Surveillance of feral cats for influenza A virus in North Central Florida

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Background   Transmission of highly pathogenic avian influenza and the recent pandemic H1N1 viruses to domestic cats and other felids creates concern because of the morbidity and mortality associated with human infections as well as disease in the infected animals. Experimental infections have demonstrated transmission of influenza viruses in cats.

Objectives   An epidemiologic survey of feral cats was conducted to determine their exposure to influenza A virus.

Methods   Feral cat sera and oropharyngeal and rectal swabs were collected from November 2008 through July 2010 in Alachua County, FL and were tested for evidence of influenza A virus infection by virus isolation, PCR, and serological assay.

Results and conclusions   No virus was isolated from any of 927 cats examined using MDCK cell or embryonated chicken egg culture methods, nor was viral RNA detected by RT-PCR in 200 samples tested. However, 0.43% of cats tested antibody positive for influenza A by commercial ELISA. These results suggest feral cats in this region are at minimal risk for influenza A virus infection.

Keywords   Cats, feline, influenza, influenza A virus, serology, surveillance.

Introduction

Migratory aquatic birds are the primary reservoir for influenza A viruses, but because of interspecies transmission, some of these viruses have adapted to and are maintained in mammalian species, such as humans, pigs, and horses.[1] Interspecies transmission is of public health and agricultural concern because of the potential for viral adaptation or reassortment between viruses affecting these varied hosts. There currently are no known influenza A viruses adapted to felids, but replication of avian (H7N3), human (H3N2, influenza B), and seal (H7N7) influenza strains in cats has been reported, albeit without pathology.[2–4] Horizontal transmission and human-to-cat transmission also have been documented with a human H3N2 strain.[2] More recently, natural infections of domestic cats with 2009 pandemic influenza (pH1N1) virus have been reported,[5–7] however other surveillance studies have found lower prevalence of pH1N1 infection.[8] Infections of domestic cats, feral cats, and large felids with highly pathogenic avian influenza virus (HPAIV H5N1) have also been reported,[9–16] and in one case report, circumstantial evidence showed horizontal transmission of HPAIV H5N1 between tigers in a Thailand zoo,[14] and another reported subclinical infections.[12] In support, several studies have shown that cats experimentally infected with pH1N1 or HPAIV H5N1 influenza develop pathology,[17–20] with horizontal transmission being confirmed for both strains.[17–19] Finally, a computational study examining transmission dynamics in cat contact networks theoretically demonstrated that cats could influence the spread, maintenance, and human transmission rates of HPAIV H5N1 during an epidemic.[21] These studies show that felids can contract and potentially spread influenza A viruses. Given the high potential for contact with humans, domesticated animals, poultry, and waterfowl, cats may represent an important bridge that facilitates interspecies transmission.

Alachua County in Florida consists of more than 93 000 acres of swamp, marsh, and open water habitats and is a resting place and wintering habitat for many migratory birds and waterfowl including teal, mallards, and wood ducks.[22] In addition, the county has a significant poultry industry generating approximately $76 000/year according to the 2007 Census of Agriculture for Alachua County. Alachua County also has a large feral cat population. At the beginning of one study, 920 feral cats were found living

in known colonies at an average of seven cats per colony.[23] The actual number of feral cats throughout the county, however, is undoubtedly much larger. Studies have shown that cats are responsible for 20–30% of collected wounded wild birds and that each cat kills approximately one bird/week.[24] This is of major concern given that a documented route of infection for cats with avian influenza is through preying on infected birds.[18,19] Therefore, feral cats in this county have a high potential for exposure to myriad avian species potentially infected with influenza A viruses, and they should be tested for current and previous exposures.

In collaboration with Operation Catnip® (University of Florida, College of Veterinary Medicine, Gainesville, FL, USA), a TNR (trap-neuter-release) feral cat control program servicing Alachua County, Florida, samples from 927 individual cats were tested by virus isolation, RT-PCR, and serum ELISA to determine whether these cats were infected or previously exposed to influenza A viruses.

