

1 Ferrets not infected by SARS-CoV-2 in a high-exposure domestic setting

2
3 Kaitlin Sawatzki¹, Nichola Hill¹, Wendy Puryear¹, Alexa Foss¹, Jonathon Stone¹ and Jonathan
4 Runstadler¹

5
6 ¹ Department of Infectious Disease and Global Health, Cummings School of Veterinary
7 Medicine at Tufts University, North Grafton, MA 01536

8 9 **Abstract**

10
11 Ferrets (*Mustela putorius furo*) are mustelids of special relevance to laboratory studies of
12 respiratory viruses and have been shown to be susceptible to SARS-CoV-2 infection and onward
13 transmission. Here, we report the results of a natural experiment where 29 ferrets in one home
14 had prolonged, direct contact and constant environmental exposure to two humans with
15 symptomatic COVID-19. We observed no evidence of SARS-CoV-2 transmission from humans
16 to ferrets based on RT-PCR and ELISA. To better understand this discrepancy in experimental
17 and natural infection in ferrets, we compared SARS-CoV-2 sequences from natural and
18 experimental mustelid infections and identified two surface glycoprotein (Spike) mutations
19 associated with mustelids. While we found evidence that ACE2 provides a weak host barrier, one
20 mutation only seen in ferrets is located in the novel S1/S2 cleavage site and is computationally
21 predicted to decrease furin activity. These data support that host factors interacting with the
22 novel S1/S2 cleavage site may be a barrier in ferret SARS-CoV-2 susceptibility and that
23 domestic ferrets are at low risk of natural infection from currently circulating SARS-CoV-2. This
24 may be overcome in laboratory settings using concentrated viral inoculum, but the effects of
25 ferret host-adaptations require additional investigation.

26 27 **Introduction**

28
29 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes COVID-
30 19, is a zoonotic member of *Coronaviridae* that emerged in 2019 as a major viral pandemic (1).
31 As of August 2020, there have been over 20 million confirmed COVID-19 cases globally and
32 approximately 761,000 deaths (2). SARS-CoV-2 uses angiotensin I converting enzyme-2
33 (ACE2) as its primary cellular receptor for host entry and infection (3-5). *In silico* analyses of
34 ACE2 genes in diverse mammalian species have shown that residues important to viral binding
35 are moderately conserved between humans and several domestic animals, and a broad range of
36 species have been demonstrated to be permissive to infection *in vitro* and *in vivo* (6-10).

37
38 It is not yet known if natural infection of animals plays a role in public health epidemiology or
39 has the potential to establish endemic reservoirs and threaten wildlife. SARS-CoV-2 has been
40 observed to be capable of natural human-to-animal reverse-zoonoses, transmitting from infected

41 individuals into mink (11), dogs (12) and felines (13-15). European mink (*Mustela lutreola*) are
42 currently the only species observed to have natural human-to-animal spillover and onward
43 transmission (11). To date, at least 27 mink farms in the Netherlands, Spain, Denmark and
44 United States have reported outbreaks, including at least one probable case of mink-to-human
45 transmission (16, 17). SARS-CoV-2 has also been shown to productively infect several species
46 including ferrets and domestic cats *in vivo* (9, 10, 18, 19). Ferrets (*Mustela putorius furo*) are of
47 special relevance to laboratory studies of respiratory viruses like *Influenza A virus* and
48 recapitulate clinical pathophysiological aspects of human disease. Given their susceptibility to
49 experimental infection and onward transmission via direct and indirect contact, ferrets have been
50 proposed as an animal model to study SARS-CoV-2 transmission. Based on *in vivo* data, we
51 expect all naïve ferrets in direct contact with an infected ferret will 1) become infected and 2)
52 have measurable viral shedding or RNA via oral swabs up to 19 days post-infection and 3)
53 seroconvert with measurable antibodies against SARS-CoV-2 receptor binding domain (RBD)
54 (18, 19).

