DETECTION OF FELINE CORONAVIRUS IN CHEETAH (*ACINONYX JUBATUS*) FECES BY REVERSE TRANSCRIPTION-NESTED POLYMERASE CHAIN REACTION IN CHEETAHS WITH VARIABLE FREQUENCY OF VIRAL SHEDDING

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DETECTION OF FELINE CORONAVIRUS IN CHEETAH (*ACINONYX JUBATUS*) FECES BY REVERSE TRANSCRIPTION-NESTED POLYMERASE CHAIN REACTION IN CHEETAHS WITH VARIABLE FREQUENCY OF VIRAL SHEDDING

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Abstract: Cheetahs (*Acinonyx jubatus*) are a highly threatened species because of habitat loss, human conflict, and high prevalence of disease in captivity. An epidemic of feline infectious peritonitis and concern for spread of infectious disease resulted in decreased movement of cheetahs between U.S. zoological facilities for managed captive breeding. Identifying the true feline coronavirus (FCoV) infection status of cheetahs is challenging because of inconsistent correlation between seropositivity and fecal viral shedding. Because the pattern of fecal shedding of FCoV is unknown in cheetahs, this study aimed to assess the frequency of detectable fecal viral shedding in a 30-day period and to determine the most efficient fecal sampling strategy to identify cheetahs shedding FCoV. Fecal samples were collected from 16 cheetahs housed at seven zoological facilities for 30 to 46 consecutive days; the samples were evaluated for the presence of FCoV by reverse transcription-nested polymerase chain reaction (RT-nPCR). Forty-four percent (7/16) of cheetahs had detectable FCoV in feces, and the proportion of positive samples for individual animals ranged from 13 to 93%. Cheetahs shed virus persistently, intermittently, or rarely over 30–46 days. Fecal RT-nPCR results were used to calculate the probability of correctly identifying a cheetah known to shed virus given multiple hypothetical fecal collection schedules. The most efficient hypothetical fecal sample collection schedule was evaluation of five individual consecutive fecal samples, resulting in a 90% probability of identifying a known shedder. Demographic and management risk factors were not significantly associated ($P \leq 0.05$) with fecal viral shedding. Because some cheetahs shed virus intermittently to rarely, fecal sampling schedules meant to identify all known shedders would be impractical with current tests and eradication of virus from the population unreasonable. Managing the captive population as endemically infected with FCoV may be a more feasible approach.

Key words: *Acinonyx jubatus*, cheetah, feces, feline coronavirus, feline infectious peritonitis virus, reverse transcription-nested polymerase chain reaction, shedding.

INTRODUCTION

Cheetahs (*Acinonyx jubatus*) are globally endangered, and the African subspecies is listed as vulnerable by the International Union for Conservation of Nature and as threatened by the Convention on the International Trade in Endangered Species-Appendix I.[4] Estimates of the number of cheetahs remaining in the wild range from 10,000 to 12,000 in Africa, with the majority in Namibia. The wild population is threatened because of habitat loss, poaching, and conflicts with humans.[23,24] To combat decline in numbers and enhance genetic diversity, reproduction of the captive cheetah population in the United States is intensively managed by the American Zoo and Aquarium Association (AZA) Cheetah Species Survival Plan (SSP). Management practices include cohousing and movement of animals between zoological facilities for breeding, practices that also provide opportunity for disease transmission. Despite concerted efforts, the captive cheetah population is not self-sustaining.[22] In the AZA Cheetah SSP population, deaths outnumber births because of poor fertility and a high prevalence of diseases, such as chronic gastritis associated with *Helicobacter* sp., amyloidosis, glomerulosclerosis, and veno-occlusive disease.[21,27,41] Cheetahs are also susceptible to infection with feline coronavirus (FCoV), and infection

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is often subclinical or causes transient mild enteritis or diarrhea.[2,6–8,27] Feline infectious peritonitis virus (FIPV), a mutated and deadly variant of feline enteric coronavirus (FECV), was the cause of an epidemic in a cheetah breeding facility, with high morbidity and mortality.[6,36] This epidemic, combined with widespread serologic evidence of FCoV exposure in the AZA Cheetah SSP population, resulted in the restriction of movement of seropositive cheetahs to prevent the spread of infection and increased risk of disease.[7] Implementation of this prevention strategy restricted movement of seropositive cheetahs between facilities, thereby preventing pairing of some breeding animals used to maintain genetic diversity.

