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DETECTION OF FELINE CORONAVIRUS INFECTION IN CAPTIVE CHEETAHS (*ACINONYX JUBATUS*) BY POLYMERASE CHAIN REACTION

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Abstract: Feline coronavirus genetic elements were detected by polymerase chain reaction from blood, fecal samples, and effusive fluid collected from 33 cheetahs in the U.S.A. Feline coronavirus-specific serum antibodies were also measured by indirect immunofluorescence. Ten cheetahs were positive for viral shedding by polymerase chain reaction, whereas 13 were seropositive by immunofluorescence. Results of serology did not consistently correlate with shedding of virus, and the capture antigen used for detection of feline coronavirus-specific antibodies had a significant impact on results. Testing of samples from one population over a 1-yr period indicated chronic infection in some animals. These relatively healthy carrier animals were a source of virus for contact animals. Screening programs in cheetah populations for feline coronavirus infection may be most reliable if a combination of serologic analysis and viral detection by polymerase chain reaction is used.

Key words: Feline coronavirus, feline infectious peritonitis, cheetah, *Acinonyx jubatus*, epidemiology, polymerase chain reaction.

INTRODUCTION

Feline coronavirus (FCoV) is an important pathogen of both domestic and nondomestic felines.[1,14] Disease resulting from infection may vary in severity, from subclinical to such severe, life-threatening disease as feline infectious peritonitis (FIP). Both host and virus-related factors may influence the severity of disease, but the specific factor remains certain.[9,16] Diagnosis is complicated by the existence of at least two antigenically distinct serotypes of FCoV, types I and II.[9,15] Spike proteins in the two types differ, with type II encoding a spike protein very similar to that of canine coronavirus.[7] Within both of these serotypes, virulent (FIP) and avirulent biotypes occur.[7,15] Serious disease may arise from FCoV mutation in the intestinal tract of infected cats.[15] Neither serology nor genetic analysis can distinguish the biotypes, so it is not possible to screen for virulent FCoV.

Cheetahs (*Acinonyx jubatus*) are especially vulnerable to FCoV-induced disease,[1,13] so the epidemiology and molecular biology of FCoV, as well as optimal screening methodology, must be understood in order to manage captive cheetah populations.

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Polymerase chain reaction (PCR) technology can detect FCoV in domestic cat populations.[4,8,10] Our report describes an assay for the detection of coronavirus genomic elements in biological samples, including feces and plasma, from cheetahs.

MATERIALS AND METHODS

We evaluated six serum samples, 26 plasma samples, nine whole blood samples, one abdominal effusion sample, and 82 fecal specimens from 33 captive cheetahs in the U.S.A. for the presence of FCoV by PCR techniques. For some animals, multiple samples were submitted, and with population F (see below), samples from multiple time points were submitted. Feces were tested in order to detect virus shedding. For six animals, only blood (and abdominal effusion from one animal of these) was submitted.

The abdominal effusion was collected from a sick cheetah that later died from histopathologically confirmed FIP. Eighteen of the cheetahs were in a collection in which two cheetahs may have died from FCoV (see description below). In this population (F), most animals were tested every 4 mo or less over a period of 1 yr, during which time the second death occurred. The first death occurred prior to this investigation. Two animals in this population were tested monthly. Single samples were obtained from the other cheetahs at the other five institutions (A–E) in the U.S.A. along with relevant individual health information.

History of population F

Two female cheetahs (32 and 33), allegedly seronegative for FCoV, arrived at the institution in

March 1995. They were quarantined for 16 mo, although three other females were housed nearby in the quarantine section. These three females were not in direct contact with the imported females until January 1997. In January 1996, the first episodes of abnormal stools (loose to overt diarrhea) were noticed in the two imported females, as well as in the three nearby resident female cheetahs. In March 1996, one of these resident females died from necrotizing colitis, but tissue samples were not available for FCoV analysis. Abnormal stools continued in the remaining resident contact females and the two imported females through 1997–1998.

A male cheetah was brought into direct contact with the imported females for the first time in January 1997, with extensive fence contact from September 1997 through January 1998. This male died in January 1998 of leukoencephalomalacia.