55
56 In March 2020, during the first wave of the SARS-CoV-2/COVID-19 pandemic in the New
57 England area, we developed a rapid response study to investigate the potential for human-to-
58 animal spillover and onward transmission in domestic, farm and wildlife species (CoVERS:
59 Coronavirus Epidemiological Response and Surveillance). The goal of CoVERS is to understand
60 if and how SARS-CoV-2 transmission is occurring at these interfaces to refine public health
61 guidelines, investigate if there are additional risks to animal or human health associated with
62 spillover and evaluate the potential for establishment of endemic reservoirs. Here, we highlight
63 one enrolled household that created an exceptional natural experiment with direct relevance to
64 our understanding of SARS-CoV-2 reverse zoonosis and animal models of disease.

65 66 **Results**

67
68 **Absence of natural SARS-CoV-2 human-to-ferret transmission in a high exposure setting**
69 A household with 29 free-roaming ferrets cared for by two adults was enrolled in the CoVERS
70 study. Individual 1 experienced fever and fatigue from March 25-April 6 and Individual 2
71 experienced a sore throat, anosmia, migraine and fatigue from March 28-April 13 (Fig. 1A).
72 Individual 2 tested positive for SARS-CoV-2/COVID-19 infection by nasopharyngeal swab and
73 RT-PCR on April 1. Individual 1 is a probable positive due to the timing and symptoms but was
74 not tested. Neither person was hospitalized, and both cared for the ferrets during the entirety of
75 their disease courses.

76
77 A two-week, in-home sample collection scheme was designed to begin during the household
78 quarantine period (Fig. 1B). The ferrets were free to move in all spaces of the home during this
79 period and handled as usual, including regular petting, feeding and grooming. The ferrets ranged
80 in age from 8 months to 7.5 years of age over 21 females and 8 males. A home sampling kit was

81 sent to the participants including material to safely collect and store ferret oral swabs. One
82 participant had significant animal handling experience and performed all sample collection to
83 standardize sampling procedures. Thirty oral swabs were collected and held in viral transport
84 media in the participants' freezer until the end of the study period. Frozen samples were directly
85 transferred to a lab member and processed.

86
87 All samples were confirmed to have viable RNA by a preliminary screen for constitutively
88 expressed β -actin (Table 1). Each sample was then tested for evidence of active or recent SARS-
89 CoV-2 infection with three established primer sets: ORF1b-nsp14 (20), Nucleocapsid (N) (14)
90 and RNA-dependent RNA polymerase (RdRP) (21). All were below the limit of detection and
91 determined to be negative for active or recent infection (Table 1).

92
93 We further took advantage of salivary immunoglobulin, which has been shown to be highly
94 sensitive and specific for SARS-CoV-2 testing (22). We tested samples for evidence of
95 antibodies against SARS-CoV-2 surface glycoprotein receptor binding domain (RBD). Twenty-
96 two ferrets (23 total samples) were confirmed to have measurable total IgG via binding to
97 recombinant protein A/G but were all negative for binding to RBD (Table 2). Therefore, there is
98 no evidence of viral infection or seroconversion in 29 ferrets living with two people with
99 COVID-19.

100
101 **Identification of two mustelid-associated mutations in SARS-CoV-2 surface glycoprotein**
102 Our observed household data support that there may be important barriers to natural infection in
103 ferrets, however, ferrets have been shown to be susceptible to infection and onward transmission
104 in experimental laboratory infections (9, 10, 18, 19). To further investigate this, we analyzed all
105 currently available genomic sequences of SARS-CoV-2 viruses of naturally infected European
106 minks and experimentally infected ferrets (32 sequences representing 24 animals, accessed:
107 2020-08-01). There are viral sequences available from two natural reverse zoonotic events in
108 European mink farms, which allowed us to infer founder-effect mutations versus acquired
109 mutations of relevance to spillover (11). We identified three mutations of interest in the surface
110 glycoprotein (S protein) coding sequence: N501T, D614G and S686G (Fig. 2A).