FCoV is a member of the family *Coronaviridae* and is an enveloped, single-stranded positive-sense RNA virus, with two serotypes, type I and type II, and two biotypes, FECV and FIPV.[33] FECV is transmitted mainly by the fecal–oral route, infects the mature apical villi of intestinal epithelium, and is shed mainly in feces and rarely in saliva.[1,34] In domestic cats, when fecal shedding of FCoV has been monitored for as long as 7 yr, there are variable patterns of shedding that have been described as absent, or of low, medium, or high frequency.[1,9,11] Initial infection may be followed by complete viral clearing without shedding. If the virus is not cleared, persistent infection and intermittent shedding may result. Intermittent shedding of virus also may occur if a cat is reinfected. Lastly, infection may be followed by continuous shedding, suggesting an inability to clear the virus. The reasons for the different patterns of fecal shedding after infection with FECV are not completely understood but may be associated with cycles of reinfection or persistence of virus in colonic enterocytes.[13,18,20] Both intermittent and continuous shedders may be asymptomatic. In cheetahs, it has been suggested that shedding of FCoV in the feces may be intermittent and that shed virus is an important source of infection to other cheetahs.[15]

In domestic cats, FECV is considered a ubiquitous intestinal virus that is common in multicat households and catteries, and it can cause mild transient enteritis and diarrhea; yet, infection is rarely fatal.[18,26,34,35] In cheetahs, unlike cats, FECV may rarely be associated with chronic ulcerative colitis[15] (Munson, unpubl. data). FIPV is a mutated form of FECV, and multiple mutations have been identified.[3,37,39] Genetic mutations in the 7a7b open reading frame of coronavirus in cheetahs also have been identified.[17] After mutation, FIPV acquires the ability to enter and replicate in macrophages and spread systemically, resulting in either the effusive "wet form," the granulomatous "dry form," or a combination of the two forms of the disease. Descriptions of the clinical manifestations and pathologic lesions of FIP are similar between domestic cats and cheetahs, including fibrinopurulent pleuritis, peritonitis, and vasculitis, as well as multifocal necrosis throughout many organs.[5,19,36] However, cheetahs are proposed to be more susceptible to viral infections, such as FCoV, due to genetic monomorphism of the major histocompatibility complex associated with a genetic bottleneck. Therefore, it has been suggested that cheetahs may be more likely to develop fatal FIP when infected with FECV.[25,30,31]

Historically, serologic testing for FCoV was mandated by the AZA Cheetah SSP to identify seropositive cheetahs before movement between zoologic facilities. However, interpretation of serologic test results is complicated because the detection of serum antibodies may only indicate previous, not current, infection with the virus, and serology does not distinguish between FECV and FIPV. In addition, as in domestic cats, seropositivity does not correlate with active infection or fecal shedding of the virus in cheetahs.[11,14–16] To use fecal detection of FCoV in cheetah feces as a complementary screening tool to identify actively shedding animals, the frequency of fecal shedding of virus needs to be more closely evaluated.

This study had three objectives: 1) to determine the optimal handling and storage procedures for detecting FCoV by reverse transcription-nested polymerase chain reaction (RT-nPCR) in fecal samples; 2) to assess the frequency of FCoV shedding in cheetahs naturally exposed to FCoV, so that a reasonable fecal sampling schedule with a high probability of identifying a cheetah shedding FCoV can be recommended; and 3) to assess potential demographic and management risk factors for association with fecal shedding of virus. This information will be used to assess whether fecal RT-nPCR for FCoV is a useful test for identifying actively shedding animals and to recommend appropriate testing protocols.

MATERIALS AND METHODS

Study population

Twenty-five AZA Cheetah SSP cheetahs, 8 males and 17 females, ranging in age from 1 to 15 yr, with a median age of 10 yr, from seven geographically distinct zoological facilities in the

Table 1. Zoological facility and individual animal information for 25 cheetahs including prior FCoV serology, prior FCoV fecal RT-nPCR, this study's FCoV fecal RT-nPCR results, and percentage of positive FCoV fecals by RT-nPCR for this study's collection period.