Also in January 1997, two male cheetahs were moved into an enclosure immediately after the two imported females were removed from it. These males subsequently had their first direct exposure to the imported females from September 1997 to March 1998. One of the males died in July 1998 from necrotizing colitis.

None of the remaining cheetahs in population F have been in contact with the imported females, the contact females and males, or any of these animals' enclosures.

Five surviving cheetahs in population F, therefore, including the imported females, formed the ''exposed'' group. All keepers cared for the entire carnivore collection. When the infection status of the exposed group was discovered, quarantine procedures were implemented, and the exposed group was not intermingled with the other cheetahs.

Within population F, the individual's virus infection status over a 12-mo period was evaluated by PCR.

RNA extraction, reverse transcription, and PCR

All biological samples were stored at −70°C. Total RNA was extracted from the specimens with Trizol LS (Gibco BRL, Baltimore, Maryland 21279, USA) according to the manufacturer's directions. The RNA was used for reverse transcription with Moloney murine leukemia virus reverse transcriptase (Gibco BRL) according to the manufacturer's recommendations with the downstream external primer.[10] Nested PCR was done with ExTaq polymerase (Intergen, Purchase, New York 10577, USA).[6] Primers encompassing the entire 7a7b open reading frame (ORF), containing approximately 1,000 nucleotides, were used for amplification.[10,11] In vitro-propagated FCoV strain WSU1143 was the positive PCR control (American BioResearch, Sevierville, Tennessee 37864, USA), and water was the negative control. Products were evaluated by electrophoresis on 1% agarose gels.

Serology

FCoV-specific antibody titers were measured by indirect immunofluorescence for all 28 cheetahs from which serum or plasma was provided.[1] A type I (UCD1) and a type II (WSU 1143) FCoV (American BioResearch) were used as capture antigens. These viruses were isolated from two separate cases of FIP. Each was propagated separately in Crandell feline kidney cells. The virus-infected cells were applied and fixed to glass slides. Twofold serial dilutions of the serum/plasma were made starting at a 1:5 dilution and proceeding to a maximum of 1:5,120. Serologic testing for FCoV-specific antibodies was performed on serial dilutions of serum starting at a 1:5 dilution in order to ensure that any antibody level was detected. Though low titers are considered insignificant in terms of FIP disease diagnosis, we were interested in detecting any previous exposure to a FCoV.[9] Antibody-positive and -negative serologic controls (VMRD, Pullman, Washington 99163, USA) were purchased. Antibody was detected with rabbit anti-feline IgG conjugated to fluorescein isothiocyanate (VMRD). The titer was reported as the reciprocal of the highest dilution in which fluorescence was observed. Antibody titers of <5 were considered negative.

RESULTS

PCR results

Results are shown in Tables 1 and 2, with a positive PCR result indicating that FCoV was detected by PCR in at least one sample from that animal. Ten animals (30%) were PCR positive. Five of 15 cheetahs (33%) from five U.S.A. institutions other than population F tested positive by PCR in biologic samples (primarily feces). One of the five cheetahs was positive in feces but negative in blood, one tested positive in blood only (no feces submitted), one in effusive fluid only (no feces submitted), and two tested positive in both feces and blood.

Five of the cheetahs in population F (28%), all either imported or exposed to the imported cheetahs, tested positive for FCoV in feces (Table 2). Three of these (17%) were positive on more than one occasion. Four of them (22%) tested positive in samples collected in July 1998, which thus appears to have been a peak shedding time.

Table 1. Health status and feline coronavirus (FCoV) polymerase chain reaction (PCR) and serologic results for cheetahs from institutions A–E.

Cheetah number	Facility	Health status	PCR results	Serology	
				FCoV-I	FCoV-II
1	A	Feline infectious peritonitis	+	>640	<5
2	A	Healthy	−	<5	<5
3	A	Healthy	−	320	40
4	B	Healthy	+	<5	<5
5	C	Healthy	−	<5	<5
6	C	Healthy	−	<5	<5
7	C	Healthy	+	<5	<5
8	C	Healthy	−	>640	<5
9	D	Gastritis	+	<5	<5
10	D	Loose feces	−	<5	<5
11	D	Chronic diarrhea	−	20	<5
12	D	Healthy	−	<5	<5
13	E	Healthy	+	160	<5
14	E	Weight loss/poor appetite	−	<5	<5
15	E	Healthy	−	ND[a]	ND

[a] ND = not done.

