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Abstract: Feline coronavirus (FCoV) infects members of the Felidae family with results ranging from seroconversion with no disease to fatal feline infectious peritonitis (FIP). Infection of nondomestic felids with FCoV is of concern, particularly in endangered populations such as cheetahs (Acinonyx jubatus). In this investigation, we tested 342 animals in the Republic of South Africa and Namibia, including 140 animals from wild populations, for evidence of FCoV infection by serology and/or reverse transcription/nested polymerase chain reaction (RT/nPCR) on feces from 1999 through 2001. Past or current infection was evaluated. Of these, 195 animals had evidence of infection and included 41 animals from wild populations. Serology (indirect immunofluorescence) did not always correlate with viral RNA detection, as seronegative animals were occasionally virus-positive, while many seropositive animals were not shedding virus. Serology indicated the infecting virus was most closely related to type I FCoV. Antibody levels in the majority of animals wre low, even in those actively infected. Ten of 48 animals tested at more than one time point by RT/nPCR were shedding virus at multiple time points possibly indicating persistent animals tested had evidence of current or previous FCoV infection. Testing by serology and RT/nPCR is recommended for screening for FCoV infection.

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# DETECTION OF FELINE CORONAVIRUS INFECTION IN SOUTHERN AFRICAN NONDOMESTIC FELIDS

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ABSTRACT: Feline coronavirus (FCoV) infects members of the Felidae family with results ranging from seroconversion with no disease to fatal feline infectious peritonitis (FIP). Infection of nondomestic felids with FCoV is of concern, particularly in endangered populations such as cheetahs (*Acinonyx jubatus*). In this investigation, we tested 342 animals in the Republic of South Africa and Namibia, including 140 animals from wild populations, for evidence of FCoV infection by serology and/or reverse transcription/nested polymerase chain reaction (RT/nPCR) on feces from 1999 through 2001. Past or current infection was evaluated. Of these, 195 animals had evidence of infection and included 41 animals from wild populations. Serology (indirect immunofluorescence) did not always correlate with viral RNA detection, as seronegative animals were occasionally virus-positive, while many seropositive animals were not shedding virus. Serology indicated the infecting virus was most closely related to type I FCoV. Antibody levels in the majority of animals were low, even in those actively infected. Ten of 48 animals tested at more than one time point by RT/nPCR were shedding virus at multiple time points possibly indicating persistent infection. Infection in free-ranging animals was also notable, as over a quarter of the free-ranging animals tested had evidence of current or previous FCoV infection. Testing by serology and RT/nPCR is recommended for screening for FCoV infection.

*Key words: Acinonyx jubatus,* cheetah, indirect immunofluorescence, polymerase chain reaction, South Africa, survey.

## INTRODUCTION

Coronavirus is a contagious and significant pathogen of nondomestic felids (Pfeifer et al., 1983; Hoskins, 1993; Kennedy et al., 2001, 2002). It has been associated with fatal systemic disease, feline infectious peritonitis (FIP), as well as enteric disease (Evermann, 1986; Evermann et al., 1989; Kennedy et al., 2001). Feline coronavirus infection and disease has been reported in a variety of species, including cheetahs (*Acinonyx jubatus*), lions (*Panthera leo*), tigers (*P. tigris*), jaguars (*P. onca*), leopards (*P. pardus*), sand cats (*Felis margarita*), mountain lions (*F. concolor*), caracals (*Caracal caracal*), and lynx (*Lynx lynx*) (Pfeifer et al., 1983; Heeney et al., 1990; Roelke et al., 1993; Watt et al., 1993; Paul-Murphy et al., 1994; Juan Salles et al., 1997; Kennedy et al., 2002). Antibodies to FCoV have been identified in free-ranging lions in Namibia (Etosha National Park) and Tanzania (Serengeti National Park, Ngorongoro Crater, and Lake Manyara region) (Spencer and Morkel, 1993; Hofman-Lehmann et al., 1996). Infection with FCoV has also been detected in captive cheetahs in Africa (Heeney et al., 1990). Outbreaks of FIP have been reported in several captive cheetah populations (Pfeifer et al., 1983; Evermann et al., 1989). In addition, FCoV enteritis has resulted in mild to severe chronic diarrhea in cheetahs and has been associated with vague signs of disease including weight loss, depression, and inappetance (Heeney et al., 1990; Kennedy et al., 2001). Control of this pathogen is complicated by the occurrence of persistent carriers that serve as important sources of the virus for the rest of the population (Kennedy et al., 2001).

