Evaluation of antibodies against feline coronavirus 7b protein for diagnosis of feline infectious peritonitis in cats

Melissa A. Kennedy, DVM, PhD; Mohamed Abd-Eldaim, BVSc, MVSc, PhD; Sarah E. Zika, DVM, MPH; Joseph M. Mankin, DVM; Stephen A. Kania, PhD

Objective—To determine whether expression of feline coronavirus (FCoV) 7b protein, as indicated by the presence of specific serum antibodies, consistently correlated with occurrence of feline infectious peritonitis (FIP) in cats.

Sample Population—95 serum samples submitted for various diagnostic assays and 20 samples from specific-pathogen–free cats tested as negative control samples.

Procedures—The 7b gene from a virulent strain of FCoV was cloned into a protein expression vector. The resultant recombinant protein was produced and used in antibody detection assays via western blot analysis of serum samples. Results were compared with those of an immunofluorescence assay (IFA) for FCoV-specific antibody and correlated with health status.

Results—Healthy IFA-seronegative cats were seronegative for antibodies against the 7b protein. Some healthy cats with detectable FCoV-specific antibodies as determined via IFA were seronegative for antibodies against the 7b protein. Serum from cats with FIP had antibodies against the 7b protein, including cats with negative results via conventional IFA. However, some healthy cats, as well as cats with conditions other than FIP that were seropositive to FCoV via IFA, were also seropositive for the 7b protein.

Conclusions and Clinical Relevance—Expression of the 7b protein, as indicated by detection of antibodies against the protein, was found in most FCoV-infected cats. Seropositivity for this protein was not specific for the FCoV virulent biotype or a diagnosis of FIP. (*Am J Vet Res* 2008;69:1179–1182)

Feline infectious peritonitis is a lethal disease affecting domestic and nondomestic felidae. It is a substantial problem in catteries, multiple-cat households, and shelters. The disease has been estimated to cost cat breeders more than $2,000/cattery per year.[1] It is also a threat to many exotic felidae, including cheetahs, an endangered species.[2,3] This disease can occur as an effusive peritonitis or pleuritis with a short course ending in death. Alternatively, a more protracted course with granulomatous lesions affecting multiple organs may occur that also invariably progresses to death.[4]

Abbreviations

FCoV	Feline coronavirus
FIP	Feline infectious peritonitis
IFA	Immunofluorescence assay

These FIP-associated lesions reflect immune-mediated damage.[5]

The agent of this disease is FCoV. The FCoVs are closely related and consist of 2 biotype groups, one that is virulent (causing FIP) and one that is typically nonvirulent. The latter group, also known as feline enteric coronaviruses, may be associated with mild enteric disease or subclinical infection in cats.[4] Infection of cats with FCoV is common and usually causes mild enteritis or subclinical infection. In environments where large numbers of cats are housed closely together, 75% to 100% of the cats may be seropositive to the virus.[6] Despite these numbers, only a small percentage of FCoV-infected cats develop FIP. Host and virus factors may be involved with the pathogenesis of FIP, but the precise mechanism of disease remains unknown.

Host factors speculated to play a role in pathogenesis include cytokine production pattern, major histocompatibility complex expression, and lymphocyte responses.[7–10] Virus factors are important to disease

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From the Department of Comparative Medicine, College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37996-4543 (Kennedy, Abd-Eldaim, Zika, Mankin, Kania); and the Department of Virology, College of Veterinary Medicine, Suez Canal University, Ismailia, Egypt (Abd-Eldaim). Dr. Mankin's present address is Small Animal Medicine and Surgery, Veterinary Teaching Hospital, School of Veterinary Medicine, Purdue University, West Lafayette, IN 47907.
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Address correspondence to Dr. Kennedy.

development because they vary in virulence.[11] Virulent FCoV may arise from mutation of the infecting FCoV during replication in infected cats.[4,11–17] Mutations in several regions of the FCoV genome have been identified and are associated with changes in tissue tropism and virulence.[4,12,13,15–17] However, no consistent genetic difference has been identified that can distinguish all virulent from nonvirulent FCoVs. As a result, antemortem diagnosis of FIP is difficult because no detection assay that is specific and sensitive for the FIP virus is available.

