Pathogenesis, transmission and response to re-exposure of SARS-CoV-2 in domestic cats

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Abstract

The pandemic caused by SARS-CoV-2 has reached nearly every country in the world with extraordinary person-to-person transmission. The most likely original source of the virus was spillover from an animal reservoir and subsequent adaptation to humans sometime during the winter of 2019 in Wuhan Province, China. Because of its genetic similarity to SARS-CoV-1, it is likely that this novel virus has a similar host range and receptor specificity. Due to concern for human-pet transmission, we investigated the susceptibility of domestic cats and dogs to infection and potential for infected cats to transmit to naïve cats. We report that cats are highly susceptible to subclinical infection, with a prolonged period of oral and nasal viral shedding that is not accompanied by clinical signs, and are capable of direct contact transmission to other cats. These studies confirm that cats are susceptible to productive SARS-CoV-2 infection, but are unlikely to develop clinical disease. Further, we document that cats develop a robust neutralizing antibody

response that prevented re-infection to a second viral challenge. Conversely, we found that dogs do not shed virus following infection, but do mount an anti-viral neutralizing antibody response. There is currently no evidence that cats or dogs play a significant role in human exposure; however, reverse zoonosis is possible if infected owners expose their domestic pets during acute infection. Resistance to re-exposure holds promise that a vaccine strategy may protect cats, and by extension humans, to disease susceptibility.

Introduction

The COVID-19 pandemic, caused by the SARS-CoV-2 (SARS2) coronavirus, originated in the Wuhan province of China, in late 2019 and within four months spread to nearly every country in the world. Sequence analysis and epidemiological investigations suggest that the virus was of animal-origin, possibly bat, and was first introduced into the human population via an intermediate animal host in the Huanan seafood market in Wuhan, China (Bogoch et al. 2020; Zhou et al. 2020). The virus quickly adapted to humans and human-to-human transmission became the almost immediate source of subsequent infections, with direct contact and aerosol droplets as the primary routes of infection (Li et al. 2020). Early indications suggested that SARS2, much like SARS-CoV-1 (SARS1), infects host cells by binding to the angiotensin-converting enzyme 2 (ACE2), a receptor that is expressed in many animal species, although notably not in mice or rats (Wan et al. 2020). Thus, while humans are almost certainly the sole source of infection to other humans, multiple early studies suggest other animals are susceptible to infection as well.

The first report of reverse zoonosis, or transmission from human to animal, was reported from Hong Kong, where a COVID patient's dog tested PCR positive for SARS2 multiple times (Sit et al. 2020). In following weeks, other reports of domestic pets becoming infected following exposure to humans were documented, including another dog in Hong Kong and a cat with clinical disease in Belgium (Chini 2020). Serologic studies so far have failed to identify domestic dogs and cats as a primary source of human infection (Deng et al. 2020). Importantly, a survey of veterinary students with confirmed COVID infection was unable to identify antibodies in their pets (Temmen et al. 2020). Despite the low probability of pet-to-human or human-to-pet transmission, it remains important to clarify what role, if any, that domestic pets play in SARS2 transmission.

The first published study involving cat experimental infections showed that cats could become infected by SARS2 and could potentially transmit to other cats via aerosols, as defined by PCR positive fecal samples from cats in cages in the same room as directly infected cats. This study also described pathology and mortality in juvenile cats euthanized at 3 and 7 days post-infection (Shi et al. 2020) Additional communications described viral shedding and direct contact transmission in cats as well as seroconversion in cats exposed to infected humans (Halfmann et al. 2020; Zhang et al. 2020). The experiments described herein expand upon existing work by providing shedding kinetics in cats over time, assessing virus neutralization, seroconversion, and exploring transmission. Furthermore, to the author's knowledge, this is the first report of protective immunity against SARS2 following repeated exposure. These studies indicate that cats may serve as a suitable animal model for studying SARS2 infection, and for furthering the development of vaccines and therapeutics for use in both animals and humans. We also confirm an earlier report that dogs do not replicate virus locally (Shi et al. 2020), but

document evidence of anti-viral neutralizing activity in post-exposure canine sera. The role of cats in zoonotic transmission remains an open question, but relatively short duration of shedding and resistance to re-exposure suggests risk of this is very low, particularly when cats are kept indoors.

Materials and Methods

Virus

SARS2 virus strain WA1/2020WY96 was obtained from BEI Resources (Manassas, VA, USA), passaged twice in E6 Vero cells and stocks frozen at -80C in Dulbecco's Modified Eagle Medium (DMEM) with 5% fetal bovine serum and antibiotics. Virus stock was titrated on Vero E6 cells using standard double overlay plaque assay (Kropinsky et al. 2008) and plaques counted 72 hours post-infection to determine plaque-forming units (pfu) per ml.

