, FIPV strain UCD1 was titrated and quantified for infectivity and by PCR. Virus titer was the reciprocal of the highest dilution of virus per unit volume that resulted in cytopathic effects in 50% of inoculated cells ($CCID_{50}$/ml). Using 1 round of amplification, ≥300,000 $CCID_{50}$/ml could be detected. However, by adding a second round of amplification using the internal primers (primers 3 and 4), ≥30 $CCID_{50}$/ml could be detected (Table 1).

Clinical samples were evaluated for the presence of FCV RNA by RT–nested PCR. A variety of samples from affected and healthy cats were subjected to RNA extraction, RT, and 2 rounds of amplification. Samples from cats suspected of having FIP were acquired from area veterinarians and included ascitic fluid, pleural fluid, serum, plasma, whole blood, and tissue samples. Fecal samples from healthy cats were obtained from an area cattery that had a history of FIP.

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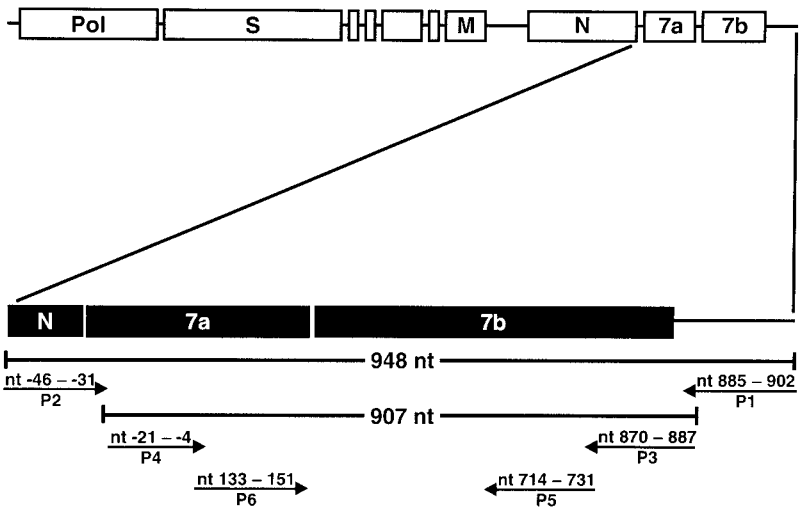

Figure 1. Feline coronavirus genome with enlargement of region amplified by the reverse transcription–nested polymerase chain reaction. Primers are denoted by arrows labeled with primer number (P1–6). Nucleotide (nt) number denotes position from initiation codon for the 7a ORF.[17] cDNA products from amplification with the indicated primer set are denoted by the bar lines, with the length of the products corresponding to the number of nucleotides indicated.

Two hundred fifty microliters of fluid samples (serum, whole blood, effusion fluid) that had been stored at −70 C were used for RNA extraction. At least 1 ml of fecal sample was diluted 1:1 (vol:vol) in cell culture medium, mixed thoroughly, and centrifuged at 600 × *g* for 5 minutes to pellet insoluble debris, and 250 μl of the supernatant fluid was used for RNA extraction. Tissue samples of at least 1 ml were homogenized in cell culture medium (1:1, vol:vol) and centrifuged at 600 × *g*, and 250 μl of the supernatant fluid was used for RNA extraction. RNA extraction of all biologic samples was done with Trizol LS[b] according to the manufacturer's directions. Ten micrograms of glycogen[g] was added to the 250 μl of biologic sample prior to the addition of Trizol LS. All RNA isolated from biologic samples was used for RT followed by PCR. The amplification products were approximately 1 kb in size (Fig. 2). Amplification products from clinical specimens were identified by nucleotide sequencing of a randomly selected portion using an automated sequencer,[h] confirming them as the 7a/7b ORFs of FCV (11 of 17 samples from PCR-positive cats; data not shown).

The clinically affected cats also were tested by additional diagnostic assays, including antigen detection by immunofluorescence, serologic assay for FCV-specific antibody, and histopathology. Antigen detection was done by standard methods on conjunctival scrapings of the third eyelid (antemortem) or tissue impressions (postmortem).[2] Results were reported as positive for antigen, negative for antigen, or inconclusive (a slight fluorescence was observed in only 1 or 2 cells, making confirmation of FIPV infection difficult). Serology for FCV-specific antibodies was done by indirect immunofluorescence by standard methods using FIPV WSU1143 propagated in Crandell feline kidney cells as the antigen.[2] Antibody titers were defined as the highest dilution that resulted in fluorescence. A titer of ≥1:640 was considered maximal, and a titer of <1:40 was considered negative. Results of histopathologic examination of a variety of tissue samples were reported as lesions diagnostic of FIP, negative for FIP, or inconclusive (lesions in 1 or few tissues that were consistent with those described in classical cases of FIP but

Table 1. Comparison of FCV detection using single and double polymerase chain reaction.

