

1 **Detection and Interspecies Comparison of** 2 **SARS-CoV-2 Delta Variant (AY.3) in Feces from a** 3 **Domestic Cat and Human Samples**

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19 **Abstract:** Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections have
20 spilled over from humans to companion and wild animals since the inception of the global
21 COVID-19 pandemic. However, whole genome sequencing data of the viral genomes that
22 infect non-human animal species has been scant. Here, we detected and sequenced a
23 SARS-CoV-2 delta variant (AY.3) in fecal samples from an 11-year-old domestic house cat
24 previously exposed to an owner who tested positive for SARS-CoV-2. Molecular testing of two
25 fecal samples collected 7 days apart yielded relatively high levels of viral RNA. Sequencing of
26 the feline-derived viral genomes showed the two to be identical, and differing by between 4
27 and 14 single nucleotide polymorphisms in pairwise comparisons to human-derived lineage
28 AY.3 sequences collected in the same geographic area and time period. However, several
29 mutations unique to the feline samples reveal their divergence from this cohort on phyloge-
30 netic analysis. These results demonstrate continued spillover infections of emerging
31 SARS-CoV-2 variants that threaten human and animal health, as well as highlight the im-
32 portance of collecting fecal samples when testing for SARS-CoV-2 in animals. To the authors'
33 knowledge, this is the first published case of a SARS-CoV-2 delta variant in a domestic cat in
34 the United States.

35 **Keywords:** COVID-19; SARS-CoV-2; coronavirus; delta variant; AY.3; cats; feline; whole
36 genome sequencing; feces; One Health

37 **1. Introduction**

38 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections have spilled
39 over from humans to numerous animal species, including domestic cats and dogs,
40 non-domestic large felids, minks, and white-tailed deer, amongst others [1-4]. Several spe-
41 cies, including domestic cats, transmit their infection to naive conspecifics under experimental
42 conditions [5]. A number of recent studies have demonstrated natural spillover infections in
43 white-tailed deer (*Odocoileus virginianus*), with likely spread amongst wild deer in the field [2,
44 6, 7]. Furthermore, human-to-mink and mink-to-human transmission has been documented in
45 mink farms in the Netherlands [4]. These findings provide evidence that SARS-CoV-2 may
46 establish itself in one or more enzootic reservoirs that threaten both non-human animal spe-
47 cies and humans.

48 Emerging SARS-CoV-2 variants have distinct host species ranges. For example, the beta
49 (B.1.351) variant infects deer mice (*Peromyscus* spp.) and laboratory mouse strains, whereas
50 the original strain cannot [8]. Tracking the natural host range of each variant can further clarify
51 potential enzootic reservoir formation and consequential secondary spillover events. This is
52 especially important with the still-widespread delta variant, which transmits readily in humans,
53 can cause severe disease, and is associated with higher rates of vaccine breakthrough in-
54 fections than lineages that emerged earlier in the epidemic [9-11]. The delta variant encom-
55 passes lineages such as B.1.617.2 and AY.3 that share common defining mutations like spike
56 L452R, P681R, and D950N [12, 13].

57 Thus far one dog in the United States contracted the SARS-CoV-2 delta variant lineage
58 AY.3 [14], and several Asiatic lions in India [15, 16] as well as three domestic cats in China [17]
59 have recently tested positive for the delta variant lineage B.1.617.2.

60 Here, we report a SARS-CoV-2 delta variant (AY.3) detected in fecal specimens from a
61 domestic house cat in the Delaware Valley region of southeastern Pennsylvania. The animal

62 had a known human COVID-19 exposure and presented to the veterinary hospital for gas-
63 trointestinal signs. Whole genome sequencing and phylogenetic analysis revealed lineage
64 AY.3 with several mutations unique among human-derived viral genomes of the same geo-
65 graphic area. To our knowledge, this is the first published case of a SARS-CoV-2 delta variant
66 in a domestic cat in the United States, and the first ever published case of lineage AY.3 in a
67 domestic cat. Our current findings add to the growing body of evidence that further spillover
68 transmission of the delta variant to non-human animals is on-going.