ID no.	Facility	Facility size	Facility FCoV	Group	Age (yr)	Sex	Origin	Cohoused	Diarrhea	Prior cheetah FCoV serology	Prior cheetah FCoV fecal shedding	Current cheetah FCoV fecal shedding (%)
C5	C	S[a]	N[b]	2[c]	10	F[d]	C[e]	N	N	Neg[f]	Neg	Neg
C6	C	S	N	2	12	M[g]	C	N	N	Neg	Neg	Neg
C24	G	S	N	2	2	M	C	Y	N	Neg	Neg	Neg
C25	G	S	N	2	2	M	C	Y	N	Neg	Neg	Neg
C4	B	S	Y[h]	1[i]	8	F	C	Y	N	NS[j]	Neg	Neg
C3	B	S	Y	1	14	M	C	Y	N	NS	Neg	Neg
C12	D	L[k]	Y	1	11	F	C	N	N	Neg	Neg	Neg
C13	D	L	Y	1	4	F	W[l]	N	N	Neg	Neg	Neg
C1	A	L	Y	2	4	F	C	N	N	Neg	Neg	Neg
C2	A	L	Y	2	14	F	C	N	N	Neg	Neg	Neg
C14	E	S	Y	1	12	F	C	Y	Y	Neg	Pos	Neg
C17	D	L	Y	1	4	F	C	Y	N	Neg	Pos	Neg
C18	D	L	Y	1	10	F	C	N	N	Neg	Pos	Neg
C20	F	S	Y	1	10	F	C	Y	N	Neg	Pos	Neg
C21	F	S	Y	2	10	F	C	Y	Y	Neg	Pos	Neg
C22	D	L	Y	2	1	M	C	Y	N	Neg	Pos	Neg
C23	D	L	Y	2	11	M	C	Y	N	Neg	Pos	Neg
C19	F	S	Y	1	10	M	C	Y	N	Pos[m]	Pos	Neg
C7	D	L	Y	1	2	F	C	Y	N	Neg	Pos	Pos (43)
C8	D	L	Y	1	2	F	C	Y	N	Neg	Pos	Pos (33)
C9	D	L	Y	1	6	F	C	N	N	Neg	Pos	Pos (13)
C10	D	L	Y	1	2	F	W	Y	N	Neg	Pos	Pos (24)
C11	D	L	Y	1	6	F	C	N	N	Neg	Pos	Pos (35)
C15	E	S	Y	1	13	M	C	Y	Y	Neg	Pos	Pos (23)
C16	E	S	Y	1	15	F	C	Y	Y	Pos	Pos	Pos (93)

[a] S, small institution with ≤15 cheetahs.
[b] N, no.
[c] 2, five fecal samples assessed.
[d] F, female.
[e] C, captive born.
[f] Neg, negative.
[g] M, male.
[h] Y, yes.
[i] 1, ≥30 days of fecal samples assessed.
[j] NS, not sampled.
[k] L, large institution with >15 cheetahs.
[l] W, wild caught.
[m] Pos, positive.

United States (Facilities A–G), were included in the study (Table 1). Two cheetahs were wild caught and 23 were captive born. Cheetahs selected for the study met one or more of the following criteria: prior positive FCoV serology (antibody titer >1:40), prior positive FCoV fecal RT-PCR, housed in or originating from an institution with endemic FCoV, or scheduled to move to a facility free of FCoV. Positive serologic results were based on previously published criteria.[14,15] In brief, FCoV-specific antibody titers were measured by indirect immunofluorescence using type I (UCD1) and type II (WSU 1143) FCoV as capture antigens. Antibody titers were defined as the highest dilution that resulted in fluorescence, and a titer of ≤1:40 was considered negative. Additional variables, including cohousing, number of cage-mates, sharing of exhibit space, and institutional information were obtained from questionnaires (Table 1).

Sample collection and storage

For each of the 25 cheetahs, fecal samples were collected daily for a minimum of 30 and a maximum of 46 days, stored at –20°C or –80°C at the participating zoological facility, and

Table 2. Results of RT-nPCR for FCoV on cheetah feces stored for increasing lengths of time, at different temperatures, with and without RNA stabilization solution for fecal samples run in triplicate.