Animals

Seven adult (1 male, 6 female, 5-8 year old) cats were obtained from a closed breeding colony held at Colorado State University in a pathogen-free environment in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International accredited animal facility. Cats were screened negative for feline enteric coronavirus antibody prior to transfer. Three dogs (female, 5-6 years old) were obtained from Ridglan Farms (Blue Mounds, WI, USA). Cats and dogs were transferred to the Animal Disease Laboratory, an Animal Biosafety Level-3 (ABSL3) facility at Colorado State University, group housed and fed dry/wet food mix with access to water ad libitum. Animals were allowed several days to acclimate before

92 temperature-sensing microchips (Lifechips, Destron-Fearing) were inserted subcutaneously in
93 the dorsum. Baseline weights, body temperatures, clinical evaluation, and oral swabs were
94 obtained prior to inoculation. All animals were in apparent good health at the onset of the study.
95
96 *Virus challenge*
97 Cats were lightly anesthetized with 30-50 mg subcutaneous ketamine hydrochloride
98 (Zetamine™) and dogs sedated with 1-3 mg xylazine. Virus diluted in phosphate buffered saline
99 (PBS) was administered to both species via pipette into the nares (500ul/nare) for a total volume
100 of 1ml; animals were observed until fully recovered from anesthesia. Virus back-titration was
101 performed on E6 cells immediately following inoculation, confirming that cats received 3.0E5
102 pfu and dogs received 1.4E5 pfu.
103
104 *Sampling*
105 Cat cohort 1 (n=3)
106 Oropharyngeal swabs were collected daily on days 1-5, 7, 10, and 14 post-infection using a
107 polyester tipped swab applicator. Swabs were placed in BA-1 medium (Tris-buffered MEM
108 containing 1% BSA) supplemented with gentamicin, amphotericin B and
109 penicillin/streptomycin. Nasal flushes were performed on 1, 3, 5, 7, 10, and 14 days post-
110 infection (DPI) by instilling 1ml BA-1 dropwise into the nares of awake or lightly anesthetized
111 cats and collecting nasal discharge into a sterile petri dish by allowing the wash fluid to be
112 sneezed out or dripped onto the dish. Blood (5 ml into serum separator tubes) was collected prior
113 to inoculation and on days 7, 14, 21, 28, 35 and 42 post-infection. At 28 DPI, cats were re-
114 challenged with 3e5 pfu of homologous virus. Oronasal sample collection was performed 1, 3,

5, 7, 10 and 14 after re-inoculation (days 29, 31, 33, 35, 38 and 42 post initial challenge), at which time cats were euthanized and tissues collected for histopathology.

Cat cohort 2 (n=4)

Two of the four cats were lightly anesthetized, and challenged with SARS2 as for Cohort 1. Forty-eight hours post-infection, two naïve cats were introduced into the room with the infected cats and sampled on the same schedule as before. The two directly challenge cats were euthanized on 5 DPI and the following tissues collected for virus isolation and histopathology: nasal turbinates, trachea, esophagus, mediastinal lymph node, lung, liver, spleen, kidney, small intestine, uterus, and olfactory bulb. Tissues were collected into BA-1 frozen at -80C and homogenized prior to plaque assay. Additional tissues collected for histopathology included heart, colon, pancreas, hemi-lung lobe, and mesenteric lymph nodes. Thoracic radiographs were also obtained for these two cats pre-challenge and just prior to euthanasia. The remaining two cats were euthanized at 30 DPI and necropsied; these cats will be referred to as contact cats hereafter.

Dogs (n=3)

Dogs were sampled at the same frequency, and using the same methods as cats in Cohort 1 for 42 days post-infection. Dogs were not re-challenged.

Clinical observations

Body temperatures were recorded daily for the duration of the study using the thermal microchips. Cats and dogs were observed twice daily for the first seven days post-challenge and

at least once daily for the duration of the study. Body weights were obtained weekly. Clinical evaluation included temperament, ocular discharge, nasal discharge, ptyalism, coughing/sneezing, dyspnea, diarrhea, lethargy, anorexia, moribund. None of the animals exhibited clinical signs of disease characterized by any of these symptoms at any time during the study.