111
112 First, N501T was observed in 11/11 experimentally infected ferrets (donor, direct and indirect
113 contact), with an increasing proportion of the virome represented through the study period,
114 supporting strong positive selection in ferrets (19). Only 1 of 13 mink viruses are N501T, which
115 supports spontaneous mutation and natural selection in the population. The measured mutation
116 rate calculated from the closest observed human-derived sequences in mink is very low, 4.2×10^{-4} ,
117 so we asked if this specific mutation is otherwise common and not unique to mustelid infection.
118 Of 9,049 high quality human-derived SARS-CoV-2 S genes, none exhibit the N501T mutation
119 (Fig. 2B). However, N501T is seen in 5/17 pangolin-derived SARS-CoV-2-like viruses. Notably,
120 the equivalent residue in SARS-CoV is a threonine (T487).

121
122 We observed a second conserved mutation, D614G, in one of the two mink clades and all ferrets.
123 However, this mutation has become prevalent in the human population (D614, 30.5%; D614G,
124 69.5%, Fig. 2B) and was observed in the ferret human donor and mink farm's closest observed
125 ancestor (Fig. 2A). We conclude that D614G mutations are due to variation in the human
126 population/donors and are not specifically associated with mustelid infection.

127
128 The third non-synonymous S protein mutation, S686G, was only observed in ferrets and is
129 located at the P1' serine residue directly adjacent to the novel S1/S2 polybasic cleavage site
130 (PRRAR↓S) (Fig. 2A). This mutation is of special interest as this cleavage site partially
131 distinguishes SARS-CoV-2 from other SARS-like viruses and allows immune evasion prior to
132 receptor binding (23-25). Like N501T, S686G was observed in 11/11 ferrets and was a minority
133 variant in the donor inoculum and increased proportional representation in the virome over time,
134 suggesting positive selection (19). We found that no other human-derived viral sequence has
135 been observed with this mutation (Fig. 2B). S686G has also not been observed in SARS-CoV-2-
136 like viruses from other carnivores (naturally infected felines and canines), all of which retained
137 the complete cleavage site and adjacent P1' serine. All mustelid-derived viruses retained the
138 second, downstream S1/S2 cleavage site motif (IAY↓TMS), as well as the S2' TMPRSS2-
139 processed cleavage site for fusion.

140
141 Host furin and furin-like proteases have been shown to cleave the S1/S2 polybasic cleavage site
142 (3, 25, 26). P1' residues are strongly favored to be serine in furin cleavage, and alternate residues
143 are restricted by size and hydrophilicity due to their location in the furin binding pocket (27).
144 Glycine is small but hydrophobic. We performed *in silico* analysis of the cleavage site to
145 compare identical sequences that differed only at position 686 using PiTou 2.0 (28). PiTou
146 scores are biologically meaningful prediction values of furin cleavage derived from binding
147 strength and solvent accessibility and can be directly compared. S686 results in a PiTou score of
148 9.19633 while S686G results in a score of 6.92387. While both are predicted to be cleaved by
149 furin, S686 is estimated to have stronger interactions in the binding pocket (P6-P2'). Therefore,
150 S686G is an unfavorable substitution for furin cleavage.

151
152 We further performed phylogenetic analysis of the proprotein convertase family that cleave
153 polybasic sites (PCSK1-7), including furin, and Cathepsin L in a number of mammals including
154 *Mustela putorius furo* and the well-annotated *Mustela erminea*. However, we found no
155 significant difference between ferrets, ermines and other carnivores.

156 157 **Discussion**

158
159 Multiple studies have now demonstrated that ferrets may be directly infected by human-derived
160 SARS-CoV-2 and, following infection, exhibit a 100% transmission rate via direct contact (9,

161 10, 18, 19). However, our data suggest that the initial barrier of human-to-ferret transmission
162 may be higher than relevant for most household pets. We calculated that a sample size of 10
163 animals was sufficient to test the hypothesis that at least one ferret was infected, given an
164 observed attack rate of 87% in mink farms (95% CI, 0.05) (29). In this natural experiment, all 29
165 ferrets had significant opportunities for direct contact with all other ferrets and had direct
166 exposure to at least one, and likely two infectious people. While we were unable to collect
167 human samples, current epidemiological knowledge of SARS-CoV-2 would lead to the
168 conclusion that both adults had an infectious period with viral shedding (30, 31).