Captive cheetah populations are espe-

cially vulnerable to serious consequences resulting from infectious diseases (O'Brien et al., 1985; Evermann et al., 1989; Murray et al., 1999). They are known to be highly susceptible to disease following infection with feline coronavirus (Evermann, 1986). A disastrous outbreak of FIP occurred in a captive cheetah population in Oregon in 1982–83 (Evermann, 1986; Evermann et al., 1989). Disease occurred in 90% of the cheetahs with mortality of 60%, one of the largest die-offs of captive cheetahs. The inherent susceptibility of cheetahs is thought to be due to their lack of genetic variation (O'Brien et al., 1985; Evermann, 1986; Heeney et al., 1990).

We used polymerase chain reaction (PCR) for detection of infection in nondomestic felids. Our studies on nondomestic felids in the USA have shown that FCoV is prevalent among captive cheetahs. as nearly one-third of the animals we tested were shedding FCoV in their feces or had virus detectable in plasma (Kennedy et al., 2001). In addition, though not proven to be causal, illness consistent with FCoV infection was noted in nearly half of the infected cheetah populations. Diseases and abnormalities reported included mild intermittent to chronic diarrhea, weight loss, decreased appetite, necrotizing colitis, and FIP. While FCoV may not be the sole agent of disease in all cases, we speculate that it is at least a contributing factor to the illnesses.

In this investigation, we used reverse transcription and PCR to detect FCoV genetic material in feces from nondomestic felids in southern Africa. When serum was available, serology for FCoV-specific antibodies was done.

## MATERIALS AND METHODS

### Sample collection

Samples (feces and/or blood) collected from 190 captive felids from two wildlife sanctuaries in the Republic of South Africa (Institution A—25°37′S, 27°57′E; Institution C—24°29′S, 31°2′E) and one in Namibia (Institution B—20°29′S, 16°39′E) from 1999 through 2001 were tested for virus detection by reverse transcription/nested polymerase chain reaction (RT/nPCR) using primers targeting the 3′-untranslated region (UTR; described below). We tested cheetah ($n$=182), black footed cat (*Felis nigripes*; $n$=1), African wild cat (*Felis lybica*; $n$=2), African lion ($n$=1), caracal ($n$=2), and African leopard ($n$=2). These included 43 cheetahs and one leopard from the wild in Namibia (20°29′S, 16°39′E) and South Africa (24°29′S, 31°2′E). Forty-eight cheetahs from one sanctuary were tested by RT/nPCR and serology at more than one time. Serum was provided for antibody detection from 151 of 190 animals. In addition, serum samples alone were submitted from 96 free-ranging lions (24°59′S, 31°36′E). Banked serum samples also were tested from 56 captive felids, including cheetahs ($n$=54) and African lions ($n$=2) from three sanctuaries in South Africa for antibodies. Ten additional cheetahs were tested using primers targeting the 7a7b genes only and not screened with 3′-UTR primers (described below).

### RNA extraction, reverse transcription, and nested polymerase chain reaction

All samples were stored at −70 C until tested. Total RNA was extracted from fecal specimens using Trizol LS according to the manufacturer's directions (Gibco BRL, Baltimore, Maryland, USA). The RNA was taken to reverse transcription using Moloney murine leukemia virus reverse transcriptase according to the manufacturer's recommendations (Gibco BRL). The downstream external primer was used for first strand synthesis as described previously (Kennedy et al., 1998). Polymerase chain reaction was done using ExTaq polymerase (Intergen, Purchase, New York, USA) as described previously with the upstream external primer (Kennedy et al., 1998). This procedure was followed by nPCR using internal primers (Kennedy et al., 1998). For the majority of samples ($n$=190), primers used encompassed the 3′-UTR, as this region is highly conserved (Herrewegh et al., 1995). The 7a7b open reading frame (ORF), the 3′-most ORF, was amplified from 36 nondomestic felid samples, 10 of which were not tested with the UTR primers (Kennedy et al., 1998). Amplification of this region was attempted on some samples in order to characterize this region genetically. The 7b region in particular has been associated with virulence of the virus (Herrewegh et al., 1995). Nucleotide sequencing of products was to be undertaken on the 7a7b products from these samples. Sensitivity and specificity of these procedures were described previously (Kennedy et al., 1998). Products were evaluated by electrophoresis on 1% agarose gels.