Viral assays

Virus isolation was performed on all oral swab, nasal flush and 5 DPI tissue samples by double overlay plaque assay on Vero E6 cells as previously described (Kropinsky et al. 2009). Briefly, 6-well plates with confluent monolayers of cells were washed once with PBS and inoculated with 100 ul of serial 10-fold dilutions of samples, incubated for 1 hour at 37ºC, and overlaid with a 0.5% agarose in MEM containing 2% fetal bovine serum and antibiotics/antifungal agents. A second overlay with neutral red dye was added at 48 hours and plaques were counted at 72 hours. Viral titers were reported as the $\log_{10}$ plaque-forming units (pfu) per ml.

Plaque reduction neutralization assays (PRNT) were performed as previously described (Perera et al. 2020). Serum was heat-inactivated for 30 mins at 56ºC, and two-fold dilutions prepared in BA-1 starting at a 1:5 dilution were aliquoted onto 96-well plates. An equal volume of virus was added to the serum dilutions and incubated for 1 hour at 37ºC. Following incubation, serum-virus mixtures were plated onto Vero E6 plates as described for virus isolation assays. Antibody titers were recorded as the reciprocal of the highest dilution in which >90% of virus was neutralized.

ELISA

Serum samples from cats were heat inactivated and tested by plaque assay to verify samples were noninfectious prior to conducting ELISA analysis. Positive control antibodies to the receptor-binding domain (RBD) and full-length spike protein were human MAb CR3022 antibody (Absolute Antibody, Oxford UK) and human IgG whole molecule (Jackson Immuno Research, West Grove PA, USA). Positive control for the nucleocapsid ELISA was SARS-CoV nucleoprotein rabbit monoclonal antibody (Sino Biological Inc, Beijing, China). Negative controls were reagent grade human sera (compared to Mab CR3022). Cat serum from specific pathogen free, naïve experimental animals, and field isolate bioarchived samples obtained prior to 2019 (Carver et al, 2005, Sprague et al, 2018). ELISA protocols were adapted from protocols for SARS CoV-2 ELISA described by Amanat et al., 2020. ELISA plates (Thermo) were coated at 2ug/ml with spike glycoprotein Receptor Binding Domain (RBD) from SARS-CoV-2, WuHan-Hu-1 recombinant from HEK293T cells (BEI), or Spike glycoprotein (Stabilized) from SARS-CoV-2, Wuhan-Hu-1, recombinant from Baculovirus (BEI). SARS CoV-2 nucleocapsid protein was a gift of Dr. Brian Geiss. Prior to running experimental cat sera, the assay was optimized using positive and negative control sera described above (data not shown.) Samples and controls were diluted 1:50 in ELISA diluent (1X PBS, tween, milk powder) and run in duplicate. Human sera controls were developed using anti-human IgG HRP (Thermo), cat sera was developed using anti-cat IgG HRP (Thermo) or anti-cat IgM (Novus Biologicals) and rabbit mAb SARS-CoV NP was detected by anti-rabbit IgG HRP (Thermo). Secondary antibodies were diluted 1:3000 and-SigmaFast OPD was prepared in WFI and added to wells. Plates were read at 490nm using a Multiskan® Spectrum spectrophotometer (Thermo Fisher). The mean of negative control sera OD490 plus three times the standard deviation of the negative control readings were used to determine cut off values for each plate.

qRT-PCR

Plaques were picked from culture plates from each cat to confirm SARS2 viral shedding. RNA extractions were performed per the manufacturer's instructions using Qiagen QiaAmp Viral RNA mini kits. RT-PCR was performed as recommended using the E_Sarbeco primer probe sequence as described by Corman and colleagues (2020) and the Superscript III Platinum One-Step qRT-PCR system (Invitrogen), with the following modification; the initial reverse transcription was at 50ºC. Standard curves were obtained by serial dilution of stock viral RNA from the original WA1/2020WY96 SARS2 isolate.

Histopathology

Tissues from cats were fixed in 10% buffered formalin for 12 days and transferred to 70% ethanol prior to sectioning for H&E staining. Slides were read by a board certified veterinary pathologist.