169
170 We found no evidence of SARS-CoV-2 transmission to ferrets based on RT-PCR and serology, a
171 finding at odds with the high transmission rates observed in ferrets and mink and infectivity of
172 SARS-CoV-2. Based on current knowledge of SARS-CoV-2 transmission and shedding in
173 ferrets, we determined that our collection time points fell within the timeframe to obtain
174 measurable viral RNA, even if transmission occurred on March 22, prior to any symptom onset
175 in the household. However, it was important to perform additional antibody testing to address
176 two concerns; first, that transmission could have occurred prior to March 22 and second, that the
177 level of infection and viral shedding was so low as to be below collection and screening
178 sensitivity. In either scenario, we still expected a robust antibody presence within days of initial
179 infection but found no evidence of RBD-specific antibodies. Despite significant and prolonged
180 exposure in the home, we have concluded that there is no evidence of SARS-CoV-2/COVID-19
181 human-to-ferret transmission in this household.

182
183 Notably, Ferret 12 (7yo) was euthanized on April 16, and had a history of adrenal disease, and
184 Ferret 16 (7yo) died unexpectedly on April 20. Both were swabbed within four days of their
185 deaths and we expect would have been RT-PCR or antibody positive had their deaths been
186 related to SARS-CoV-2 infection.

187
188 Viral host receptors are often a key factor in determining host range. European minks and ferrets
189 share identical ACE2 residues with known viral S protein interactions, therefore we expect these
190 species to have similar natural susceptibility (7). N501T is in the receptor binding motif of the
191 SARS-CoV-2 surface glycoprotein, which interacts with ACE2 primarily at Y41, but also K353,
192 G354 and D355 (32, 33). Of these, mustelids only differ from humans at ACE2 G354R (7). Both
193 the viral mutation (N501T) and receptor difference (G354R) are observed in both mink and
194 ferrets which suggest that mustelid ACE2 might provide some barrier to infection or requirement
195 for host adaptation upon infection. However, as mink were initially infected by virus without this
196 viral mutation and there have now been dozens of independent human-to-mink spillover events,
197 we propose that ACE2 provides a limited host barrier to SARS-CoV-2 entry in mustelids. While
198 acquisition of N501T may increase viral fitness in mustelids, it is not necessary for interspecies
199 transmission.

200

201 The absence of transmission in the high-exposure home described in this paper contrasts with
202 multiple human-to-mink spillover events, suggesting additional host barriers specific to ferrets.
203 S686G is a particularly intriguing mutation as it lies directly adjacent to a motif that is likely to
204 enhance virulence (25). To date, S686 is perfectly conserved in 9189/9189 human sequences,
205 indicating strong purifying selection. S686G changes a neutral polar residue to a non-polar one,
206 which we estimated to decrease furin efficiency. Furthermore, S686 completes a novel
207 glycosaminoglycan (GAG)-binding motif (XBBXB \underline{X} /PRRARS \underline{S}) that enhances binding and the
208 two flanking serines in the S1/S2 site (SPRRAR \underline{L} SV) have been shown to be permissive to host
209 phosphorylation and consequent down regulation of furin activity, (26, 34). We were surprised to
210 see evidence of positive selection over time for this potentially unfavorable mutation in ferrets as
211 described by Richard *et al* for these reasons (19). If there is further evidence of S686G selection
212 in experimentally or naturally infected ferrets, it is essential to fully investigate changes in viral
213 fusion activity, kinetics and pathology to determine if ferrets are an appropriate model for human
214 disease.