TABLE 1. Results of testing cheetahs by reverse transcription/nested polymerase chain reaction (PCR) with or without serology (indirect immunofluorescence).

| Institution | Number positive by PCR/number tested | Number serologically positive/number tested |
|---|---|---|
| A | 15/39 | 19/20 |
| B | 3/39 | 19/39 |
| C | 48/104 | 86/87 |
| Total | 66/182 | 124/146 |

### Serology

Detection of FCoV-specific antibodies was done for all animals from which serum or plasma were provided ($n$=302). Serology was done by indirect immunofluorescence as previously described (Kennedy et al., 1998). Briefly, a type I (UCD1) and a type II (WSU 1143) FCoV were propagated separately in Crandell feline kidney cells (American Bioresearch, Sevierville, Tennessee, USA). The virus-infected cells were applied and fixed to glass slides for use as capture antigens. Two-fold serial dilutions of the serum/plasma were made starting at a 1:5 dilution and proceeding to a maximum of 1:640. Antibody was detected with anti-feline IgG conjugated to fluorescein isothiocyanate (VMRD, Pullman, Washington, USA; The Binding Site Limited, Birmingham, UK). Antibody titer was reported as the reciprocal of the highest dilution in which fluorescence was still present. Antibody titers of <5 were considered negative. The 151 serum samples from animals tested by RT/nPCR were assayed for antibodies to type I and II FCoV. The 152 samples that were not tested by RT/nPCR were screened with only type II FCoV due to availability of capture antigen. The 96 free-ranging lions were screened at a single serum dilution of 1:20 because only limited quantities of banked sera were available.

## RESULTS

Sixty-six of 182 (36%) fecal samples from cheetahs tested positive by RT/nPCR for FCoV (Table 1). Cheetahs from institution C had the highest percentage of positive animals (45%) followed by institution A (35%) and B (7.5%). Among the species other than cheetahs that were tested ($n$=8), all were negative by RT/nPCR. Seven of the RT/nPCR-positive cheetahs were captured and sampled upon arrival at the submitting institution (Table 2). All animals tested with the 7a7b primers ($n$=36), including eight samples RT/nPCR-positive using the UTR primers, were negative. Ten of 48 animals tested at multiple time points were positive at more than one time point (20%) with intervals ranging from 1–12 mo (Table 3). All ten were negative on at least one sample.

For cheetahs tested by RT/nPCR from which serum was provided ($n$=146), 123 were seropositive (84%) (Table 4). Forty-six of 152 animals (30%) tested by serology alone were positive for antibodies to FCoV. In addition, one lion, two caracals, and one leopard from one institution that were negative by RT/nPCR were seropositive. Thus, 57% of all animals tested for FCoV-specific antibodies were positive. Again, institutions A and C had the highest percent of seropositive animals (96% and 99%, respectively) as compared to institution B (47.5%) (Table 1). Of the 49 RT/nPCR-positive cheetahs from which serum was provided, three were seronegative (Table 2). Eighty seropositive animals were negative by RT/nPCR. The antibody levels of seropositive animals ranged from 10–320. However, most seropositive samples were at levels ≤40. Of the 151 animals tested by RT/nPCR and serology, only nine (6%) had titers over 40 to either serotype. Six of these nine were also RT/nPCR positive at a minimum of one time point, with three positive at more than one time point (Table 2 and 3). Fifty-four of 127 (42%) seropositive animals had two-fold higher titers to type I than to type II FCoV. Three animals had a higher titer to type II than to type I. Of the ten animals testing positive by RT/nPCR at more than one time point, only three had a titer of ≥1:80 at any point during the testing period (Table 3). The remaining animals had titers of 1:40 or less at every time point tested. Antibody titers decreased in two animals (Number 2 and 4) despite converting from RT/nPCR negative to positive.