Condition	Temp (°C)	Days to extraction	RNA*later*	RT-nPCR result–chronic shedder	RT-nPCR result–intermittent shedder
A	20–25	0	N[a]	+[b]	+/−[c]
B	4	3	N	+	+/−
C	4	5	N	+	+
D	4	7	N	+	−
E	−20	7	N	+	+/−
F	−80	7	N	+	+/−
G	20–25	7	Y[d]	+/−	+
H	4	7	Y	+/−	+/−
I	−20	7	Y	+	+/−

[a] N, no.
[b] +, all three replicates positive.
[c] +/−, one or two replicates positive.
[d] Y, yes.

shipped on dry ice to the University of California, Davis. Before collection of fecal samples, a variety of storage scenarios were assessed to determine what storage conditions would allow for optimal detection of FCoV in feces in the event −80°C storage was not available. Feces from two cheetahs known to shed FCoV persistently were collected fresh and shipped immediately on dry ice to the laboratory. Each sample was thoroughly mixed and separated into different aliquots that were then subjected to variable temperatures and times in storage, with and without RNA stabilization solution (RNA*later*®, Invitrogen, Carlsbad, California 92008, USA). RT-nPCR was done, in triplicate, as described for the study samples (see next section). Results indicated that FCoV was detectable in these fecal samples when processed immediately, and when processed after storage at 4°C, −20°C, and −80°C, for up to 7 days (Table 2). In addition, positive control feces stored at both −20°C and −80°C for 4 yr consistently had detectable virus by RT-nPCR. The decision was made that for the study samples, normally voided feces could be stored at −20°C or −80°C at the zoological facility, depending on the facility's capabilities, and then shipped overnight on dry ice and stored at −80°C until samples were processed.

Sample preparation, RNA extraction, RT-nPCR, and sequence analysis

Fecal samples were defrosted and then thoroughly mixed. A 1.0-ml aliquot was suspended in a 1:2 (vol:vol) of Dulbecco's modified Eagle's medium with 5% fetal bovine serum (Invitrogen), and the sample was then homogenized by vortexing, and insoluble particles were allowed to settle. Total RNA was extracted using TRIzol® LS (Invitrogen) according to the manufacturer's instructions for biological fluids. Reverse transcription was done using the Moloney murine leukemia virus reverse transcriptase kit (Invitrogen), replacing the oligo(dT) with a previously published primer (primer 211), and nPCR was done as described previously with primers of high sensitivity and specificity for FCoV, encompassing a highly conserved, 177-base pair (bp) region of the 3′ untranslated region of the FCoV genome.[12,14] Amplification was performed using AmpliTaq polymerase (Applied Biosystems, Foster City, California 94404, USA) in a Gene Amp PCR System 9700 thermocycler (Applied Biosystems) with the following cycling conditions for each of the two amplification reactions: one cycle of 1.5 min at 90°C, followed by 30 cycles of 0.5 min at 50°C, 1 min at 72°C, and 1 min at 94°C, and completed with 2 min at 50°C and 5 min at 72°C. Positive controls for RT-nPCR were feces from a persistently shedding cheetah and in vitro–propagated FCoV strain WSU1143 (American BioResearch, Sevierville, Tennessee 37864, USA). Water (Invitrogen) was the negative control. A 10-µl sample of amplified product was separated by electrophoresis on a 1.5% agarose gel and visualized with UV light after staining with ethidium bromide (Bio-Rad Laboratories, Hercules, California 94547, USA). PCR product identity was confirmed by direct sequencing of a subgroup ($n = 10$) of 177-bp amplification products. PCR products were purified using a Centricon 100 column (Millipore, Bedford, Maryland 01730, USA), and nucleotide sequencing was done using an ABI 3730 capillary electrophoresis

Table 3. Mean number and proportion of seven cheetahs with known detectable FCoV in feces correctly identified given 12 hypothetical schedules of fecal sample collection and assessment by RT-nPCR.