215
216 Our results suggest that virus and host genetic barriers significantly limit natural infection in
217 ferrets, and these are only likely to be overcome by a concentrated and/or diverse inoculum of
218 human-derived virus. To date, experimental ferret infections have been successful 6×10^5 and
219 $10^{5.5}$ TCID₅₀, and at least one inoculum contained a minority of virus with the S686G variant
220 (18, 19). These limitations may negatively affect ferrets as a disease and/or transmission model
221 and should be further investigated. We are, however, optimistic that the lack of spillover in this
222 household supports that there is a very low risk of human-to-ferret SARS-CoV-2 transmission in
223 domestic settings.

224

225 **Materials and methods**

226

227 **Study enrollment and sample collection**

228 The study participants were enrolled under a protocol approved by Tufts University Institutional
229 and Animal Care and Use Committee and Health Sciences Institutional Review Board (#G2020-
230 27). A self-administered sampling kit was sent to the enrollees' residence with sterile standard
231 polyester tipped applicators (Puritan, Guilford, ME), vials with 800ul M4RT viral transport
232 media (Remel, Lenexa, KS), instructions, a data sheet and secondary containment bags. Oral
233 swabs were obtained using gloves and a mask in the home and held in a home freezer until
234 transfer to a lab member via a cooler.

235

236 **RNA extraction and RT-PCR**

237 Samples were vortexed and 50ul aliquoted for MagPlate OMEGA extraction following
238 manufacturer protocols. RNA was tested by semi-quantitative real time reverse transcription
239 polymerase chain reaction (RT-PCR) on the StepOnePlus platform (ABI, Beverly, MA) with
240 qScript XLT 1-Step RT-PCR ToughMix, using five primer sets: one for internal controls

241 (ACTB) and three for SARS-CoV-2 (ORF1b, N1, E, RdRP). CoVERS-ACTB, F:
242 GATGCAGAAGGAGATCAC, R: CTAGAAGCATTGCGGTG, Probe: HEX-
243 CTCCTGCTTGCTGATCCACA-TAM; HKU-ORF1, F: TGGGGYTTTACRGGTAACCT, R:
244 AACRCGCTTAACAAAGCACTC, P: FAM-TAGTTGTGATGCWATCATGACTAG-TAM;
245 2019-nCoV_N1 [CDC], F: GACCCCAAATCAGCGAAT, R:
246 TCTGGTACTGCAGTTGAATCTG, P: FAM-ACCCCGCATTACGTTTGGTGGACC-TAM;
247 RdRP_SARSr, F: GTGARATGGTCATGTGTGGCmGG, R:
248 CARATGTAAASACACTATTAGCAmTA, P: FAM-
249 CAGGTGGAACCTCATCAGGAGATGC-TAM. All plates were run with negative VTM
250 controls and positive control (NR-52285, Genomic RNA from SARS-Related Coronavirus 2,
251 Isolate USA-WA1/2020, BEI Resources, Manassas, VA).

252

253 **ELISA**

254 Oral swabs were tested for total IgG and IgG against SARS-CoV-2 receptor binding domain with
255 minor modifications to an established protocol (35). Briefly, Immulon 2 HB plates were coated
256 with 2µg/ml Pierce recombinant protein A/G (ThermoFisher catalog no: 77677) or purified
257 SARS-CoV-2 receptor binding domain (provided by Florian Krammer, available as NR-52366,
258 BEI Resources, Manassas, VA) and incubated 2 days at 4°C. After washing, plates were blocked
259 with PBS supplemented with 0.1% Tween-20 (PBS-T) and 3% milk at room temperature for 2
260 hours. All samples were heat inactivated at 56°C for 1 hour. Ferret samples were diluted 1:5 in
261 PBS-T with 1% milk. Positive controls were serum from S protein immunized alpacas (provided
262 by Charles Shoemaker), and diluted 1:5 in PBS, then to final dilution of 1:50 in PBS-T with 1%
263 milk. Following blocking, 100µl diluted samples were incubated at room temperature for 2
264 hours. Plates were washed and 50µl Pierce recombinant protein A/G with peroxidase (Thermo
265 Fisher catalog no: 32490) added at 1:10,000 in PBS-T with 1% milk as a secondary and
266 incubated 1 hour at room temperature. Plates were washed and developed for 10 minutes with
267 SigmaFast OPD solution (Sigma-Aldrich catalog no: P9187), stopped with 50ul 3M HCl and
268 read at an absorbance of 490nm on a BioTek Synergy 4 Multidetector plate reader (Winooski,
269 VT). VTM was tested at 1:2 and 1:5 and confirmed to not affect results.