Results

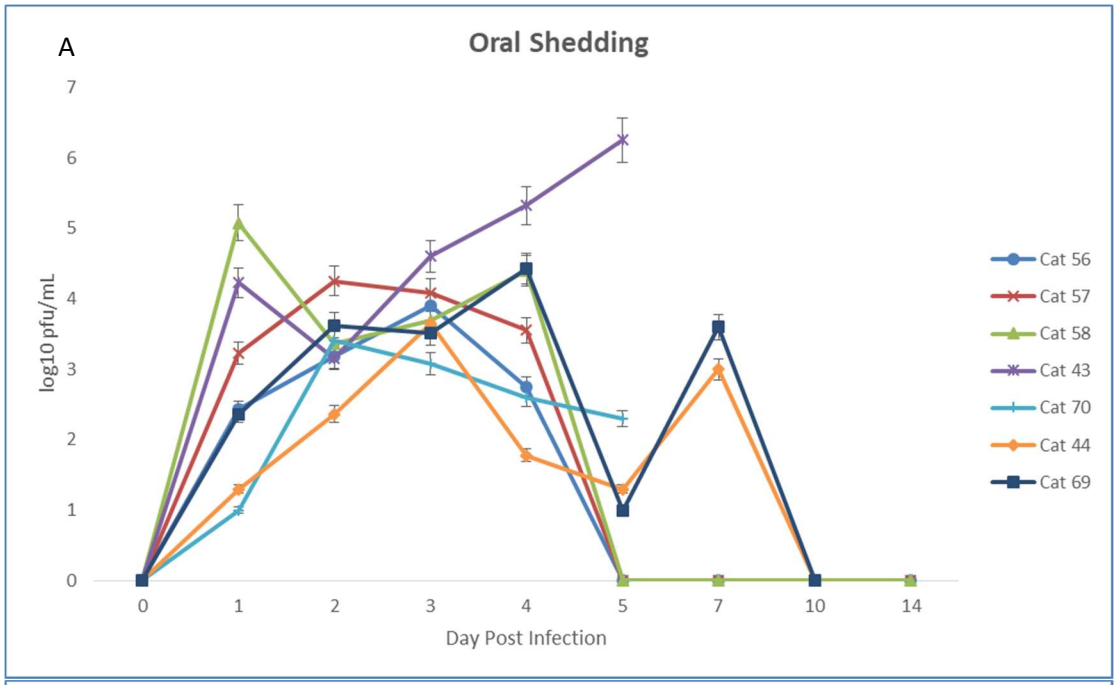
Clinical Disease

None of the cats in either cohort displayed any clinical signs of disease and remained afebrile throughout the study. Body weights were maintained over time. Radiographs were taken pre-challenge and at 5 DPI just prior to euthanasia for the experimentally inoculated cats in Cohort 2. No evidence of lung involvement or any other radiographically-detectable abnormalities were

noted (images not shown). Similarly, dogs inoculated with SARS2 remained clinically normal and afebrile.

Viral shedding

In Cohort 1, all three cats shed virus both orally and nasally for up to 5 days post-infection, with peak titers achieved from nasal shedding at day 3. Nasal titers were approximately 1 log higher than oral swabs collected at the same time (Figure 1). There was some variability in titer over the course of infection that is likely attributable to sample collection (i.e. quality of sneezes), but overall the data demonstrates clear presence of infectious virus in both the nasal cavity and the oropharynx for multiple days post-infection. In Cohort 2, the inoculated cats shed virus for 5 days post-infection both orally and nasally, with a similar pattern to Cohort 1. The contact cats, however, shed infectious virus orally by 24 hours post-exposure and the duration of shedding was prolonged compared to the inoculated cats, with peak shedding occurring at 7 days post-exposure (Figure 1). Virus was isolated from trachea, nasal turbinates, and esophagus from cats in Cohort 2 necropsied on day 5. Infectious virus was not found in the lung or other organs of either cat. Viral shedding was not detected from any of the dogs at any point post-infection.