The product of the 7b open reading frame, which is the gene nearest the 3′ end of the RNA genome, may be a virulence factor.[4,12,16] The product is a nonstructural protein of unknown function that appears to be highly conserved among strains of FCoV. Deletional mutations occurring in this gene have been found to lead to decreased virulence in experimental infections.[12] Investigators have theorized that 7b expression may be suppressed in many infections; therefore, mutational events that lead to removal of this suppression may lead to increased virulence.[17]

If expression of the 7b protein consistently leads to disease, cats infected with virulent FCoV would be expected to have measurable antibodies against this virus protein, whereas cats infected with the nonvirulent FCoV would not. This would allow differentiation of cats infected with virulent FCoV from those infected with a nonvirulent strain. To investigate this possibility, the purpose of the study reported here was to determine specific antibody concentrations against the 7b protein in cats with FIP or other diseases and healthy cats.

Materials and Methods

7b gene cloning and expression—Feline coronavirus from ascitic fluid of a kitten with FIP was used for 7b production. The genetic regions encompassing the 7a7b open reading frames first were amplified from the virus by use of reverse transcription–PCR techniques and cloned by use of a PCR cloning system[a] used in a previous investigation.[13] The recombinant plasmid was used as a template for amplification of the 7b gene alone. Primers were designed from this sequence data to encompass the 7b gene beginning at the start codon (nucleotide 1) and continuing to the stop codon (nucleotide 621), including the reverse primer 5′–GGAAGCTTGCC**TTA**TAACTCAGTT–3′ and the forward primer 5′–GGGAATTC**ATG**ATTGTTGTAACCCTT–3′ (bold indicates stop and start codons, respectively). Restriction enzyme sites were engineered into the 5′ end for directional cloning into the expression vector.

The 7b gene of FCoV was then amplified by use of the PCR assay. The resultant product was purified via gel purification[b] and directionally cloned into the prokaryotic expression system.[c] The vector contains a 6X histidine affinity tag for specific detection and ease of purification. The recombinant plasmid was transformed into bacterial competent cells,[d] after which the nucleotide sequence of the expression recombinant was determined to verify the identity and correct orientation of the gene for transcription.

The protein expression protocol was performed according to the manufacturer's directions.[c] Briefly, transformed *Escherichia coli* were propagated in Luria broth, and expression was induced with isopropyl β-D-1-thiogalactopyranoside. The expressed protein was extracted from the bacteria by use of protein extraction reagent.[e] The product was identified by use of PAGE[f] staining with Coomassie blue to detect the protein and verified via western blot analysis with specific anti-His reagent.[g] Western blot analysis with feline anti-FCoV serum[h] was also used to detect the location of the 7b gene protein, which was in the inclusion body insoluble sediment, not the soluble fraction (supernatant). The purification process from the sediment was performed with His bind kits[i] according to the manufacturer's protocol.

Serum samples—Ninety-five serum samples submitted for various diagnostic assays were used for serologic analysis. Nineteen cats were confirmed to have FIP via results of a combination of diagnostic assays (antemortem) or histopathologic lesions (postmortem). Eleven cats had no health problems (samples were submitted for FCoV antibody screening). Nine cats had diseases other than FIP (hypertrophic cardiomyopathy [n = 2], diabetes mellitus, lymphosarcoma, fibrosarcoma, FIV infection, hemobartonellosis, heartworm disease, and toxoplasmosis). In addition, samples collected from 11 cheetahs (*Acinonyx jubatus*) housed at 2 institutions were tested. One of the cheetahs died of FIP, whereas the rest housed at a separate institution were healthy. Forty-five samples were from cats that had a variety of clinical signs, some suggestive of FIP, but for which the final diagnosis was unavailable. Twenty additional samples from specific-pathogen–free cats were tested as negative control samples.[j]

Western blot analysis—Purified 7b protein was loaded onto 10% Tris-HCl 2-well PAGE,[f] transferred to a nitrocellulose membrane, and cut into individual strips. Each individual strip was incubated with the cat's serum. The reaction was developed by use of peroxidase-labeled antibody against cat IgG,[k] followed by addition of substrate.[l] A strong positive result was recorded if the protein band was easily visible in the nitrocellulose strip. A weak positive result was recorded if the band was faint and negative if no band was evident.