270

271 **Viral sequence collection and assembly**

272 High quality SARS-CoV-2 surface glycoprotein sequences were curated using NCBI Virus and
273 GISAID EpiCoV databases as follows. 9,664 full length S nucleotide sequences were collected
274 from NCBI Virus and aligned using ClustalΩ 1.2.4. Sequences were trimmed to coding region
275 sequence (CDS), translated and realigned. Sequences with >10% unknown residues were
276 excluded. All non-human animal-derived SARS-CoV-2 and SARS-CoV-2-like viral sequences
277 were collected from GISAID EpiCoV. To collect viral genomes from experimental ferret
278 infection, sequencing reads were downloaded from 23 Illumina and Minion sequencing runs
279 uploaded to NCBI Sequence Read Archive (PRJNA641813). Reads were confirmed to be post-
280 quality control by Prinseq and mapped to the human donor sequence (hCoV-

281 19/Germany/BavPat1/2020|EPI_ISL_406862|2020-01-28) using BWA (Illumina) and Pomoxis
282 mini_align (Minion). Consensus was called using Samtools and replicate Illumina/Minion
283 libraries were compared to confirm consistency.

284

285 **Mammalian gene collection, assembly and phylogenetic analysis**

286 PCSK1-7 and CTSL sequences were collected from NCBI Orthologs from *Homo sapiens*, *Pan*
287 *troglodytes*, *Sus scrofa*, *Ovis aries*, *Bos Taurus*, *Canis lupus familiaris*, *Vulpes vulpes*, *Felis*
288 *catus*, *Panthera tigris altaica*, *Phoca vitulina*, *Mustela erminea*, *Myotis lucifugus*, *Eptesicus*
289 *fuscus* and *Rousettus aegyptiacus*. *Mustela putorius furo* orthologs were inconsistent with related
290 species by preliminary RAxML ortholog analysis. Seven publicly available RNAseq run from
291 *Mustela putorius furo* (SRR11517721-SRR11517724, SRR391982, SRR391968, SRR391966)
292 were downloaded and putative PCSK1-7/CTSL reads were extracted using BLAST. Reads were
293 assembled using Pomoxis mini_assemble with ermine references. Reads were then mapped back
294 to the proposed ferret assembly with BWA and well-supported consensus sequences were called
295 using Samtools. Ortholog collections were analyzed using maximum-likelihood phylogenetics
296 via RAxML (JTT γ using empirical base frequencies, 5000 bootstraps).

297

298 **Acknowledgements**

299

300 This work was supported by NIAID grant HHSN272201400008C/AI/NIAID. We thank Dr.
301 Florian Krammer for directly providing RBD and Dr. Charles Shoemaker for providing alpaca
302 control serum for ELISAs. We thank Drs. Jennifer Graham and Elizabeth Rozanski for their
303 assistance in connecting us to ferret-owners for the CoVERS study. We thank Lynne
304 Christiansen and Mary Andersen for providing us masks to safely collect samples. We are
305 especially grateful to our enrolled household who were exceptional participants during a
306 challenging time.