Samples were available from only one of the dead animals, the male that died of necrotizing colitis in January 1998. PCR tests on feces collected monthly for the 3 mo preceding its death were negative. However, the cheetah's FCoV-specific antibody level rose from negative in 1996, to 625 in March 1997 (2 mo postexposure to imported females), to 3,125 in June 1998, 1 mo prior to death.

Table 2. Health status and feline coronavirus (FCoV) polymerase chain reaction (PCR) and serologic results for captive cheetahs from institution F.

Cheetah number	Facility	Health status	PCR results	Serology	
				FCoV-I	FCoV-II
16	F	Healthy	−	<5	<5
17	F	Healthy	−	<5	<5
18	F	Healthy	−	<5	<5
19	F	Healthy	−	<5	<5
20	F	Healthy	−	ND[a]	ND
21	F	Healthy	−	40	<5
22	F	Healthy	−	10	<5
23	F	Healthy	−	<5	<5
24	F	Healthy	−	<5	<5
25	F	Healthy	−	ND	ND
26	F	Healthy	−	ND	ND
27[b]	F	Healthy	−	20	<5
28[b]	F	Intermittent abnormal feces	+	320	<5
29[b]	F	Intermittent abnormal feces	+	10	<5
30[b]	F	Intermittent abnormal feces[c]	−	ND	ND
31[b]	F	Intermittent abnormal feces	+	20	<5
32[d]	F	Intermittent abnormal feces	+	>640	<5
33[d]	F	Intermittent abnormal feces	+	>5,120	640

[a] ND = not done.
[b] Exposed to imported females.
[c] Death due to necrotizing colitis July 1998.
[d] Imported females.

This testing, done at another reference laboratory as part of the routine monitoring of the cheetah population, is not included in our other data.

Fecal samples from the two imported females collected monthly from May to August 1998, in December 1998, and in March 1999 were positive by PCR for FCoV, showing constant shedding from these animals. The individuals may be chronically FCoV infected.

FCoV serology

Serology with type I FCoV as the capture antigen identified 13 cheetahs (39%) with detectable antibody levels (Tables 1, 2). However, when type II FCoV was used, only two cheetahs (6%) were seropositive. Five cheetahs (15%) had antibody titers >80 to type I FCoV but were seronegative for type II FCoV.

Three of the 10 PCR-positive cheetahs (33%) were seronegative to both types I and II FCoV (Table 1). Six of the 13 seropositive cheetahs (46%) were negative by PCR (Tables 1, 2). Serologic screening did not identify all cheetahs shedding virus. Conversely, PCR did not detect all animals previously exposed to FCoV.

All five PCR-positive cheetahs from population F were seropositive to type I FCoV (Table 2). Two of these resident females had low titers to type I, whereas a male had a titer of 320 to type I. The two imported females had titers ≥640. Four of the five were seronegative to type II. One of the imported females with a titer of 640 to type I was seronegative to type II. The other imported female had a titer of 5,120 to type I and 640 to type II. The contact male that died in July 1998 had a rising antibody level in March 1997 and June 1998 (testing done at another laboratory).

Cheetah health status

Three deaths occurred within population F since the importation of two females in 1995: one from leukoencephalomalacia, and two from necrotizing colitis that may have involved FCoV, although only one of the cheetahs with colitis was tested. This animal was negative by PCR with the 7a7b primer set three times between May and July 1998, when it died. The remaining cheetahs from population F were healthy, though abnormal stools were noted on repeated occasions in five animals. Each of these was PCR positive in at least one sample.

Of the remaining cheetahs (not members of population F), one was verified by histopathology to have died of FIP. This animal's abdominal effusion was PCR positive. One cheetah at another institution was suffering from chronic diarrhea, and although this animal was PCR negative, a contact cheetah was PCR positive. A third PCR-positive cheetah was from a population in which a contact animal had experienced weight loss and decreased appetite and another contact had suffered diarrhea. The remaining two PCR-positive cheetahs and their contacts are healthy to date.