Sampling schedule no.	Description	No. of fecal samples	No. of days needed for sampling	Mean no. of shedding cheetahs detected	Proportion of shedding cheetahs detected (mean no. detected/7)
1	3 consecutive samples	3	3	5.33	0.76
2	5 consecutive samples	5	5	6.33	0.90
3	Every third sample for 30 days	11	30	7.00	1.00
4	Every fifth sample for 30 days	6	30	5.00	0.71
5	Every other sample for 30 days	15	30	7.00	1.00
6	Every seventh sample for 30 days	4	30	5.67	0.81
7	Once a month on a random day	1	1	3.33	0.48
8	3 consecutive samples, twice, 1 wk apart	6	13	6.33	0.90
9	5 consecutive samples, twice, 1 wk apart	10	17	6.33	0.90
10	Every third sample for 14 days	5	14	6.00	0.86
11	Every fifth sample for 14 days	3	14	5.67	0.81
12	Every seventh sample for 14 days	2	14	5.33	0.76

genetic analyzer and BigDye terminator v.31 cycle sequencing (University of California, Davis, California 95616, USA). Subsequent amplification products visualized at 177 bp were interpreted as positive.

One subset of cheetahs ($n = 16$), referred to hereafter as group 1, had 30 to 46 days of feces analyzed. A second subset of cheetahs ($n = 9$), referred to hereafter as group 2, had five consecutive fecal samples analyzed to assess the use of five consecutive individual samples for detection of a shedding animal.

Statistical analysis

The presence or absence of detectable FCoV in consecutive fecal samples as detected by RT-nPCR was the outcome variable. To determine the probability of correctly identifying a cheetah shedding FCoV by analyzing fewer than 30 samples/cheetah, 12 hypothetical schedules of fecal sample collection and analysis by RT-nPCR were applied to the known daily fecal RT-nPCR results of the seven cheetahs with detectable FCoV in their feces. The proportion of the seven cheetahs that would be correctly identified as fecal shedders with each collection schedule was calculated and compared with the number of samples needed for that schedule. The schedule correctly identifying the highest proportion of shedding cheetahs using the smallest number of samples over the fewest sampling days was considered the most efficient. The first day a fecal result was to be assessed was assigned using a random number generator (Microsoft Office 2007, Excel, Microsoft, Redmond, Washington 98052, USA). Scenarios were run in triplicate and the results averaged (Table 3). If the day(s) to be assessed had no feces collected, the result from the next consecutive sample was used.

Fisher's exact test (EpiInfo, v.3.5.1, Centers for Disease Control and Prevention, Atlanta, Georgia 30333, USA) was used to assess the association between potential risk factors and the presence or absence of FCoV in feces in group 1. Risk factors evaluated included age (dichotomized into juvenile [$\leq$2 yr] or adult [$>$2 yr]), sex, facility size (dichotomized into small [$\leq$15 cheetahs] or large [$>$15 cheetahs]), wild-caught or captive born status (termed "origin"), presence or absence of diarrhea at the time of fecal collection, cheetah's prior FCoV serologic result, cheetah's prior FCoV fecal RT-PCR result, presence or absence of cage-mates, cage-mate's FCoV fecal RT-PCR result, presence or absence of cheetahs shedding FCoV at the facility, and the potential for contact with feral cats. A $P \leq 0.05$ was significant.

RESULTS

Fecal samples ($n = 764$), historical information about potential risk factors, and zoological facility information were collected for 25 AZA Cheetah SSP cheetahs (Table 1). Fifteen of 25 (60%) cheetahs had at least one historically positive FCoV fecal RT-PCR. Two of 23 (8.7%) cheetahs had at least one historically positive FCoV titer. These same two cheetahs also had a history of positive fecal RT-PCR. Of the 10 cheetahs with no prior positive FCoV serologic result or fecal RT-

Day	Persistent shedder	Intermittent shedder	Rare shedder
1	+[a]	-[b]	-
2	+	-	-
3	+	+	-
4	+	-	NS[c]
5	+	-	-
6	+	-	-
7	+	+	NS
8	+	-	NS
9	+	-	NS
10	NS	-	+
11	+	-	NS
12	+	-	NS
13	+	-	-
14	+	-	-
15	+	-	+
16	+	-	-
17	+	+	NS
18	NS	-	-
19	+	+	-
20	+	+	NS
21	+	-	-
22	+	+	-
23	+	+	-
24	+	+	+
25	-	+	-
26	+	-	-
27	+	-	-
28	+	-	-
29	-	+	-
30	+	-	-
31		+	-
32		-	-
33		-	-
34		+	-
35		+	NS
36		-	+
37		-	NS
38		+	-
39		-	NS
40		+	-
41		-	-
42		-	NS
43		-	NS
44			-

Figure 1. The frequency of FCoV shed in cheetah feces as detected by RT-nPCR from fecal samples collected over 30 to 44 consecutive days.