Materials and methods

Sample processing

Swabs and sera were collected from 927 cats admitted to the monthly TNR program for feral cats in Alachua County, Florida (Operation Catnip®). This program routinely admits 150–250 cats each month for sterilization surgery. Randomly selected samples from 40–60 cats/month

were received from November 2008 through July 2010. No samples were received during August 2009 or April 2010. Age, sex, health status, retroviral infection status, location where trapped, and habitat characteristics were recorded for each sampled cat (Figure 1). While under anesthesia for sterilization surgery, oropharyngeal swabs and rectal swabs were collected from each cat and placed into tubes containing viral transport medium (VTM) consisting of Hank's balanced salt solution buffered to maintain a pH of 7.3 ± 0.2 and supplemented with bovine serum albumin, sucrose, glutamic acid, gelatin, amphotericin B (4 μg/ml), colistin (7·5 μg/ml), vancomycin (100 μg/ml), and cryoprotectants. Blood (3 ml) was collected in clot tubes from each cat and serum prepared by centrifugation. The swabs and sera were shipped overnight on cold packs to the processing laboratory at the University of Georgia College of Veterinary Medicine. Samples were stored at −20°C (serum) and −80°C (swabs). Sample collection was approved by the University of Florida Institution for Animal Care and Use Committee.

Virus isolation methods

All procedures were conducted under guidelines approved by the Institutional Animal Care and Use Committee of the University of Georgia. Madin-Darby canine kidney cells (MDCK; CCL-34, ATCC, Manassas, VA, USA) were propagated in 12-well tissue culture plates (Corning Inc.,
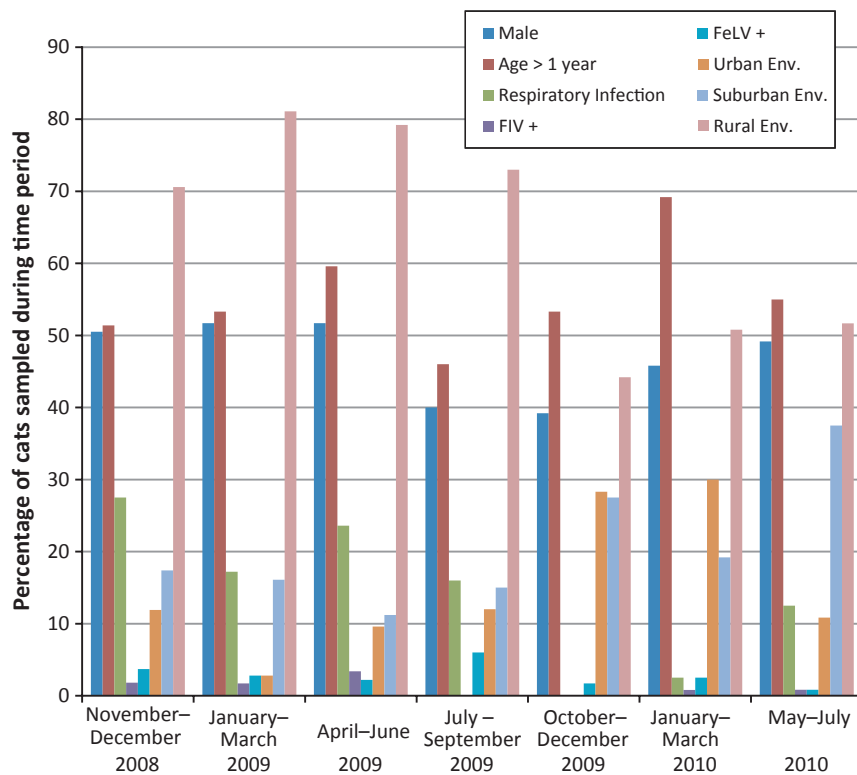

Figure 1. Characteristics of cats sampled stratified by the quarter of year in which samples were collected. Between 40 and 60 samples were collected every month between November 2008 and July 2010, except for August 2009 and April 2010. Urban, suburban, and rural environments reflect the general location where the cats were trapped.