307

308 **Citations**

309

- 310 1. Wang C, Horby PW, Hayden FG, Gao GF. A novel coronavirus outbreak of global health
311 concern. *Lancet*. 2020;395(10223):470-3.
- 312 2. Coronavirus disease (COVID-19) Situation Report – 209. World Health Organization;
313 2020.
- 314 3. Walls AC, Park YJ, Tortorici MA, Wall A, McGuire AT, Veesler D. Structure, Function,
315 and Antigenicity of the SARS-CoV-2 Spike Glycoprotein. *Cell*. 2020;181(2):281-92 e6.
- 316 4. Zhou P, Yang XL, Wang XG, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak
317 associated with a new coronavirus of probable bat origin. *Nature*. 2020;579(7798):270-3.
- 318 5. Xu H, Zhong L, Deng J, Peng J, Dan H, Zeng X, et al. High expression of ACE2 receptor
319 of 2019-nCoV on the epithelial cells of oral mucosa. *Int J Oral Sci*. 2020;12(1):8.
- 320 6. Xu X, Chen P, Wang J, Feng J, Zhou H, Li X, et al. Evolution of the novel coronavirus
321 from the ongoing Wuhan outbreak and modeling of its spike protein for risk of human
322 transmission. *Sci China Life Sci*. 2020;63(3):457-60.

- 323 7. Damas J, Hughes GM, Keough KC, Painter CA, Persky NS, Corbo M, et al. Broad Host
324 Range of SARS-CoV-2 Predicted by Comparative and Structural Analysis of ACE2 in
325 Vertebrates. *bioRxiv*. 2020.
- 326 8. Zhao X, Chen D, Szabla R, Zheng M, Li G, Du P, et al. Broad and differential animal
327 ACE2 receptor usage by SARS-CoV-2. *J Virol*. 2020.
- 328 9. Schlottau K, Rissmann M, Graaf A, Schön J, Sehl J, Wylezich C, et al. SARS-CoV-2 in
329 fruit bats, ferrets, pigs, and chickens: an experimental transmission study. *The Lancet*
330 *Microbe*.
- 331 10. Shi J, Wen Z, Zhong G, Yang H, Wang C, Huang B, et al. Susceptibility of ferrets, cats,
332 dogs, and other domesticated animals to SARS-coronavirus 2. *Science*.
333 2020;368(6494):1016-20.
- 334 11. Oreshkova N, Molenaar R-J, Vreman S, Harders F, Munnink BBO, Hakze R, et al. SARS-
335 CoV2 infection in farmed mink, Netherlands, April 2020. *bioRxiv*.
336 2020:2020.05.18.101493.
- 337 12. Sit THC, Brackman CJ, Ip SM, Tam KWS, Law PYT, To EMW, et al. Infection of dogs
338 with SARS-CoV-2. *Nature*. 2020.
- 339 13. Newman A, Smith D, Ghai RR, Wallace RM, Torchetti MK, Loiacono C, et al. First
340 Reported Cases of SARS-CoV-2 Infection in Companion Animals - New York, March-
341 April 2020. *MMWR Morb Mortal Wkly Rep*. 2020;69(23):710-3.
- 342 14. Yoo HS, Yoo D. COVID-19 and veterinarians for one health, zoonotic- and reverse-
343 zoonotic transmissions. *J Vet Sci*. 2020;21(3):e51.
- 344 15. Patterson EI, Elia G, Grassi A, Giordano A, Desario C, Medardo M, et al. Evidence of
345 exposure to SARS-CoV-2 in cats and dogs from households in Italy. *bioRxiv*. 2020.
- 346 16. Cahan E. COVID-19 hits U.S. mink farms after ripping through Europe. *Science*. 2020.
- 347 17. COVID-19 update (209): Netherlands (NB) farmed mink, animal-to-human, cat, epid
348 [Internet]. *ProMED International Society for Infectious Diseases*. 2020.
- 349 18. Kim YI, Kim SG, Kim SM, Kim EH, Park SJ, Yu KM, et al. Infection and Rapid
350 Transmission of SARS-CoV-2 in Ferrets. *Cell Host Microbe*. 2020;27(5):704-9 e2.
- 351 19. Richard M, Kok A, de Meulder D, Bestebroer TM, Lamers MM, Okba NMA, et al. SARS-
352 CoV-2 is transmitted via contact and via the air between ferrets. *Nature Communications*.
353 2020;11(1):3496.
- 354 20. Chu DKW, Pan Y, Cheng SMS, Hui KPY, Krishnan P, Liu Y, et al. Molecular Diagnosis
355 of a Novel Coronavirus (2019-nCoV) Causing an Outbreak of Pneumonia. *Clin Chem*.
356 2020;66(4):549-55.
- 357 21. Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DK, et al. Detection of
358 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill*. 2020;25(3).
- 359 22. Randad PR, Pisanic N, Kruczynski K, Manabe YC, Thomas D, Pekosz A, et al. COVID-19
360 serology at population scale: SARS-CoV-2-specific antibody responses in saliva. *medRxiv*.
361 2020.
- 362 23. Shang J, Wan Y, Luo C, Ye G, Geng Q, Auerbach A, et al. Cell entry mechanisms of
363 SARS-CoV-2. *Proc Natl Acad Sci U S A*. 2020;117(21):11727-34.
- 364 24. Coutard B, Valle C, de Lamballerie X, Canard B, Seidah NG, Decroly E. The spike
365 glycoprotein of the new coronavirus 2019-nCoV contains a furin-like cleavage site absent
366 in CoV of the same clade. *Antiviral Res*. 2020;176:104742.