DISCUSSION

The 7a7b genes, the 3′-most ORFs of the FCoV genome, were targeted for amplification. These genes were used for several reasons, including the consistent success our laboratory has had in amplifying FCoV genetic material regardless of virus strain, the proposed association of this region with virulence, and the possibility that the avirulent form of FCoV does not express the 7b protein.[15,16] Although the functions of the 7a7b gene products are unknown, this region is associated with virulence because deletions that occur in this region in some virus isolates have been shown to lead to decreased virulence of the virus.[8,15,16]

PCR proved to be a sensitive assay for detection of virus shedding. FCoV is prevalent among captive cheetahs in the U.S.A. because nearly one-third of the animals we tested were shedding FCoV in their feces or had detectable virus in plasma. Although not all infected animals exhibited characteristic FIP, disease consistent with FCoV infection had occurred in four of the six infected cheetah populations. FCoV may be a factor in cheetah gastrointestinal diseases, particularly those exhibiting such vague signs as abnormal stools, decreased appetite, and/or weight loss.

Our results indicate that a combination of serologic analysis and PCR detection of virus shedding may be needed to detect FCoV infection. Serology results do not consistently correlate with PCR results for detection of virus shedding, as in FCoV-infected domestic cat populations.[5,10] Seronegative animals were occasionally virus positive, whereas the converse was also true. Thus, although serology can detect previous exposure to the virus, PCR is more sensitive for detecting viral shedding. Additionally, the capture antigen used for detection of FCoV-specific antibodies had a significant impact on the results, with type II FCoV frequently leading to false-negative results. Type II FCoV biotypes are more closely related to canine coronavirus antigenically than to type I FCoV.[7] Although types I and II FCoV cross-react, their antigenicities are sufficiently different that low titers to one type may be missed when using the other to measure virus-specific antibody. Thus, serologic screening that uses only one serotype is inherently flawed. In addition,

one isolate of cheetah FCoV is antigenically distinct from FCoV of domestic cats.[2,6] This antigenic diversity impacts serology results and may explain our findings. The antigenic disparity between the infection and assay viruses may result in failure of antibody detection.

The epizootiology of captive population F was examined. From the history and PCR data, the two imported females were probably chronically infected with FCoV when they arrived at this institution. Chronic carrier states lasting a period of months occur in domestic cats.[12,15] Infection of additional cheetahs probably occurred by direct and/or indirect exposure, primarily through shared enclosures and through-the-fence contact. As noted, peak shedding in the population occurred in July 1998. Male stimulation trials, involving across-the-fence access of these males to females for the purpose of stimulating reproductive activity in the female and selection of male mates, began in May 1998. Resulting stress may have induced viral shedding or predisposed the cheetahs to infection. Similar patterns of shedding have been noted in closed populations of domestic cats, with waxing and waning of both infection and viral shedding.[3] Recurrent abnormal feces, occasionally severe, were noted in this group. Two cheetahs died of necrotizing colitis, possibly related to FCoV infection. Samples were available from only one of these and were PCR negative, possibly due to viral nucleotide sequence variation, including deletions in the genomic region amplified, that we have documented in population F, leading to failure of amplification due to loss of primer binding sites (unpubl. results).

FCoV is clearly an important contagious pathogen of captive cheetahs. Carrier animals may be an important source of infection through direct and indirect contact with susceptible animals, and serious disease may occur in some infected cheetahs. Screening for infection only with serology, especially if only one serotype is used, is not ideal. The optimal screening methodology uses both serologic analysis and PCR fecal virus detection.

Total RNA extraction, reverse transcription, and nested PCR were used to amplify the 7a7b ORFs of the FCoV genome. This region amplifies consistently in samples from domestic cats.[9] We also evaluated the level of FCoV-specific antibodies by indirect immunofluorescence in the majority of animals tested by PCR. Comparison of virus infection by PCR for virus detection vs. serology for virus-specific antibody was done. Finally, a zoologic collection of cheetahs was tested by PCR over a period of 1 yr in order evaluate the epidemiology of infection in a captive population.

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