Of 342 animals tested by RT/nPCR and/or serology, 194 (57%) had evidence of in-

TABLE 2. Feline coronavirus (FCoV) reverse transcription/nested polymerase chain reaction positive cheetahs tested by indirect immunofluorescence for FCoV-specific antibodies.

| Animal | Feline coronavirus serology[a] | |
|---|---|---|
| | Type I | Type II |
| 1 | 20 | 10 |
| 2[b] | 20 | 40 |
| 3[b] | <5 | <5 |
| 4[b] | <5 | <5 |
| 5 | 20 | 20 |
| 6 | 20 | 20 |
| 7 | 20 | 20 |
| 8 | 10 | 10 |
| 9 | 80 | 80 |
| 10 | 10 | 10 |
| 11 | 20 | 20 |
| 12 | 20 | 10 |
| 13 | 10 | 10 |
| 14 | 160 | 80 |
| 15 | 20 | 20 |
| 16 | 10 | 10 |
| 17 | 10 | 10 |
| 18 | 80 | 40 |
| 19 | 20 | 10 |
| 20[b] | 10 | 10 |
| 21[b] | 20 | 20 |
| 22[b] | 40 | 20 |
| 23 | 20 | 20 |
| 24 | 20 | 20 |
| 25 | 20 | 10 |
| 26 | 10 | 10 |
| 27 | 10 | 10 |
| 28 | 10 | 10 |
| 29 | 20 | 20 |
| 30 | 10 | 10 |
| 31 | 10 | 10 |
| 32 | 40 | 40 |
| 33 | 10 | 10 |
| 34 | 10 | 10 |
| 35 | 10 | 10 |
| 36 | 20 | 20 |
| 37 | 10 | 10 |
| 38 | 160 | 80 |
| 39 | 10 | <5 |
| 40 | 40 | 20 |
| 41 | 40 | 40 |
| 42 | 20 | 20 |
| 43 | 10 | 10 |
| 44 | 20 | 20 |
| 45 | 10 | 10 |
| 46 | 10 | 10 |
| 47 | 10 | 10 |
| 48 | 10 | 10 |
| 49[b] | <5 | <5 |

[a] Antibodies reported as reciprocal of serum dilution.
[b] Wild-caught cheetahs.

fection with FCoV. Of 140 free-ranging animals tested, 16 lions were seropositive, seven cheetahs were positive by RT/nPCR, and an additional 18 cheetahs, while RT/nPCR-negative, were seropositive (29%).

## DISCUSSION

Our previous investigations have determined that FCoV is prevalent among captive felids in the USA. Over 50% of animals tested had evidence of infection with FCoV (Kennedy et al., 2002). In this investigation, over 50% of the animals from Southern Africa tested by serology and RT/nPCR had evidence of infection. These results were not limited to captive animals, as 41 of 140 animals originating in wild populations also may have been infected with FCoV. While no conclusions about prevalence or significance of FCoV in wild populations can be made, because some animals were tested after arrival at the captive institution, the source in all cases was the wild indicating its presence in these populations.

There was a significant difference between the prevalence of antibody positive and RT/nPCR-positive cheetahs at the South African institutions as compared with the Namibian institution. This may be because all the Namibian animals were wild-caught, while most of the South African cats were bred in captivity. Housing and management also differed. In Namibia, the cheetahs are kept in small groups in camps ranging from 10–50 ha with little or no contact with neighbors. In South Africa, the housing is much more intensive and animals are in contact with neighboring animals through fences. In addition, movement of animals to enclosures previously inhabited by other cheetahs is continuous. It is notable that there is a low prevalence of FIP in the institutions in southern Africa. Only one case has been reported in Institution C, despite a high incidence of FCoV infection. A possible explanation for this is that the virus occurring in cheetahs in southern Africa is largely nonpathogenic.

TABLE 3. Results of multiple reverse transcription/nested polymerase chain reaction (PCR) assays and indirect immunofluorescence serology (IFA) on individual cheetahs.

| Animal number | 11/99[a] | | 8/00 | | 11/00 | | 3/01 | | 4/01 | | 9–11/01[b] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PCR | IFA[c] | PCR | IFA | PCR | IFA | PCR | IFA | PCR | IFA | PCR | IFA |
| 1 | + | ND[d] | ND | 10/10 | + | ND | − | ND | − | 40/40 | ND | ND |
| 2 | ND | ND | − | 40/40 | ND | ND | ND | ND | + | 160/80 | + | 80/40 |
| 3 | + | ND | − | 10/10 | − | ND | + | ND | − | ND | ND | ND |
| 4 | + | ND | + | 40/40 | + | ND | − | ND | − | 40/40 | + | 20/10 |
| 5 | ND | ND | + | 10/10 | ND | ND | + | ND | − | 80/40 | − | 20/10 |
| 6 | + | ND | + | 40/20 | − | ND | + | ND | ND | ND | ND | ND |
| 7 | ND | ND | + | 20/20 | − | ND | + | ND | + | 40/40 | − | 80/40 |
| 8 | + | ND | + | 20/10 | − | ND | − | ND | − | 20/20 | ND | ND |
| 9 | + | ND | − | <5/<5 | ND | ND | ND | ND | ND | ND | + | 10/10 |
| 10 | − | ND | ND | ND | ND | ND | ND | ND | + | 10/10 | + | 10/10 |

[a] Dates of testing by month and year.
[b] Tested once during the period.
[c] Reciprocal antibody titers for FCoV types I/II.
[d] ND = not done.