69 **2. Materials and Methods**

70 *2.1 Animal and Human Subjects*

71 Local human-derived viral sequences were gathered as described in a previous publica-
72 tion where sequence data can be accessed [18]. The University of Pennsylvania Institutional
73 Review Board (IRB) reviewed the human research protocol and deemed the limited data
74 elements extracted with positive human SARS-CoV-2 specimens to be exempt from human
75 subject research per 45 CFR 46.104, category 4 (IRB #848605). Informed owner consent was
76 provided for all procedures involving the cat. The University of Pennsylvania Institutional
77 Animal Care and Use Committee (IACUC) and Privately Owned Animal Protocol (POAP)
78 Committee approved the protocol (IACUC/POAP #806977). Consent was obtained from the
79 state animal health officials to collect specimens from the cat for SARS-CoV-2 testing, and for
80 submission of “non-negative” specimens to the National Veterinary Services Laboratory
81 (Ames, IA) for confirmation of a positive test.

82

83 *2.2 SARS-CoV-2 Clinical Testing*

84 RNA was extracted from specimens using a QIAamp Viral RNA Mini Kit (Qiagen, Ger-
85 mantown, MD). Testing for SARS-CoV-2 was performed at the university microbiology la-
86 boratory using the CDC 2019 Novel Coronavirus (2019-nCoV) Real-Time Reverse Tran-
87 scriptase (RT)–PCR Diagnostic Panel (IDT, Coralville, IA). The university microbiology la-

88 boratory is a member laboratory of the Food and Drug Administration (FDA) Veterinary La-
89 boratory Investigation and Response Network (Vet-LIRN). As part of this network, the uni-
90 versity microbiology laboratory completed an Inter-Laboratory Comparison Exercise (ICE) of
91 SARS-CoV-2 Molecular Detection Assays Being Used by Veterinary Diagnostic Laboratories
92 in August 2020.

93

94 *2.3 SARS-CoV-2 Whole Genome Sequencing*

95 The POLAR protocol was used for sequencing genomes [19] . Specifically, 5 µl of viral
96 RNA, 0.5µl of 10mM dNTPs Mix (Thermo Fisher, 18427013), 0.5 µl of 50 µM Random
97 Hexamers (Thermo Fisher, N8080127), and 1 µl water was heated at 65°C for 5 minutes.
98 Reverse transcription was performed with a reaction containing 6.5 µl from the previous step,
99 0.5 µl of RNaseOut (Thermo Fisher, 18080051), 0.5 µl of 0.1M DTT (Thermo Fisher,
100 18080085), 0.5 µl SuperScript III Reverse Transcriptase (Thermo Fisher, 18080085), and 2 µl
101 of 5X First-Strand Buffer (Thermo Fisher, 18080085). This mixture was heated at 42°C for 50
102 minutes, then incubated at 70°C for 10 minutes. ARTIC-nCoV2019 version 4 primers were
103 used (IDT) to amplify the product by PCR in a reaction containing 2.5 µl of the product from the
104 previous step, 0.5 µl of 10 mM dNTPs Mix (NEB, N0447S), either 4.0 µl of primer set 1 or 3.98
105 µl of primer set 2, 0.25 µl Q5 Hot Start DNA Polymerase (NEB, M0493S), 5 µl of 5X Q5 Re-
106 action Buffer (NEB, M0493S), and water to bring to 25 µl. The mixture was amplified with 1
107 cycle at 98°C for 30 seconds, then 25 cycles at 98°C for 15 seconds and 65°C for 5 minutes.
108 Products from primer set 1 and 2 were combined and then brought to a concentration of 0.25
109 ng/µl. The Nextera XT Library Preparation Kit (Illumina, FC-131-1096) and the IDT for Illumina
110 DNA/RNA UD Indexes (Illumina, 20027213, 20027214, 20027215, 20027216) were used for
111 library prep. Each sample was quantified with the Quant-iT PicoGreen dsDNA quantitation kit
112 (Invitrogen, P7589). The samples were then pooled and sequenced on an Illumina NextSeq.

113

114 *2.4 SARS-CoV-2 Whole Genome Sequencing*

115 Sequences were trimmed and aligned to the Wuhan reference sequence (NC_045512.2).
116 Alignment used the BWA aligner tool (v0.7.17) [20]. Samtools package (v1.10) was used to
117 remove reads that did not align to the reference [21]. To accept a genome as high quality, we
118 required that coverage must be ≥ 5 read depth for $\geq 95\%$ of the genome. The Bcftools package
119 (v1.10.2-34) was used to call the variant positions [22]. The Pangolin lineage software
120 (Pangolin version 3.1.17 with the PangoLEARN 2021-12-06 release) was used to assign
121 variants. A pipeline developed by Everett et al. was used to assign point mutations [23].