Amount of virus in sample ($CCID_{50}$/ml)	PCR results	
	Round 1	Round 2
3×10^8	+	+
3×10^7	+	+
3×10^6	+	+
3×10^5	+	+
3×10^4	−	+
3×10^3	−	+
3×10^2	−	+
3×10	−	+
0	−	−

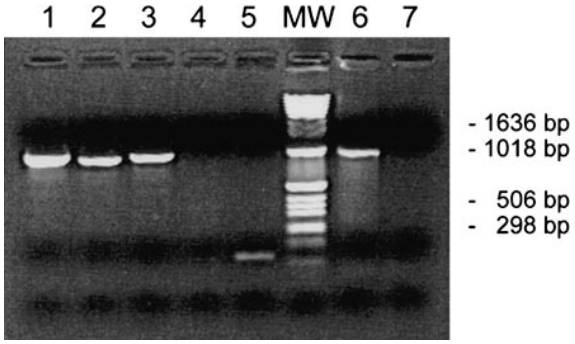

Figure 2. Agarose gel electrophoresis of amplification products from biologic samples taken from clinically ill cats. Lanes 6 and 7 are positive and negative controls, respectively. Lanes 1–4 are amplification products from ascites, plasma, whole blood, and serum, respectively, from cat 21. Lane 5 is the amplification product from plasma from cat 15. Molecular weight marker is indicated.

Table 2. Comparison of results* from histopathology, serology, immunofluorescence (IFA), and reverse transcription-nested polymerase chain reaction (RT/nPCR) for clinically ill cats.

				RT/nPCR				
Cat no.	Histopathology	Serology†	IFA‡	Effusion	Plasma	Serum	Whole blood	Tissue
1	ND	<1:40	−	−	−	ND	−	ND
2	ND	1:320	ND	ND	−	ND	ND	ND
3	ND	1:1,280	ND	ND	ND	−	ND	ND
4	ND	1:40	ND	ND	−	ND	ND	ND
5§	ND	<1:40	ND	ND	+	ND	ND	ND
6	ND	ND	−	+	ND	ND	ND	ND
7	No FIP‖	<1:40	ND	−	−	ND	−	ND
8	No FIP	1:40	−	−	ND	ND	−	−
9	No FIP	1:80	ND	ND	−	ND	−	−
10	No FIP	1:320	−	ND	−	ND	ND	ND
11	NC	<1:40	NC	−	ND	ND	−	ND
12	NC	1:5,120	−	+	+	−	+	ND
13	NC	1:40	ND	−	ND	ND	ND	ND
14	NC	1:320	−	ND	+	−	ND	ND
15	NC	1:160	ND	ND	−	−	ND	ND
16¶	NC	1:640	ND	+	−	−	ND	ND
17	NC	1:80	ND	ND	ND	−	ND	ND
18	FIP	<1:40	−	+	ND	−	ND	ND
19	FIP	1:160	ND	+	+	+	+	ND
20	FIP	<1:40	ND	+	−	−	−	ND
21	FIP	>1:5,120	ND	+	+	−	+	ND
22	FIP	1:640	ND	ND	+	ND	ND	ND
23	FIP	1:320	ND	ND	ND	−	ND	ND
24#	No FIP	ND	ND	ND	ND	ND	ND	−
25	No FIP	ND	ND	−	ND	−	ND	ND
26	ND	ND	−	−	ND	ND	−	ND
27	FIP	ND	ND	+	ND	ND	ND	ND

* ND = not done; NC = not conclusive.
† FCVirus-specific antibody titers determined by indirect immunofluorescence.
‡ Assay results from nictitating membrane conjunctiva (antemortem) or tissue impressions (postmortem).
§ Bengal tiger.
‖ FIP = feline infectious peritonitis.
¶ Snow leopard.
# Serval.

that lacked some significant diagnostic features).[9] Feces from 1 cat (Table 2, cat 5) were examined by electron microscopy following preparation by standard methods.[i] This cat had numerous coronavirus-like particles present in the feces.

The results of the nested PCR on samples from affected cats are given in Table 2. Of the 27 cats, 11 were positive for FCV by PCR. Unfortunately, samples available for some of the cats were limited.