Corning, NY, USA) to approximately 80% confluency in growth media [Dulbecco's modified Eagle medium (DMEM; Thermo Scientific, Logan, UT, USA) supplemented with 1% l-glucose and 5% fetal bovine sera]. The growth media were removed and the cells washed with 1× phosphate-buffered saline (PBS; Thermo Scientific). Each well was inoculated with 100 μl of swab VTM along with 300 μl of infection media [MEM (Thermo Scientific), l-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK)-treated trypsin (1 μg/ml; Worthington, Biochemical Corporation, Lakewood, NJ, USA), antibiotic cocktail (10 μg/ml penicillin, 10 μg/ml streptomycin, and 25 μg/ml amphotericin B; Mediatech, Inc., Manassas, VA, USA), and gentamycin (10 μg/ml; MP Biomedicals, Solon, OH, USA)]. Plates were incubated at 37°C for 1–3 hours, and 1 ml of infection media was added to each well. The plates were incubated for 5 days at 37°C in a humidified incubator.

Specific pathogen-free, embryonated chicken eggs (ECE) were received from the Poultry Diagnostic Research Center in Athens, GA after 9–10 days of incubation. Eggs were inoculated in duplicate with oropharyngeal or rectal swab VTM as previously described.[25] Embryos were evaluated for viability at days 1, 3, and 5 post-inoculation (pi).

Hemagglutination assays (HA) were performed on all virus isolation experiments as previously described.[26] The established threshold for a potentially positive specimen was a serum dilution of 1:2. Potentially positive cell supernatants or egg allantoic fluid was re-inoculated into MDCK cells or embryonated chicken eggs in quadruplicate. Positive samples (HA > 2) from this second screen were tested by RT-PCR.

RT-PCR

Total RNA was isolated from HA-positive MDCK culture supernatants and egg allantoic fluid using the RNeasy kit (Qiagen Inc., Valencia, CA, USA). RNA from oropharyngeal VTM samples was isolated using Purelink Viral RNA/DNA Mini and 96 Kits [Invitrogen (Life Technologies), Grand Island, NY, USA]. RNA extractions were kept at −20°C for short-term storage and −80°C for long-term storage. RT-PCR were performed using the Stratagene MX3000P and MX3005P systems, utilizing Qiagen's One-Step RT-PCR kit. Reactions utilized M gene-specific primers (forward: 5′-GACCRATCCTGTCACCTCTGAC-3′; reverse: 5′-AGGGCATTYTGGACAAAKCGTCTA-3′) and a FAM fluorescent probe (5′-FAM-TGCAGTCCTCGCTCACTGG-GCACG-BHQ1-3′) targeting the region of the M gene conserved across all influenza A viruses, as formulated by the CDC (Biosearch Technologies, Inc. http://www.who.int/csr/resources/publications/swineflu/CDCrealtimeRTPCR protocol_20090428.pdf). This primer/probe set was selected to identify both avian and pH1N1 viruses. For confirming potential culture positives, RNA samples from MDCK supernatant or egg allantoic fluid were prepared using the manufacturer's protocol.

Two hundred oropharyngeal samples (10–11 chosen randomly from each sampling month) were tested by RT-PCR for the presence of influenza A viral RNA. The RNA samples extracted from swab VTM were prepared utilizing the following optimized procedure. Each reaction contained 5·0 μl 5× PCR buffer, 0·5 μl enzyme mix, 0·5 μl dNTP mix, 0·5 μl each of forward and reverse primers (2 μm), 0·5 μl FAM probe (0·2 μm), 14·5 μl nuclease-free water, and 3 μl sample. The thermal cycle program utilized a single cycle of 50°C for 30 minutes, followed by 95°C for 15 minutes, and completed with 45 cycles of 95°C for 15 seconds and 55°C for 30 seconds per cycle. Fluorescence data were collected during the 55°C amplification step. The C_t cutoff value for positivity was set at 35.