PCR result, six cheetahs were housed at institutions with a history of FCoV infection in cheetahs at the time feces was collected, two originated from a facility with a history of FCoV infection in cheetahs (Gaffney, data not shown) and two were sampled before transportation to a new zoological facility. Sixteen of 25 (64%) cheetahs had cage-mates at the time of fecal collection. Of those cheetahs with cage-mates, nine cheetahs had a single cage-mate, and seven cheetahs had two or more cage-mates (Gaffney, data not shown). Of the nine cheetahs without cage-mates, six cheetahs had the opportunity for contact with other cheetahs or cheetah fluids, either directly through a fence or indirectly through shared common space, two cheetahs shared common space without the opportunity for fence contact with another cheetah, and one cheetah had no opportunity for direct or indirect contact with another cheetah (Gaffney, data not shown).

Frequency of FCoV fecal shedding

Seven of 16 (44%) cheetahs in group 1 had detectable FCoV in feces at a minimum of four time points and a maximum of 26 time points in a 30- to 44-day period. The percentage of positive samples for individual cheetahs ranged from 13% (4/31) to 93% (26/28) (Table 1). The frequency at which virus was shed varied greatly. One animal (C16) had detectable FCoV in 93% (26/28) of fecal samples and no more than one negative sample between positive samples; this animal was termed a persistent shedder over the 30-day period. Four animals (C7, C8, C10, and C11) had detectable FCoV in 43% (16/37), 33% (14/42), 24% (9/38), and 35% (15/43) of fecal samples with at least two, and up to five, consecutive positive samples, and from two to nine consecutive negative samples; these animals were termed intermittent shedders over the 30-day period. Two animals (C9 and C15) had detectable FCoV in 13% (4/31) and 23% (5/22) of fecal samples, and positive samples were never consecutive; these two animals were termed rare shedders over the 30-day period. Representative patterns are depicted in Figure 1.

Probability of identifying a cheetah shedding FCoV in feces

Applying 12 hypothetical schedules of fecal sample collection to the subset of group 1 cheetahs with detectable FCoV in their feces ($n = 7$) yielded variable proportions of cheetahs correctly identified as shedding virus (Table 3). One hundred percent (7/7) of the cheetahs known to have shed FCoV in feces were correctly identified in the two sampling schedules requiring the highest number of fecal samples over the longest collection period (schedules 3 and 5). Only 48% (3.33/7) of the cheetahs known to have shed FCoV in feces were correctly identified if only one fecal sample was to be evaluated over 30 days (schedule 7). Ninety percent (6.33/7) of the cheetahs known to have shed FCoV in feces were correctly identified in schedules 2 and 8, requiring five and six fecal samples, respectively.

Based on these results, sampling schedule 2, collecting and testing five individual consecutive fecal samples starting on a random day in a 30-day period, was considered to be the most efficient, providing the highest positive percentage with the fewest number of fecal samples over the shortest sampling period. This schedule was adopted and evaluated using data from group 2. Evaluation of feces ($n = 45$) from these cheetahs ($n = 9$) for detectable FCoV by RT-nPCR yielded all negative results, suggesting that there is 90% chance these cheetahs were not actively shedding detectable FCoV in feces during the sampling period.

None of the potential risk factors examined were significantly associated ($P > 0.05$) with the presence of detectable FCoV in cheetah feces. Cheetahs without detectable FCoV in feces tended to be older than 2 yr ($P = 0.063$). Cheetahs that had previous detection of FCoV in feces by RT-PCR tended to have detectable FCoV in feces in this study ($P = 0.088$).