All cats that were histologically negative for FIP ($n = 6$) also were negative by PCR. Six of 7 cats in which FIP was confirmed by histopathology were positive by PCR. Serum was the only sample available for the single PCR-negative, histopathology-positive cat. Of 8 cats inconclusive for FIP by histopathology, 3 were positive by PCR, and 2 of these 3 had significant antibody titers. Of the remaining 5 cats (PCR negative, histopathology inconclusive), antibody titers for the 4 that were tested were insignificant.

The results of the nested PCR assay using effusive fluid correlated with histopathology results, i.e., cats with histopathologic changes diagnostic of FIP were PCR positive and cats with no FIP-specific lesions were PCR negative. Positive PCR results were more consistently obtained with effusive fluid than with any other sample, closely followed by results with plasma and whole blood (Table 2). Serum yielded positive PCR results from only 1 of the 7 PCR-positive cats from which serum was available. The data indicate that serum is a poor sample for PCR and often is negative for FCV-infected cats.

Tissue immunofluorescence did not correlate with PCR or histopathology results; results were negative or inconclusive in all cases. FIP-specific antibody levels were significant (titers > 1:320) for only 4 of the 9 PCR-positive cats for which serology results were available. Antibody titers were significant for only 2 of the 6 histopathology-positive cats for which serology results were available. Insignificant or undetectable antibody titers were noted for 4 of 4 histopathology-negative cats for which serology results were available. One PCR-positive, seronegative animal for which histopathology results were not available was confirmed as FCV infected by electronmicroscopy. This finding confirms data collected over several years in the Clinical Virology laboratory at the University of Tennessee College of Veterinary

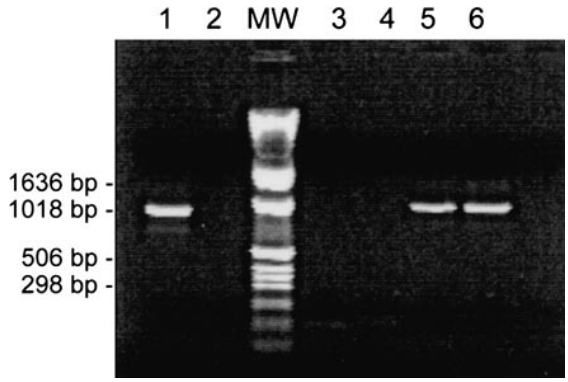

Figure 3. Agarose gel electrophoresis of amplification products from feces of healthy cats. Lanes 1 and 2 are positive and negative controls, respectively. Lanes 3–6 are products from amplification using feces from healthy cats.

Medicine that low FCV-specific antibody levels must be interpreted with caution (unpublished). Other investigators have also questioned the value of serology.[4,15] The PCR assay on effusion fluid or plasma could offer a useful antemortem test for FIP with the accuracy of histopathology but without the risks of biopsy.

Fourteen fecal samples from healthy cats housed in a cattery with a history of FIP-related deaths (confirmed by histopathology; data not given) also were tested by RT–nested PCR. Six of these cats were positive, and the products were the same size as products from cats suspected to have FIP (Fig. 3). A previous report identified a deletion of approximately 250 nucleotides in the 7b ORF of a putatively avirulent FCV (strain 79-1683).[17] If this deletion in FECV 7b ORF represented a consistent feature of avirulent FCVs, it would allow distinction between FIPV and FECV. However, subsequent investigation has indicated that this deletion does not occur in all FECV isolates,[6] based on sequence comparison of 7 virulent and 5 avirulent FCV laboratory strains. Sixteen field strains, 12 of which were associated with FIP, also were examined, and deletions in the 7b ORF were not detected. This observation was confirmed by the data presented here; all PCR products from biologic samples regardless of the health status of the cats sampled were approximately 1 kb in length. Products derived from clinically ill cats, healthy cats, and nondomestic felids were of similar size. No conserved genomic deletions were detected. Sequence analysis of the amplification products of the 7a/7b ORF from clinical samples is ongoing to determine whether conserved nucleotide sequence differences may exist between virulent and avirulent FCV biotypes. Analysis of amplification products from clinical samples would avoid spurious data introduced by in vitro propagation of the virus.