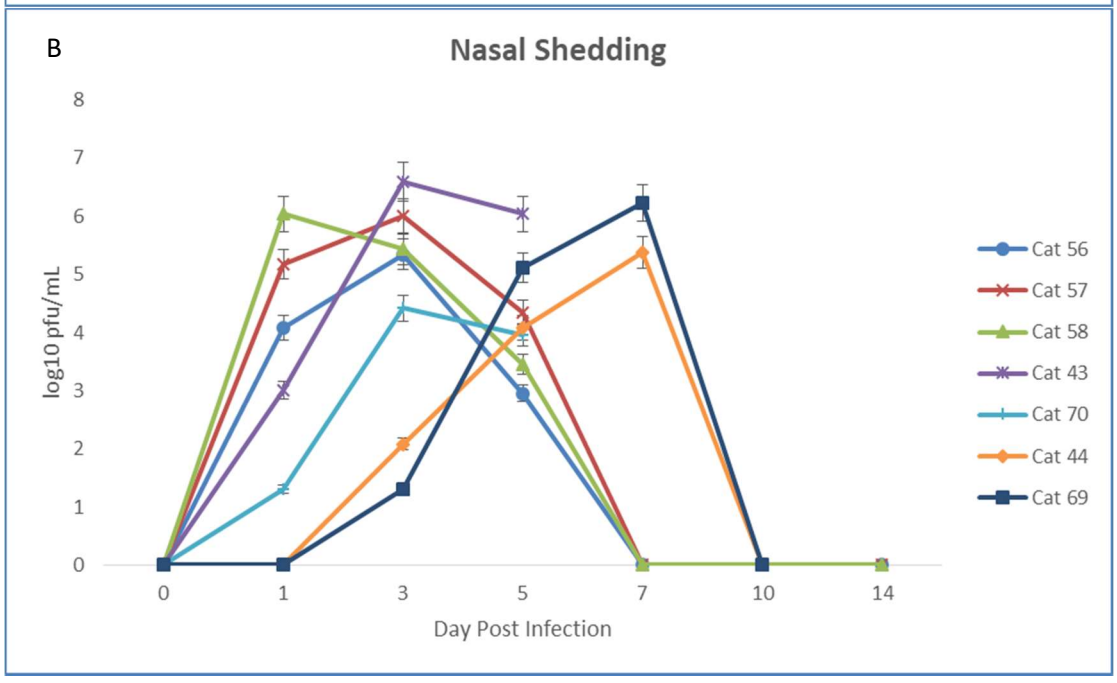


Figure 1: Inoculation and exposure with SARS2 leads to oral and nasal shedding in cats. SARS2 virus is detected by plaque assay from (A) oral and (B) nasal secretions of cats 1-5 days post infection. Viral titers expressed as $\log_{10}$pfu/ml. Cats 56, 57, and 58 represent Cohort 1. Cats 43, 70, 44, and 69 represent Cohort 2. Cats 43 and 70 were euthanized on 5 DPI. Cats 44 and 69 were introduced to the infected cats in Cohort 2 on 2 DPI.

Pathology

Gross lesions were not observed in any of the necropsied cats. Histologically, in both cats sacrificed at 5 DPI from Cohort 1, moderate ulcerative, suppurative lymphoplasmacytic rhinitis was observed in the nasal turbinates along with mild lymphoplasmacytic tracheitis. These cats also had minimal alveolar histiocytosis with edema and one of the cats had rare type II pneumocyte hyperplasia (Figure 2). All three cats from Cohort 1 sacrificed 42 DPI had mild lung changes, including mild interstitial lymphocytic pneumonia with peribronchiolar and perivascular lymphocytic cuffing and alveolar histiocytosis. Two of these cats also had minimal tracheitis, but largely the lesions in the upper respiratory tract appear decreased in comparison to the early timepoint cats, while lung pathology was more evident in these animals compared to those sacrificed during acute infection. Dogs were not euthanized at the time of this report.