122

123 *2.5 SARS-CoV-2 Whole Genome Sequencing*

124 To construct phylogenetic trees, NextClade was used for alignment [24], IQ-Tree
125 (v1.6.12) was used to generate the phylogenetic tree [25-28], and FigTree v1.4.4 was used to
126 visualize the tree.

127 **3. Results**

128 *3.1. Case Description*

129 In September 2021, an 11-year-old indoor-only female spayed domestic shorthair cat
130 (*Felis catus*) was presented to the Ryan Veterinary Hospital Emergency Service at the Uni-
131 versity of Pennsylvania School of Veterinary Medicine following several days of anorexia,
132 lethargy, soft stools, and vomiting as well as a known COVID-19 exposure. One of the cat's
133 owners tested positive for SARS-CoV-2 prior to onset of the cat's clinical signs. At the time of
134 sample collection, the cat had been isolated from the infected human for 11 days and was
135 cared for by another household member who repeatedly tested negative.

136 The cat had a medical history of presumptive chronic enteropathy, which had been
137 successfully managed with a hydrolyzed protein diet and for which further diagnostics were
138 not performed, as well as hypertrophic obstructive cardiomyopathy that was treated with
139 atenolol.

140 On physical examination, the cat's heart rate, respiratory rate, and temperature were
141 within normal limits, with normal lung sounds on cardiothoracic auscultation. She was mildly
142 uncomfortable on abdominal palpation. The remainder of her physical examination was un-
143 remarkable.

144 A fecal sample was submitted for polymerase chain reaction (PCR) testing for infectious
145 agents associated with feline gastrointestinal disease: Feline *parvovirus*, *Tritrichomonas*
146 *foetus*, *Campylobacter jejuni/coli*, *Cryptosporidium* spp., *Cryptosporidium felis*, *Salmonella*
147 spp., *Giardia* spp., *Clostridium difficile* toxin A/B, and *Clostridium perfringens* enterotoxin. All
148 tests were negative.

149 *3.2 Molecular Detection and Sequencing*

150 The fecal sample was tested for SARS-CoV-2 using the Centers for Disease Control 2019
151 Novel Coronavirus real time PCR (RT-PCR) Diagnostic Panel. The sample tested positive for
152 both viral nucleocapsid targets with cycle threshold (Ct) values of 26.3 and 27.7. The
153 oropharyngeal swab sample was negative. To comply with reportable disease mandates, an
154 aliquot of the fecal sample was sent to the National Veterinary Services Laboratory (NVSL)
155 (Ames, IA) and confirmed as positive. A second fecal sample collected seven days later was
156 positive with Ct values of 27.7 and 28. Attempts to isolate replication-competent virus were
157 unsuccessful.

158 We performed SARS-CoV-2 whole genome sequencing (WGS) on the two samples from
159 the cat. We received 99.7% and 98.3% coverage with a mean coverage of 1,843 and 374
160 reads for the two samples respectively [18]. WGS performed by NVSL yielded nearly identical
161 results using slightly different techniques. Differences between our groups' sequencing results
162 are attributed to differences in primers used at the time of sequencing [29].

163 *3.3 Comparison to Known Sequences in the Delaware Valley*

164 The feline-derived SARS-CoV-2 genome was identified as delta variant lineage AY.3. The
165 sequences obtained from the fecal specimens on days 1 and 8 were identical, and therefore

166 stable over a 7-day period. In addition to the mutations associated with known human-derived
167 AY.3 sequences, our sample has several that are uncommon or unique (Table 1). Out of over
168 4,200 human samples that we have sequenced from our geographic region, the Delaware
169 Valley in Pennsylvania, 10 single nucleotide polymorphisms (SNPs) found in the fe-
170 line-derived samples have been identified in less than 5% of them (“Percent in Human Da-
171 taset” column of Table 1). 7 of these 10 nucleotide mutations were silent mutations. The 3
172 rarer non-silent mutations include an I3731V mutation in ORF1ab (Nsp6 protein), N2426T
173 mutation in ORF1ab (Nsp16 protein), and D80N in Spike.