ELISA

ELISAs were performed on all 927 serum samples utilizing an Avian Influenza Virus Antibody Test kit (MultiS-Screen; IDEXX Laboratories, Westbrook, Maine) according to manufacturer's protocols and were analyzed on a BIO-TEK PowerWave XS reader. This ELISA is an epitope-blocking ELISA that tests for antibodies to influenza nucleoprotein.[27] Samples were run in duplicate. Endpoint data were formulated by dividing the average sample value by the negative control average (S/N values). Officially validated in avian species, this kit has been shown to be sensitive and specific to influenza from pig isolates.[27] The kit S/N positive cutoff was <0·5. However, a ROC analysis of swine isolates gave an optimized cutoff of 0·673.[27] This assay has not been formally validated with feline influenza samples, but the assay detected a positive serum obtained from a cat naturally infected by HPAIV H5N1,[11] while providing negative results for uninfected cat sera. The epitope-blocking ELISA assay was also tested using sera from six cats experimentally infected with LPAIV. The epitope-blocking ELISA generated S/N values below pre-immune serum samples (all > 0·9) in all six animals with two of six serum samples falling below the recommended cutoff and clearly positive (S/N range 0·3–0·8). A ROC curve could not be created to determine the optimal cutoff values, but taking the results of a dilution series of positive cat serum into context with the swine study, the cutoff value was set to 0·6 (data not shown).

The ELISA-based SNAP FIV/FeLV Combo Test (IDEXX Laboratories, Westbrook, Maine) was utilized to test all cats for feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) according to the manufacturer's protocol.

Hemagglutination inhibition assay

Sera designated as positive by ELISA were tested by hemagglutination inhibition (HI) assay in an attempt to subtype

previous viral exposures. Sera samples first treated with a receptor-destroying enzyme (Accurate Chemical, Westbury, NY, USA) were incubated overnight at 37°C and then heat inactivated. HI assays were performed with chicken RBCs by using homologous virus and antisera using standardized techniques (WHO Manual on Animal Influenza Diagnosis and Surveillance, (http://www.who.int/entity/vaccine_research/diseases/influenza/WHO_manual_on_animal-diagnosis_and_surveillance_2002_5.pdf).

A control HI was performed with serum from HPAIV H5N1 experimentally infected cat,[11] and the procedure proved sensitive and specific for cat serum (data not shown). For each experiment, positive antisera and PBS-only and uninfected cat serum controls ensured viable data. Hemagglutinin subtypes tested include 1a–c, 2a, 3a–c, 4, 5, 6a, 7a–b, 8, 9, 10, 11a–b, 12, 14, and 15.

Statistical methods

Pearson's chi-squred test for independence was performed to determine whether the observation of temporal relationship of positive sera was statistically significant. The 95% confidence intervals (CI) for screening test results were constructed using the exact binomial test.

Results

Figure 1 shows that most cats are healthy, free of retroviral infections, rurally located, and over 1 year of age, with an equal number of males and females. No influenza A virus was isolated from any of the oropharyngeal or rectal swab samples tested. Oropharyngeal samples from all 927 unique cats were tested by MDCK cell and ECE culture methods (95% CI: 0–0·4%). Owing to practical constraints, rectal samples from all 927 cats were tested by MDCK cell culture methods (95% CI: 0–0·4%), but only 237 rectal swab samples were tested by ECE culture methods (95% CI:

0–1·5%). No influenza A viral RNA was detected in 200 oropharyngeal swab samples tested by RT-PCR (95% CI: 0–1·8%). Of the 927 serum samples tested for antibodies to influenza A, only 4 (0·43%; 95% CI 0·12–1·1%) were positive. The individual and habitat characteristics of the seropositive cats are detailed in Table 1. Hemagglutinin inhibition assays were performed on the ELISA-positive samples, but the HA antibodies were unable to be subtyped by the assay (data not shown). Of all 927 cats, 1·4% were positive for feline immunodeficiency virus (FIV) and 2·7% were positive for FeLV.