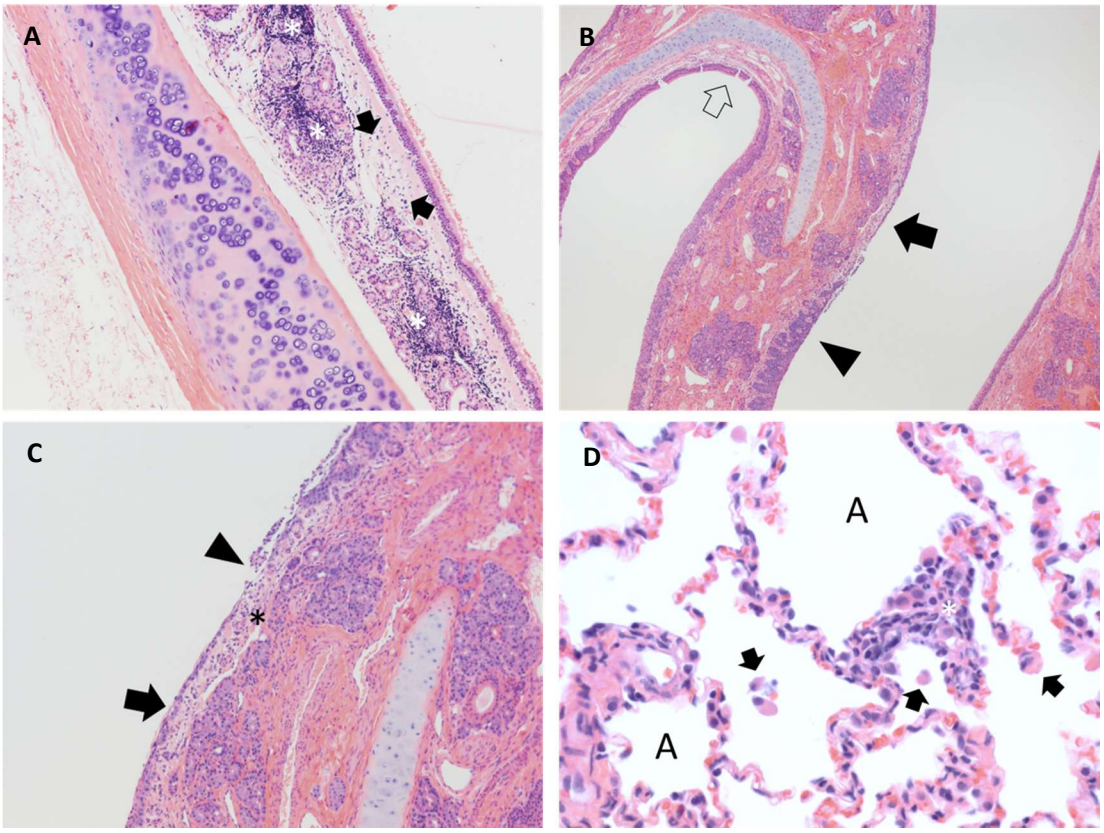


Figure 2: SARS2 exposure results in acute upper respiratory inflammation and mild lung infiltrates during later courses of infection.

Panel A: **Cat 43, trachea 5 DPI.** The submucosa is expanded by edema (arrow) and abundant lymphocytic inflammatory infiltrates (asterisks) which dissect and disrupt submucosal glands. Hematoxylin & eosin stain. 100x magnification.

Panel B: **Cat 70, nasal turbinates 5 DPI.** Normal thickness respiratory mucosa is present in the section (open arrow). Nasal respiratory epithelium ranges from hyperplastic (filled black arrow) to ulcerated (arrowhead). The submucosa in regions of ulceration is edematous and infiltrated by scattered neutrophils and mononuclear cells. Hematoxylin & eosin stain. 40x magnification.

Panel C: **Cat 70, nasal turbinates, 5 DPI.** Nasal respiratory epithelium ranges from attenuated (arrow) to ulcerated (arrowhead) with overlying remnant cellular debris. The submucosa (asterisk) in regions of ulceration is edematous and infiltrated by scattered neutrophils and mononuclear cells. Hematoxylin & eosin stain. 100x magnification.

Panel D: **Cat 56, lung, 42 DPI.** Alveolar spaces (A) contain scattered mononuclear cells (arrow). The alveolar wall is expanded by mixtures of mononuclear cells and occasional neutrophils (asterisk). Hematoxylin & eosin stain. 400x magnification.

Seroconversion

Cats in both Cohort 1 and the direct contact cats developed neutralizing activity as measured by PRNT as early as 7 DPI. Neutralizing titers in all cats reached or exceeded 1:2560 by 14 DPI and either maintained or increased in titer between 28 and 42 DPI. Cats re-challenged at 28 DPI displayed a moderate increase in PRNT titer in the 14 days following exposure (Table 1). Dogs

264 developed neutralizing antibodies by 14 DPI and peaked at 21 DPI with titers between 1:40-1:80
265 (Table 1).
266
267 **Table 1: PRNT90 values for cats and dogs**

Animal	0 DPI	7 DPI	14 DPI	21 DPI	28 DPI	42 DPI
Cat 56 (Cohort 1)	<50%	320	5120	2560	2560	10240
Cat 57 (Cohort 1)	<50%	80	5120	2560	2560	5120
Cat 58 (Cohort 1)	<50%	80	2560	2560	1280	5120
Cat 43 (Cohort 2)	<50%					
Cat 70 (Cohort 2)	<50%					
Cat 44 (Cohort 2)	<50%	NT	2560	5120	5120	
Cat 69 (Cohort 2)	<50%	NT	2560	1280	10240	
Dog 46	<50%	<10	10	40	40	80
Dog 47	<50%	10	80	20	20	40
Dog 48	<50%	<10	20	40	20	20

268
269 IgG antibodies responses exceeding OD490 cut off values were detected at day 7 PI against both
270 the complete spike glycoprotein and RBD in all experimentally inoculated cats, and
271 seroconversion against NP was detected in 2 of 3 cats at this time. By day 14 all five cats had
272 OD values that neared upper limit of detection in the Spike ELISA; RBD and NP OD saturation

was obtained by day 21 and did not increase following re-exposure (Figure 3). Rates of seroconversion and absorbance levels were similar between contact cats and experimentally infected cats. Seroconversion to spike protein was most rapid and robust, and the specificity of response to RBD exceeded that of NP. Seroconverted cat OD values for all three antigens exceeded absorbances of SPF or field domestic cats, and background was highest for NP. IgM antibodies against RBD were detected at days 7 and 14 but not at day 28. IgG responses were much more robust than IgM (Figure 3). ELISA assays were not performed on dog sera.