DISCUSSION

In this study of 25 AZA cheetah SSP cheetahs, 44% of cheetahs from environments with the potential for FCoV infection shed detectable FCoV in their feces. When FCoV was shed in feces, it was shed at different frequencies by different cheetahs, and shedding was classified as persistent, intermittent, or rare over the sampling period. In light of the high sensitivity of the RT-nPCR assay and the documented adequacy of the storage conditions used for the feces, the variable frequency with which virus was detected in feces in different cheetahs is believed to demonstrate true variability in shedding frequency and not a function of false negative results. Probability estimates based on proposed fecal sampling schedules demonstrated that the variable frequency of FCoV shedding within a 30-day period make it necessary to analyze more than one fecal sample to obtain a >50% chance of identifying a cheetah shedding virus. Probability

estimates also demonstrated that analysis of five consecutive fecal samples by RT-nPCR for FCoV correctly identified 90% of the cheetahs in this study as shedding detectable FCoV in their feces. Cheetahs incorrectly identified as not shedding FCoV were those that shed virus rarely over the collection period. No significant risk factors for shedding of FCoV in feces were identified. Evaluation of multiple sample storage conditions indicated that viral RNA was preserved in fecal samples frozen at −20°C, providing an alternative for facilities that lack −80°C storage capabilities.

The frequency of shedding of FCoV in feces as detected by RT-PCR in domestic cats naturally infected with FCoV or from endemic environments is well studied.[1,9,11] In studies following cats exposed to FCoV for many years, several fecal shedding patterns of the virus were identified, including cats that never shed, cats that cease to shed after a period of shedding, intermittent shedders that are reinfected after viral clearing, and persistent shedders. The 30–46 days of cheetah feces examined for the presence of FCoV in this study is short compared with studies done in cats and cannot be used to fully assess long term fecal viral shedding patterns in cheetahs. However, the examination of 30 or more days of cheetah feces mimics the conditions under which samples could be collected while a cheetah is in quarantine before and after shipment to a new facility. For this reason, examining the frequency of viral shedding over 30 days provides practical and useful information. Knowing there could be variable frequency in fecal shedding of FCoV in cheetahs over 30 days aids in the interpretation of fecal FCoV RT-nPCR results that would be obtained while a cheetah is in quarantine and in the design and implementation of a screening program to identify cheetahs actively shedding FCoV. Although long-term studies are needed, the results of this study, combined with the results of previous studies examining cheetah feces for FCoV by RT-PCR, suggest that captive cheetahs may have similar shedding patterns to domestic cats from multicat households.[1,15,32] Long-term and systematic monitoring would be required to further characterize shedding patterns of cheetahs; however, the controlled environments utilized in many cat studies may not be as feasible in all zoological facilities.

Multiple randomized fecal sampling and testing schedules were applied to the actual known fecal RT-nPCR results. The most practical and feasible sampling schedule was considered to be the collection and RT-nPCR testing of five individual consecutive fecal samples, which identified 90% of the shedding cheetahs in the present study. Although this schedule of sampling (schedule 2) correctly identified the same proportion of shedding cheetahs as two other sampling schedules (schedules 8 and 9), it required fewer samples over fewer days with the simplest collection protocol. Other sampling schedules showed testing of more fecal samples did not increase the proportion of shedding cheetahs correctly identified until the number of samples tested was >10 (schedules 3 and 5). The failure to accurately identify more shedders with more samples, without more than doubling the number of samples tested, is attributable to the variable shedding frequency of FCoV in the feces of some cheetahs and demonstrates that true fecal shedders can be missed. The sampling schedules (schedules 3 and 5) that correctly identified 100% of the cheetahs with known FCoV in feces required testing of the most samples over the longest sampling period. These were not considered cost efficient or practical sampling schedules for use as a quarantine screening tool. Even with the high sensitivity of the RT-nPCR test, unlimited funds and access to cheetahs, false-negative results would still result in virus shedding cheetahs being missed. Many factors contribute to false-negative results and include low concentration of FCoV in feces, heterogeneous distribution of FCoV throughout an individual fecal sample, degradation of virus, and fecal inhibitors of the PCR assay. Heterogeneous distribution and virus degradation can be minimized by homogenizing the fecal sample and storing the feces appropriately; however, these factors cannot be eliminated.