Immunofluorescence assay (IFA)—Antigen slides were made by use of type II FCoV (WSU 1143) propagated in Crandell feline kidney cells.[m] Serial dilutions (1:40 to 1:640) of the serum-plasma samples were made in PBS solution, applied to virus-infected cells, and incubated. Bound antibody was detected with anti-feline IgG conjugated to fluorescein isothiocyanate.[k] The antibody titer was reported as the reciprocal of the highest dilution in which fluorescence was still present. Antibody titers < 40 were considered negative, titers of 40 to 640 were considered low to moderate, and titers > 640 were considered high.

Results

Expression of the 7b protein in the prokaryotic expression system produced a product of the appropriate

molecular weight (**Figure 1**). The IFA and western blot results revealed that the sera from the specific-pathogen–free cats did not have FCoV- and 7b-specific antibodies, respectively.

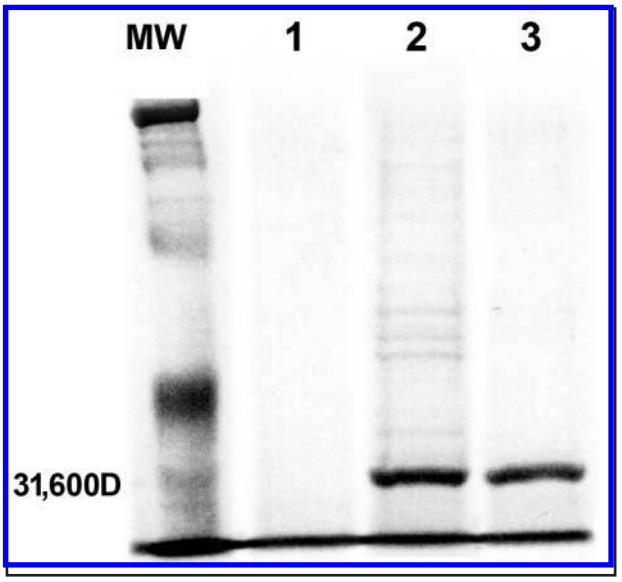

Figure 1—Results of western blot analysis of cat serum by use of specific anti-His reagent to detect 7b gene product from a prokaryotic expression system[c] (appropriate molecular weight of approx 35 kd). MW = Molecular weight marker. Lane 1 = Protein extraction prior to induction of expression. Lanes 2 and 3 = Protein extraction after induction of expression.

Table 1—Results of western blot analysis of clinical samples for FCoV 7b protein in cats with various health statuses.

Health status	Strong positive	Weak positive	Negative
FIP	19	0	0
Other conditions	2	4	3
Healthy			
FIP housemate	4	0	0
Other	5	0	2
Cheetahs			
FIP	1	0	0
Known PI	2	0	0
Healthy	0	0	8
Cats of unknown status	18	18	9
Total	**51**	**22**	**22**

PI = Persistently infected.

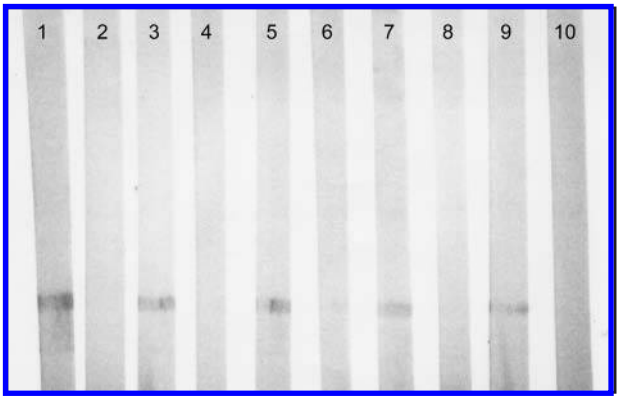

Figure 2—Results of western blot analysis of cat serum by use of FCoV antiserum against 7b protein (strips 1, 3, 5, 7, and 9 are strongly positive; remaining strips are negative).