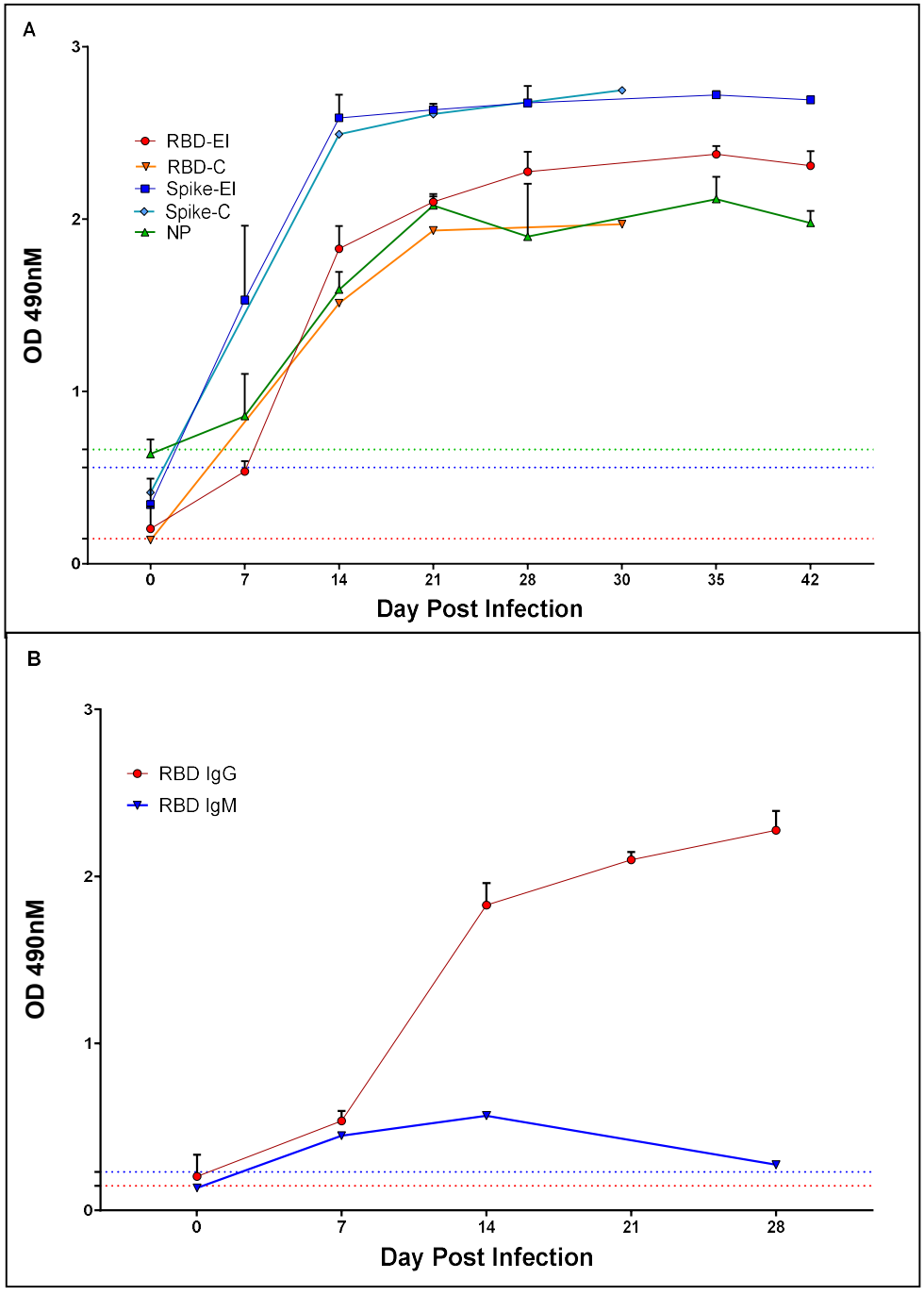


Figure 3. Cats infected with SARS CoV-1 rapidly develop antibodies against viral antigens. (A) Sera from cats with intranasal inoculation of SARS CoV-2 (n=3, 'EI') or exposed to inoculated cats (n=2, 'C') were evaluated for seroreactivity to receptor binding domain (RBD), Spike, or nucleocapsid protein (NP) for 30-42 days post exposure. IgG Reactivity to Spike and RBD was evident at day 7, and all animals had clearly seroconverted by day 14. (B) IgM against RBD was transiently detected at low levels relative to IgG on days 7 and 14 post exposure (experimentally inoculated animals, n=3). Bars represent 1 SE of the mean.