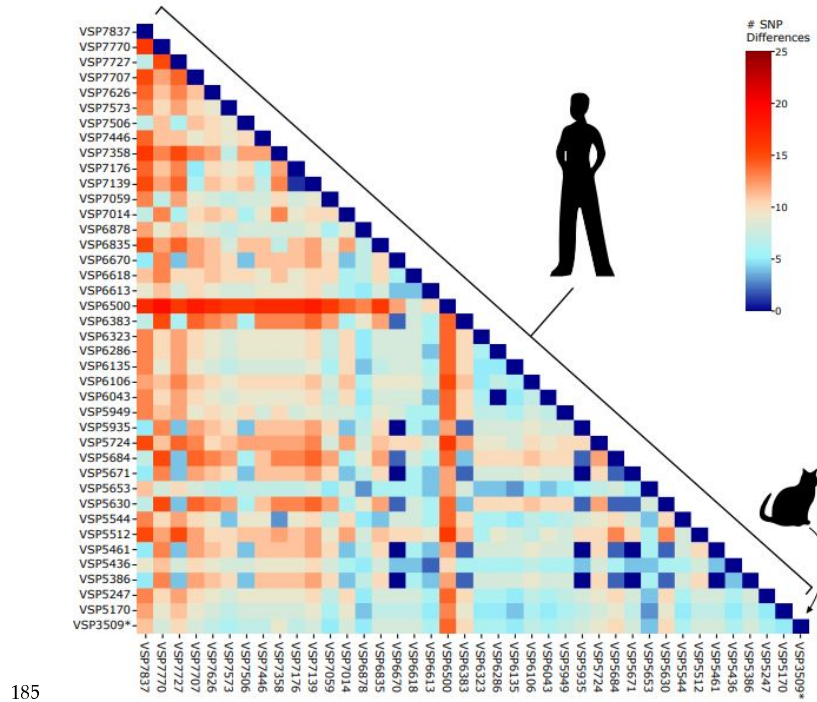
174 **Table 1.** Mutation table outlining the 45 mutations detected in the feline fecal samples. Both
175 samples collected from the cat (VSP3509 and VSP3510) contained the same mutations.
176 These mutations are compared to the random dataset consisting of 4,250 human-derived
177 genomes representing the geographical area of residence for the cat described here. The
178 original Wuhan isolate (NC_045512.2) was used as a reference.

179

Cat Mutations

Genomic Position	Gene	Affected Protein	Protein Mutation	Nucleotide Mutation	Reference Nucleotide	Percent in Human Dataset
210	intergenic			T	G	40%
241	intergenic			T	C	99.6%
3037	ORF1ab	nsp3	silent	T	C	99.95%
4181	ORF1ab	nsp3	A1306S	T	G	34.64%
6402	ORF1ab	nsp3	P2046L	T	C	34.73%
7124	ORF1ab	nsp3	P2287S	T	C	34.64%
8140	ORF1ab	nsp3	silent	T	C	1.46%
8986	ORF1ab	nsp4	silent	T	C	34.68%
9053	ORF1ab	nsp4	V2930L	T	G	34.66%
9080	ORF1ab	nsp4	silent	T	C	0.02%
10029	ORF1ab	nsp4	T3255I	T	C	38.16%
11201	ORF1ab	nsp6	T3646A	G	A	34.68%
11332	ORF1ab	nsp6	silent	G	A	34.71%
11456	ORF1ab	nsp6	I3731V	G	A	4.66%
14408	ORF1ab	nsp12 (RdRp)	P314L	T	C	99.53%
14520	ORF1ab	nsp12 (RdRp)	silent	T	C	0%
15451	ORF1ab	nsp12 (RdRp)	G662S	A	G	39.36%
16466	ORF1ab	nsp13 (Hel)	P1000L	T	C	39.32%
19220	ORF1ab	nsp14 (ExoN)	A1918V	T	C	34.59%
20744	ORF1ab	nsp16 (2'-O-MT)	N2426T	C	A	0%
21618	S	spike	T19R	G	C	39.86%
21800	S	spike	D80N	A	G	0%
21987	S	spike	G142D	A	G	13.04%
22029	S	spike	del 6	delAGTTCA	GAGTTCA	39.13%
22917	S	spike	L452R	G	T	41.58%
22995	S	spike	T478K	A	C	40.45%
23284	S	spike	silent	C	T	2.47%
23403	S	spike	D614G	G	A	99.98%
23604	S	spike	P681R	G	C	40.07%
24410	S	spike	D950N	A	G	40.02%
25339	S	spike	silent	T	C	2.64%
25469	ORF3a	ORF3a	S26L	T	C	40.05%
26767	M	membrane	I82I	C	T	41.55%
27638	ORF7a	ORF7a	V82A	C	T	39.29%
27752	ORF7a	ORF7a	T120I	T	C	39.6%
27874	ORF7b	ORF7b	T40I	T	C	34.42%
28248	ORF8	ORF8	del 6	delGATTTC	AGATTTC	38.87%
28271	intergenic		del 1	delA	TAAAA	62.19%
28461	N	nucleocapsid	D63G	G	A	39.41%
28881	N	nucleocapsid	R203M	T	G	39.91%
28916	N	nucleocapsid	G215C	T	G	34.49%
29050	N	nucleocapsid	silent	A	G	4.64%
29402	N	nucleocapsid	D377Y	T	G	42.21%
29509	N	nucleocapsid	silent	T	C	4.78%
29742	intergenic			T	G	37.01%