Discussion

Few feline influenza surveillance studies have been performed to date; however, an early study found that 6 of 28 (21·4%) domesticated cats were seropositive to a circulating 1968 H3N2 strain.[2] A study of domestic cats collected at the height of the 2009 H1N1 pandemic found 21·8% of 78 domestic cats seropositive to pH1N1,[7] and an unpublished study of 500 feral cats from Indonesia in 2007 reported 20% seropositivity to H5N1.[28] Experimental studies have shown that felines are susceptible to infection with H5N1,[18–20] pH1N1,[17] as well as a variety of other influenza viruses that have been tested.[2–4] These studies support the notion that cats are susceptible to influenza A virus; however, not all experimental inoculations resulted in infection[18] and some infected cats fail to seroconvert by HI.[4,12] These studies are also consistent with epidemiological studies showing natural infection of domestic and large felids with H5N1 [9,10,13–16] and domestic cats with pH1N1,[6] as well as anecdotal evidence from countries where HPAIV H5N1 is common also supports experimental findings, where increases in felid morbidity and mortality have occurred during HPAIV outbreaks to the point that local Javanese farmers have a colloquial name for it, roughly

Table 1. Characteristics of cats seropositive for influenza A antibodies by nucleoprotein-blocking ELISA

Cat ID#	Collection date	S/N value (SD*)	Sex	Age	Health status	Feline leukemia virus (FeLV)	Feline immunodeficiency virus (FIV)	Habitat where trapped
F8-3152	11/2/08	0·574 (0·136)	F	6 month–1 year	Healthy	N	N	Rural, residential, lakes within 0·5 mile
F9-750	1/11/09	0·463 (0·062)	F	>1 year	Healthy	N	N	Rural, near a feed store business
F10-415	3/28/10	0·450 (0·078)	M	6 month–1 year	Healthy	N	N	Rural, farm, lakes, and ponds within 0·5 mile
F10-427	3/28/10	0·559 (0·098)	M	>1 year	Healthy	N	N	Suburban, residential, wooded

*Standard deviation of three replicated experiments, each in duplicate.

translated as 'argh, plop'.[29,30] These epidemiological studies show that felids can be infected with influenza during particular epidemic situations when the prevalence of influenza is abnormally high or where the outbreak is caused by a recently emerged or emerging virus. These findings also highlight that infection and seroconversion can be variable. The study reported here is unique because it provides a detailed longitudinal evaluation of any influenza A infection as determined by virus isolation, RT-PCR, and ELISA techniques in a large feral cat population in an area not sustaining an avian influenza outbreak during the collection interval.

In this study, virus was unable to be isolated. Because not all samples were tested by molecular methods, it is possible that samples positive for viral nucleic acid were missed. However, that possibility seems unlikely, because a significant subset spanning the entire collection period was tested by RT-PCR, and no positive samples were found. Statistical analysis confirms that the frequency of potential positive samples is <2%, which is in line with serological results. As some of the samples tested were collected during the 2009 pandemic, a PCR primer/probe set was selected to include the detection of pH1N1; however, we failed to detect reverse zoonosis.

Despite potentially extensive contact with avian species, the results from this study also show that only a small percentage of the feral cats do contact influenza A viruses and seroconvert. Four of the 927 cats surveyed were positive for influenza A antibodies as measured by epitope-blocking ELISA. All 4 were captured from November through March when migratory avian species are present in Alachua County. This temporal relationship, however, was not statistically significant ($P = 0.059$). There was no association of seropositivity with age, health status, or infection by immunosuppressive viruses such as FeLV or FIV. The inability to subtype HA antibodies in the positive samples by HI assay does not disprove them, because the two tests measure different antibody responses, with the competitive ELISA measuring immunodominant antibody responses to conserved viral proteins and the HI measuring functional antibody responses to variable, strain-specific viral antigens. One potential issue with HI tests is that a strain with antigenic differences in the HA from the given antigen can cause false-negative results.[31] Given a surveillance where the potential infecting strains of influenza are unknown, it is possible for false-negative results by HI assay. Moreover, experimentally infected cats have failed to seroconvert as measured by HI.[4] The epitope-blocking ELISA used here has not been fully optimized for felines. We tested this ELISA assay with sera from naïve and experimentally infected cats, as well as with serum from a cat naturally infected with H5N1 influenza.[11] Serum from the naturally infected cat effectively blocked the signal (resulting in a