The RT–nested PCR technique is a sensitive and specific tool for detecting FCV in biologic samples. The findings presented here support those of a recent study,[5] in which FCV was detected in feces, tissues, and body fluids of cats using RT-nPCR targeting the highly conserved 3′-untranslated region. In the present study, the 7a/7b ORF was amplified in an attempt to determine if consistent genomic differences occur in this region between FCV biotypes. The previous assay was of similar sensitivity and specificity to the assay reported here. In addition, the test described here included heterologous viruses that may be present in clinical samples so that its specificity for FCV could be confirmed. The data reported here also support the results of the previous study in that the type of sample submitted is an important determinant of the PCR results. No consistent difference between FECV and FIPV could be identified, indicating that this assay is unable to distinguish these viruses. However, the test may be useful for FCV detection as an aid to suspected FIP diagnosis. To confirm the utility of this procedure, large numbers of plasma and effusion samples from cats in different geographic locales must be tested by RT–nested PCR and the results compared with those of histopathology. In addition, this method is useful for genomic characterization of FCV isolates and for molecular epidemiology. This and other regions of the FCV genome in isolates from domestic and nondomestic cats are the targets of current research.

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Sources and manufacturers

a. American BioResearch, Seymour, TN.
b. GIBCO BRL, Gaithersburg, MD.
c. Oncor, Gaithersburg, MD.
d. Invitrogen, San Diego, CA.
e. DuPont NEN, Boston, MA.
f. University of Tennessee College of Veterinary Medicine Clinical Virology Laboratory, Knoxville, TN.
g. Sigma Chemical Co., St. Louis, MO.
h. Applied Biosystems, Foster City, CA.
i. Mallinckrodt, Chesterfield, MO.

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Adenovirus infection in Spanish ibex

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Numerous adenovirus types have been described in birds,[2,14] sheep,[3,7,18] swine,[10] horses,[12] and cattle.[17] In ruminants, 6 serotypes of ovine adenovirus (OAV 1–6), 9 bovine serotypes, some serotypes associated with pathologic processes in goats,[1,20] and 2 caprine adenovirus serotypes have been identified.[13]

Adenovirus infections are transmitted by feces, aerosols, or possibly fomites to susceptible animals (usually sucklings or recently weaned young) and are highly host specific.[20,26] Infection is most often subclinical, and disease seems to occur more commonly in immunocompromised individuals.[20]

Different strains and serotypes of OAV appear to differ in their pathogenicity and predilection for the intestinal tract, respiratory tract, or other organ systems. Experimental infection of lambs with adenoviruses usually produces lesions that are confined to the respiratory tract.[7,8] Occasional mild enteritis,[11] focal hepatocellular necrosis and cholangitis,[23] renal tubular necrosis, and interstitial nephritis[5] also occur.

Only 5 reported cases of adenovirus infection have occurred in goats: in association with outbreaks of peste des petit ruminants in Senegal[20] and Nigeria,[13] in a goatex with respiratory disease in Israel,[1] in a pygmy goat kid with hepatic disease in California,[26] and in a kid with diarrhea in the Canary Islands.[21] Neither disease nor serologic surveys of antibodies related to adenovirus infection have been reported in the Spanish ibex (*Capra pyrenaica hispanica*).

Seven Spanish ibex kids 5–6 months old were captured alive in the Sierra Nevada National Park (Spain) as part of a protection and repopulation program for this species. Forty-five days after capture, coinciding with weaning, three of the captured kids exhibited hyperthermia (41.5 C), asthenia, anorexia, intense dyspnea with abdominal respiration, cough, and bronchial rales. All three died within 24–48 hours following onset of the process. Necropsy revealed hydrothorax, moderate hydropericardium, and pulmonary congestion; focal necrosis was visible in the adrenal cortex. No relevant gross changes were observed in other organs.

Lung samples were submitted for microbiologic analysis. Bacterial isolation was performed using routine media, including 5% sheep blood agar, MacConkey agar,[a] TKT agar,[b] Baird-Parker medium,[c] and tryptose broth with 5% serum.[c] All samples were incubated in aerobic conditions, with the exception of blood agar (5% CO_2). Bacteria were identified by their growth in sets of metabolic substrates; these procedures were both manual[d] and automated.[d] Sterile swabs soaked in tissue obtained following incision of the cauterized lung surface were placed in test tubes containing sterile medium[c] for mycoplasma isolation. Attempts to isolate bacterial agents or mycoplasmas were negative.

Samples of digestive tract, liver, spleen, kidney, bladder, lung, mediastinal lymph node, adrenal glands, heart, and central nervous system were fixed in 10% formalin and submitted to the diagnostic service of Veterinary Faculty of Córdoba.

Formalin-fixed samples were routinely processed in paraffin for light microscopic examination. Tissue sections 4 μm thick were stained with hematoxylin and eosin (HE) and Feulgen to detect DNA and Fraser-Lendrum to detect fibrin.[19]

For electron microscopy, formalin-fixed adrenal cortex tissues were cut into 1-mm cubes, washed overnight in 0.1 M

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