181 The feline-derived sample (VSP3509) differed by between 4 and 14 SNPs in pairwise
182 comparisons with human samples drawn from a random sampling of human-derived lineage
183 AY.3 sequences from the Delaware Valley collected between 6/21/2021 and 11/18/2021
184 (Figure 1).



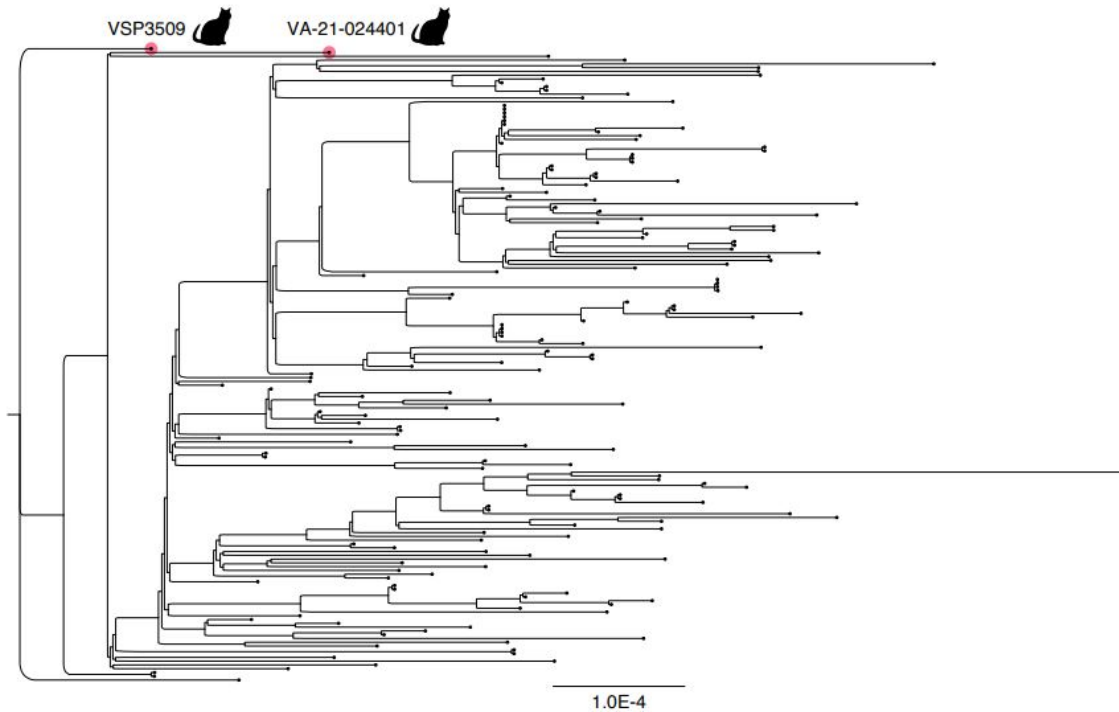
185

186 **Figure 1.** Pairwise distances between AY.3 sequences in the Delaware Valley. Included are
187 the feline-derived sequence (VSP3509) and human-derived sequences. The number of SNPs
188 separating each pair of lineages is shown by the color code (key to the right of the figure).

189

Phylogenetic analysis reveals that the cat-derived sequence, as well as another feline-derived SARS-CoV-2 lineage AY.3 genome found on GISAID, is divergent from the human sequences (Figure 2). Therefore, while there are few SNPs that differentiate the cat-derived samples from the human-derived samples nearest in sequence, the unique SNPs (Table 1) cause the cat samples to appear more distant on the phylogenetic tree. Some of these mutations may be enriched in samples from cats, however a larger dataset is

necessary to draw this conclusion.



190 **Figure 2.** Phylogenetic tree depicting the distances of AY.3 genomes. Included are the cat
191 specimen discussed in this article in addition to an AY.3 cat-derived genome previously col-
192 lected on 8/5/2021 in Virginia, USA (EPI_ISL_5761527) compared to a random sampling of
193 SARS-CoV-2 human-derived genomes in the Delaware Valley.