The cheetah SSP attempted to decrease the risk of FIP by applying stringent testing and quarantine before and after movement of cheetahs to new facilities as well as restricting the movement of FCoV seropositive cheetahs. However, in 2005, the seroprevalence of FCoV in the SSP cheetah population was estimated to be as high as 20%, and FCoV was considered endemic in the population.[28] Thus, the restrictions on animal movements did not prevent spread of the virus within the population. In addition, results from the present study and those of Kennedy et al.[14,15,16] identified seronegative animals that shed virus in feces, suggesting that the prevalence in the population is likely higher than the 20% indicated by seropositivity alone. Despite the endemic nature of FCoV within the captive cheetah population, between 1988 and 2005, only 6/344 (1.7%) of deaths were attributed to FIP in the AZA Cheetah SSP population[28] (Munson, unpubl.

data). The low number of deaths attributable to FIP, despite widespread seroprevalence to FCoV, suggests that the propensity for cheetahs to develop FIP when infected with FECV is less than originally proposed. As the risk to the population is not as great as previously anticipated, the results of this study support the current Cheetah SSP recommendations to manage the captive population as endemic for FCoV, not to restrict animal movement based on seropositivity, but to continue monitoring seroprevalence and fecal shedding in quarantine before and after movement between facilities.

FCoV is difficult to eliminate from the environment of an endemically infected population. In domestic cats, one proposed way to decrease the risk of FIPV in an environment with endemic FCoV is to eliminate chronic shedders.[10] However, eliminating chronic shedders, either by euthanasia or permanent isolation, to decrease the risk of a disease that causes <2% mortality in cheetahs does not seem warranted.

No significant risk factors were identified in cheetahs that shed FCoV in feces compared with those that tested negative. The power to detect statistically significant differences was low because of the limited sample size and the similarity in cheetah management practices between facilities; however, two trends were noted. A large number of study cheetahs that did not shed virus were adults, a finding similar to what has been reported in domestic cats; however, this also may be a function of this study's population that included three times as many adults as juveniles. Cheetahs that previously had FCoV detected in their feces were more likely to also be positive in this study. This may be due to an individual's propensity for more frequent viral shedding, for higher virus load, or for an inability to clear the FCoV infection, or it may be attributable to reinfection. It also has been suggested that cheetahs may be subject to a repetitive cycle of infection and reinfection due to contact between infected animals or contamination of the environment, culminating in intermittent shedding.[15] The majority of cheetahs in this study had cage-mates or had the opportunity to contact other cheetahs through a fence, providing an opportunity for reinfection. However, no association was found between cage-mates and shedding. It is possible that infection with and shedding of FCoV in cheetahs has more to do with individual animal immunity or viral virulence than species susceptibility, environmental factors, or management practices. Another possible contributing element to viral shedding and susceptibility to infection is stress. Although stress was not a variable examined in this study, chronic stress and other extrinsic factors affect the patterns of disease in captive cheetahs compared with their wild, but genetically similar, counterparts, and it is a possible contributing variable that warrants further investigation.[29,38,40] The roles and relationship of FCoV, chronic stress, immunosuppression, and the presence of common concurrent infectious agents in cheetahs, such as *Helicobacter* sp. and feline *herpesvirus*-1, are not known.

CONCLUSIONS

Cheetahs with detectable FCoV in feces should be correctly identified approximately 90% of the time by examination of five individual consecutive fecal samples by RT-nPCR. Samples can be stored before shipment for testing at either −20°C or −80°C. Examination of fecal samples combined with serology for FCoV is useful in identifying the majority of the cheetahs infected with FCoV and those actively shedding detectable FCoV in feces. However, identification of 100% of infected and actively shedding cheetahs is not feasible given the variability in frequency of shedding of virus. With the widespread seroprevalence to FCoV in the AZA Cheetah SSP population, lack of a significant number of fatal cases of FIP since 1988, and the likely difficulty in eliminating FCoV from the population, it seems more feasible to manage the captive cheetah population as endemically infected with FCoV rather than limiting breeding pairings and movement of cheetahs based on FCoV test results. Continued population surveillance with both serology and fecal RT-PCR would contribute to the understanding of FCoV epidemiology in captive cheetahs.

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