Reinfection

Re-challenged cats in Cohort 1 were sampled for oral and nasal shedding for 7 days post-exposure by viral isolation, and shedding was not detected in any cat at any timepoint following rechallenge.

Discussion

The COVID19 pandemic represents the first global pandemic of an emerging zoonotic disease in this century. The SARS2 virus is one of three emergent zoonotic coronaviruses capable of causing significant disease in humans in the last two decades, following SARS1 and MERS (Guarner 2020). The overall trend of disease emergence favors viral spillover from animals to human, and land use and wildlife encroachment are just two of the factors contributing to this phenomenon (Olival et al. 2017). The continued presence of live animal markets provides optimal conditions for emergence of zoonoses (Wang and Eaton, 2007). As with SARS1 and MERS, SARS2 is of probable bat origin based on phylogenetic analysis (Zhou et al. 2020), but unlike its predecessors, SARS2 has rapidly evolved for highly efficient human-

316 to-human transmission (Chan et al et al. 2020). While animals, including domestic animals and
317 pets, are frequently implicated as the source of emerging pathogens, reverse zoonosis of SARS2
318 is more probable, as human cases are far more prevalent than domestic animals. Similar results
319 were seen with SARS1, where domestic cats exposed to the virus by infected humans became
320 infected, and cats experimentally infected shed virus for several days (Martina et al. 2003, van
321 den Brand et al. 2008) There have been several cases of pets becoming infected by SARS2
322 following exposure to infected humans in New York, Hong Kong, Belgium, Germany, Spain,
323 France and Russia (Sit et al. 2020; as communicated by ProMed). Other animal exposures from
324 infected humans include farmed mink, which appear to display respiratory symptoms following
325 infection (ProMed). In several of these cases, including nondomestic felids at the Bronx Zoo and
326 pet cats in New York and Europe, animals displayed signs of respiratory disease and/or
327 conjunctivitis. None of the cats or dogs in this study exhibited any clinical signs of disease, but
328 individual animal health status, age and comorbidities may be responsible for this variability.
329 Pathological changes in cats suggest that mild subclinical disease in otherwise healthy animals
330 occurs but is not recognizable symptomatically. This is not altogether different from human
331 infections, where the majority of cases are relatively mild but more severe disease tends to occur
332 in older patients with significant comorbidities (Nikolich-Zugich et al. 2020). In a recent
333 serosurvey of cats in Wuhan, China, nearly 14.7% of sampled animals were seropositive for
334 SARS2 by RBD ELISA, suggesting that the cat population in areas with high human
335 transmission is also likely to be exposed to the virus (Zhang et al. 2020) Considering that the
336 number of human infections have reached the millions and yet only a handful of animals have
337 tested PCR positive, it seems unlikely that domestic pets are a significant source of infection or
338 are at serious risk for developing severe disease. Importantly, infected cats shed for no more than

5 days following exposure, suggesting that cats, if exposed to infected humans, will develop and clear infection rapidly. In comparison, humans typically have an incubation period of approximately 5 days and can shed virus for more than three weeks (Lauer et al. 2020, Noh et al. 2020). Thus, if symptomatic humans follow appropriate quarantine procedures and stay home with their pets, there is minimal risk of a potentially exposed cat infecting another human. Infected pet cats should not be allowed outdoors to prevent potential risk of spreading infection to other outdoor cats. More research into the susceptibility of wildlife species and potential for establishment of infection in outdoor cat populations is necessary to identify risk factors and mitigation strategies to prevent establishment of reservoir infections in feral cats.

The development of animal models for studying SARS-2 is an important step in research methodologies. Rhesus macaques, hamsters, and ferrets are all suitable models for replicating asymptomatic or mildly clinical disease, and, while not often used as a traditional animal model, this work demonstrates that cats may serve as an alternative model (Kim et al. 2020, Chan et al. 2020, Munster et al. 2020) The cats in this study developed subclinical pathological changes in the upper respiratory tract early in the course of infection with more lower respiratory tract pathology later following viral clearance, which suggests that, while subclinical, viral infection of cats is not completely benign, and may make their utility as an animal model more relevant to mild human disease. Additionally, the relatively high-titer viral shedding produced by cats and the rapidity of transmission may make them an ideal model for simulation of aerosols. As such, cat models may be quite useful for understanding the shed/spread kinetics of SARS2. Perhaps most importantly, cats develop significant neutralizing antibody titers, and are resistant to re-infection, which could prove a useful measurement for subsequent vaccine trials for both human and animal vaccine candidates.

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