194 **4. Discussion**

195 To date, published reports on the SARS-CoV-2 delta variant lineage AY.3 have described
196 infection of humans and one domestic dog [14-16, 18]. Here, we report delta variant lineage
197 AY.3 in fecal samples from a domestic cat who was exposed to a human with SARS-CoV-2.

198 Two feline fecal samples collected seven days apart both had Ct values between 26-28,
199 quantities sufficient for WGS, indicating relatively high levels of genomic replication. Fur-
200 thermore, the cat had been isolated from the infected owner for 11 days and 18 days by the

201 dates of the first and second positive SARS-CoV-2 tests, respectively, reducing likelihood that
202 the cat sample was falsely positive (for example, due to pass-through contamination from the
203 infected owner during self-grooming). However, we cannot determine whether the cat's clin-
204 ical signs are attributable to COVID-19, a flare-up of chronic enteropathy, or a combination.
205 Anorexia, diarrhea, and vomiting are among the clinical signs observed in feline patients who
206 test positive for SARS-CoV-2 by RT-PCR on fecal samples [30]. Prior to the COVID-19 ex-
207 posure, however, the cat's enteropathy had been managed successfully with a prescription
208 diet for months with no clinical signs.

209 The discovery of a delta variant lineage AY.3 sequence in a feline sample, taken together
210 with detection of delta variant lineage B.1.617.2 in non-human animal species, suggests that
211 interspecies transmission of SARS-CoV-2 occurs among multiple delta variants. Recently,
212 identical sequences of lineage B.1.575 were discovered in a pet dog and cat and their owner
213 [31], demonstrating that minimal viral evolution is required to overcome species barriers in at
214 least one variant. Because we do not have the infected owner's SARS-CoV-2 sequence, we
215 cannot determine whether the mutations found in the feline-derived sequence originate from
216 the presumptive infective human, or whether they arose with the species barrier jump.

217 While fecal samples from the infected cat contained relatively high levels of viral genetic
218 material, SARS-CoV-2 was not detected on the oropharyngeal swab collected on the day of
219 presentation to the veterinary hospital. This has been reported once previously in companion
220 animals [31]. Transmission and pathophysiology appear to differ among species and may be
221 responsible for this discrepancy, although one study found that over half of human patients
222 infected with SARS-CoV-2 continued to test positive on fecal samples for approximately 11
223 days after respiratory tract samples tested negative [32]. Therefore, we may have missed the
224 window for detecting SARS-CoV-2 in respiratory samples from our feline patient. Regardless,
225 our data underscores the importance of taking fecal samples in addition to oropharyngeal or
226 nasal swabs for maximal sensitivity when testing for the virus in non-human animals.

227 Since domestic felines can support relatively efficient replication of SARS-CoV-2 viral
228 genomes similar to those that infect humans, can transmit SARS-CoV-2 viruses to naïve

229 conspecifics, and frequently have a high degree of contact with humans, they have the po-
230 tential to become an enzootic reservoir for the virus. Cat population dynamics contribute to this
231 potential, as owned indoor-outdoor cats may mingle with each other as well as free-roaming
232 unowned cats and various wildlife species, creating an unseen network between households,
233 free-roaming community cats, and wildlife populations. Transmission and reservoir formation
234 of SARS-CoV-2 in any non-human animal species poses a threat to domestic animal, wildlife,
235 and human health. This highlights the need to closely track SARS-CoV-2 variants of concern
236 in domestic house cats to better understand the intertwined nature of animal and human
237 health in this global pandemic.

238

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241 curation, A.D.M.; writing—original draft preparation, O.C.L., A.D.M., E.M.L., F.D.B.; writ-
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249 **Institutional Review Board Statement:** Ethical review and approval were waived for this
250 study. The University of Pennsylvania Institutional Review Board (IRB) reviewed the human
251 research protocol and deemed the limited data elements extracted with positive human
252 SARS-CoV-2 specimens to be exempt from human subject research per 45 CFR 46.104,
253 category 4 (IRB #848605). Informed owner consent was provided for all procedures in-volving
254 the cat. The University of Pennsylvania Institutional Animal Care and Use Committee (IACUC)

255 and Privately Owned Animal Protocol (POAP) Committee approved the protocol
256 (IACUC/POAP #806977).

257 **Informed Consent Statement:** Not applicable.

258

259 **Data Availability Statement:** The cat-derived viral genome sequences acquired in this study
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