Serologic tests using FCoV types I and II isolated from domestic cats detected relatively low levels of FCoV-specific antibody, even in animals actively shedding virus as indicated by RT/nPCR. As with our previous investigation, serology did not necessarily correlate with virus shedding, as some animals seropositive for FCoV-specific antibodies were negative by RT/nPCR. Seropositive animals may have successfully cleared the virus. Conversely, three animals positive by RT/nPCR were seronegative. Additionally, animals testing positive for virus shedding at multiple time points maintained relatively low antibody levels, with two animals experiencing declining antibody levels despite conversion from virus-negative to virus-positive status. Low or negative antibody levels in virus-infected animals may be due to the presence of low levels of virus. A more likely explanation however, is that cheetahs may be infected with an antigenically distinct strain of FCoV. In a previous investigation (Heeney et al., 1990), virus was detected in feces by electronmicroscopy but the same animals were negative for FCoV-specific antibody. The authors speculated that there may be several immunologically distinct strains of feline coronaviruses. Our investigations have also shown a disparity in serologic and RT/nPCR results (Kennedy et al., 2001, 2002). Results of our study suggest that the FCoV of nondomestic felids we tested is more closely related antigenically to FCoV type I than type II. Type II FCoV is antigenically more similar to canine coronavirus than type I FCoV (Herrewegh et al., 1998). Significant genetic heterogeneity seems to be due to geographic variation rather than variation in virulence. That is, viruses from the same geographic locale are more closely related than FCoVs from different locales regardless of biotype (Vennema et al., 1995). It is likely that antigenically distinct strains exist in Africa.

TABLE 4. Results for cheetahs tested by reverse transcription/nested polymerase chain reaction (PCR) and indirect immunofluorescence (IFA) FCoV serology.

| Institution | PCR+/IFA+ | PCR+/IFA− | PCR−/IFA+ | PCR−/IFA− |
|---|---|---|---|---|
| A | 1 | 0 | 17 | 1 |
| B | 1 | 2 | 18 | 19 |
| C | 45 | 1 | 41 | 0 |
| Total | 47 | 3 | 76 | 20 |

Some animals were positive for viral shedding at more than one time point. Positive results were obtained at intervals ranging from 1–12 mo. This may indicate

infection followed by clearance and reinfection. Alternatively, some animals may remain infected for significant periods of time as was shown in our previous investigation (Kennedy et al., 2001). These persistently infected animals may be an important source of infection for contact animals.

Samples from 36 animals were tested by RT/nPCR with primers targeting the 7a7b region. This genetic region and its potential association with virulence have been the subject of analysis in coronavirus from domestic cats (Herrewegh et al., 1995). This region has been associated with virulence of FCoV and may play a role in disease production; additionally, mutations may occur in this region (Herrewegh et al., 1995; Vennema et al., 1998). Because cheetahs appear to be more susceptible to severe disease following infection with FCoV than other felids, we hoped to clone this region of the virus from cheetahs and determine the genetic sequence and to investigate the occurrence of mutations in this region that might correlate with virulence. Characterization of this region in FCoV of nondomestic felids in the USA has shown that deletional mutations occur in this region in a manner similar to that seen in the virus of domestic cats (Kennedy et al., 2000, 2001). This region was not successfully amplified in any of the samples tested, even in the eight samples that were positive using primers targeting the UTR. It is likely that mutations have occurred in this region and resulting in loss of one or more primer-binding sites.

In conclusion, FCoV is prevalent among nondomestic felids in southern Africa. Over half of the animals tested were seropositive; in addition, over a third of those tested by RT/nPCR may have been shedding virus in feces. Samples collected from animals originating in wild populations also had evidence of infection with FCoV. Some animals remained infected for significant periods indicating persistent infection and virus shedding. Results of serology and viral RNA detection by RT/nPCR did not always agree because seronegative animals were occasionally virus positive and seropositive animals were not always shedding virus. We recommend a combination of these tests for FCoV screening of animals.

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