All 19 serum samples from cats with confirmed FIP yielded a strong positive reaction to the 7b protein with the western blot test (**Table 1**; **Figure 2**). In addition, serum from the cheetah with FIP also reacted strongly. Although most of these animals had markedly high FCoV-specific antibody concentrations via IFA (titer, > 640), 2 had low concentrations (titer, 80), and 4 had undetectable concentrations. Of serum samples from 9 cats confirmed to have diseases other than FIP, 2 yielded strong positive results for 7b protein via western blot analysis (IFA titer, 160 and 5,120), 4 yielded weak positive results (IFA titer, negative to 320), and 3 yielded negative results (IFA titer, 80). Of serum samples from 11 cats with no health problems, 9 yielded strong positive results for 7b protein via western blot analysis. For the latter samples, IFA titers ranged from 640 to 1,280. Four of these were from cats that were housemates of cats that had died from FIP, 4 were from a laboratory animal facility, and 1 was a healthy kitten. Of the 10 healthy cheetahs tested, 2 yielded strong positive results via western blot analysis. The latter 2 cheetahs were known to be persistently infected with FCoV and had IFA titers > 640.[2,3]

The remaining 45 cats tested had a variety of health problems, but because their samples were submitted from non–University of Tennessee veterinarians, the final diagnosis remained unknown. Of those, 18 yielded strong positive results for 7b protein via western blot analysis (IFA, negative to > 5,120). Only 9 yielded negative results, 7 of which were also negative via IFA testing. The remaining samples were weakly positive by 7b western blot (IFA, negative to > 5,120).

Discussion

Detection of antibodies specific for the 7b protein of FCoV has been speculated to be a means for distinguishing infection with FIP virus from enteric FCoV strains. At least 1 commercial laboratory uses this assay as a means to confirm a diagnosis of FIP. The present study revealed that most cats tested had antibodies specific for the 7b protein, including healthy cats and cats that were seronegative via routine IFA testing. The fact that 2 cats that were seronegative to 7b protein were seropositive via IFA testing may indicate that this protein is expressed in most but not all FCoV infections because the sensitivity of the western blot test is generally higher than that of the IFA test for antibody detection. Alternatively, the immunologic response to this protein may vary among cats.

Interestingly, all cats with FIP yielded strong positive results via western blot analysis, including 4 cats that were seronegative via IFA testing. However, cats with conditions other than FIP often were seropositive, as were healthy cats. Thus, although a negative result may be helpful for ruling out FIP, a positive result is not specific for FIP. Four of the healthy seropositive cats were from households that had experienced FIP, and the 2 healthy cheetahs that were seropositive were known to be persistently infected with FCoV.[2,13] Persistent infection with FCoV may contribute to emergence of virulent mutants because the virus continues to rep-

licate in such cats, allowing mutations to arise.[4] Persistently infected cats are known to be important sources of the virus in multi-cat environments.[2,13] However, it is unknown whether the 7b protein plays a role in persistence of FCoV in infected cats.

Findings suggested that this assay may be of questionable value to confirm a diagnosis of FIP, because although all cats with FIP were strongly seropositive, cats with other conditions as well as healthy cats were also seropositive. It is possible that cats without FIP but seropositive for the 7b protein could have developed the disease at a later date because cats were not followed over time. However, because most cats tested had 7b antibody and yet FIP occurs in only a small percentage of FCoV-infected cats, this possibility seems unlikely. Seronegative status may give support to elimination of FIP as a diagnosis, but seropositive status to the 7b protein cannot confirm a diagnosis of FIP and should never lead to euthanasia of an otherwise healthy cat.

a. TA Cloning System, Invitrogen Life Technologies, Carlsbad, Calif.
b. Qiaquick gel extraction kit, Qiagen, Valencia, Calif.
c. pPROEX HT Prokaryotic Expression System, GibcoBRL Life Technologies, Gaithersburg, Md.
d. One Shot BL21 Star Competent Cells, Invitrogen Life Technologies, Carlsbad, Calif.
e. BugBuster protein extraction reagent, Novagen, Madison, Wis.
f. 2D-PAGE/PrepComb, Bio-Rad Laboratories, Hercules, Calif.
g. INDIA His Probe-HRP, Pierce, Rockford, Ill.
h. VMRD, Pullman, Wash.
i. His bind kits, Novagen, Gibbstown, NJ.
j. Liberty Research, Indianapolis, Ind.
k. Kirkegaard-Perry Laboratories, Gaithersburg, Md.
l. 1-step chloronaphthol [4 CN], Pierce, Rockford, Ill.
m. Crandell-Reese feline kidney (CRFK), American Type Culture Collection, Manassas, Va.

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