low S/N value), while serum from seven naïve animals consistently resulted in S/N values close to the no serum control (S/N > 0·9), suggesting that the assay was specific. Sera from experimentally infected cats consistently blocked some antigen binding in the assay, although only 2 of 6 samples blocked sufficient antigen binding to be scored as positive seroconversion as per the manufacturer's guidelines (S/N ranged from 0·3–0·8; data not shown). With limited sensitivity and specificity data for this assay for detecting feline antibodies to influenza, it is possible that these results are false positives. Additional assessment of this assay is needed. Nevertheless, while serologic analysis is imperfect, these data support the paucity of feline infection with AIVs.

The primary reservoirs of avian influenza are wild aquatic birds of the orders Charadriiformes (shorebirds, gulls) and Anseriformes (waterfowl),[32] but the avian influenza prevalence for such birds in this area is unknown. For comparison, a surveillance of avian influenza viruses in hunter-killed waterfowl during the 1986–87 hunting season in the Louisiana southwest coastal zone found prevalence estimates of AIV in ducks sampled during September, November, and December through January to be 3·1%, 2·0%, and 0·4%, respectively.[33] One may speculate a similar level of AIV prevalence in ducks of Alachua County because both areas have similar waterfowl wintering habitats. Other studies of AIV in waterfowl in wintering habitats in North Carolina,[34] Arkansas,[35] Texas,[36] and Georgia, Alabama, and Florida [37] also show low prevalence of AIV. A study of Charadriiformes was able to isolate the virus from 290 birds, but only 8 were isolated away the Delaware Bay area, and ruddy turnstones accounted for 87% of the isolates.[38] This shows that the prevalence of AIV in shorebirds and gulls can be highly species and location dependent, with low AIV prevalence in areas that are not 'hot spots' like Delaware Bay.[39] Therefore, the prevalence of AIV in the birds of the Charadriiformes and Anseriformes orders in wintering areas, such as Alachua County, is expected to be low.

One would expect the influenza A prevalence in feral cats to be lower than the already low AIV prevalence in the wild aquatic bird species. Importantly, the birds that cats are most likely to hunt and come into contact with are members of the order Passeriformes.[24] The prevalence of influenza infection in passerines is a point of contention, with a new study suggesting that the prevalence of AIV in passerines is higher than previously thought.[40,41] In addition, LPAIVs rarely cause morbidity in birds, but in epidemics of HPAIV, large numbers of birds may become ill or die. Cats may be more likely to catch and eat moribund or dead birds, leading to exposure to high amounts of virus. Thus, the low antibody prevalence observed in this study relative to previously referenced epidemiological studies may be because the environment in North Central

Florida provides a decreased chance of exposure compared to habitats with higher baseline influenza prevalence, current outbreaks, or emerging subtypes.

Conclusion

Felids have been shown to be susceptible to influenza A viruses, but feral cats from Alachua County, Florida, do not seem to have a significant role in the natural history or epidemiology of influenza A viruses. No virus was isolated from 927 cats, and viral RNA was not detected in any of the 200 cats sampled. While serologic analysis identified 4 of 927 sera as positive for prior influenza A exposure or infection, the assay has not been validated for feline serum and the 0·43% seropositive rate could not be confirmed with HI assay, raising the possibility of a false-positive result. Nevertheless, feral cats do not appear at this time to pose a substantial public health threat as a potential bridging species in this region of Florida. Populations of felids from different environments should still be studied to further understand the role cats may have in the natural history of influenza A viruses. Additional work is also needed to develop validated tools for serosurveillance of felids, as well as for use as a veterinary diagnostic.

Acknowledgements

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