- 367 25. Hoffmann M, Kleine-Weber H, Pohlmann S. A Multibasic Cleavage Site in the Spike
368 Protein of SARS-CoV-2 Is Essential for Infection of Human Lung Cells. *Mol Cell*.
369 2020;78(4):779-84 e5.
- 370 26. Örd M, Faustova I, Loog M. Biochemical evidence of furin specificity and potential for
371 phospho-regulation at Spike protein S1/S2 cleavage site in SARS-CoV2 but not in SARS-
372 CoV1 or MERS-CoV. *bioRxiv*. 2020:2020.06.23.166900.
- 373 27. Millet JK, Whittaker GR. Host cell proteases: Critical determinants of coronavirus tropism
374 and pathogenesis. *Virus Res*. 2015;202:120-34.
- 375 28. Tian S, Huajun W, Wu J. Computational prediction of furin cleavage sites by a hybrid
376 method and understanding mechanism underlying diseases. *Sci Rep*. 2012;2:261.
- 377 29. Kevany S. A million mink culled in Netherlands and Spain amid Covid-19 fur farming
378 havoc. *The Guardian*. 2020 17th July.
- 379 30. He X, Lau EHY, Wu P, Deng X, Wang J, Hao X, et al. Temporal dynamics in viral
380 shedding and transmissibility of COVID-19. *Nat Med*. 2020;26(5):672-5.
- 381 31. Madewell ZJ, Yang Y, Longini IM, Halloran ME, Dean NE. Household transmission of
382 SARS-CoV-2: a systematic review and meta-analysis of secondary attack rate. *medRxiv*.
383 2020.
- 384 32. Lan J, Ge J, Yu J, Shan S, Zhou H, Fan S, et al. Structure of the SARS-CoV-2 spike
385 receptor-binding domain bound to the ACE2 receptor. *Nature*. 2020;581(7807):215-20.
- 386 33. Yi C, Sun X, Ye J, Ding L, Liu M, Yang Z, et al. Key residues of the receptor binding
387 motif in the spike protein of SARS-CoV-2 that interact with ACE2 and neutralizing
388 antibodies. *Cell Mol Immunol*. 2020;17(6):621-30.
- 389 34. Kim SY, Jin W, Sood A, Montgomery DW, Grant OC, Fuster MM, et al. Characterization
390 of heparin and severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2)
391 spike glycoprotein binding interactions. *Antiviral Res*. 2020:104873.
- 392 35. Stadlbauer D, Amanat F, Chromikova V, Jiang K, Strohmeier S, Arunkumar GA, et al.
393 SARS-CoV-2 Seroconversion in Humans: A Detailed Protocol for a Serological Assay,
394 Antigen Production, and Test Setup. *Curr Protoc Microbiol*. 2020;57(1):e100.
395

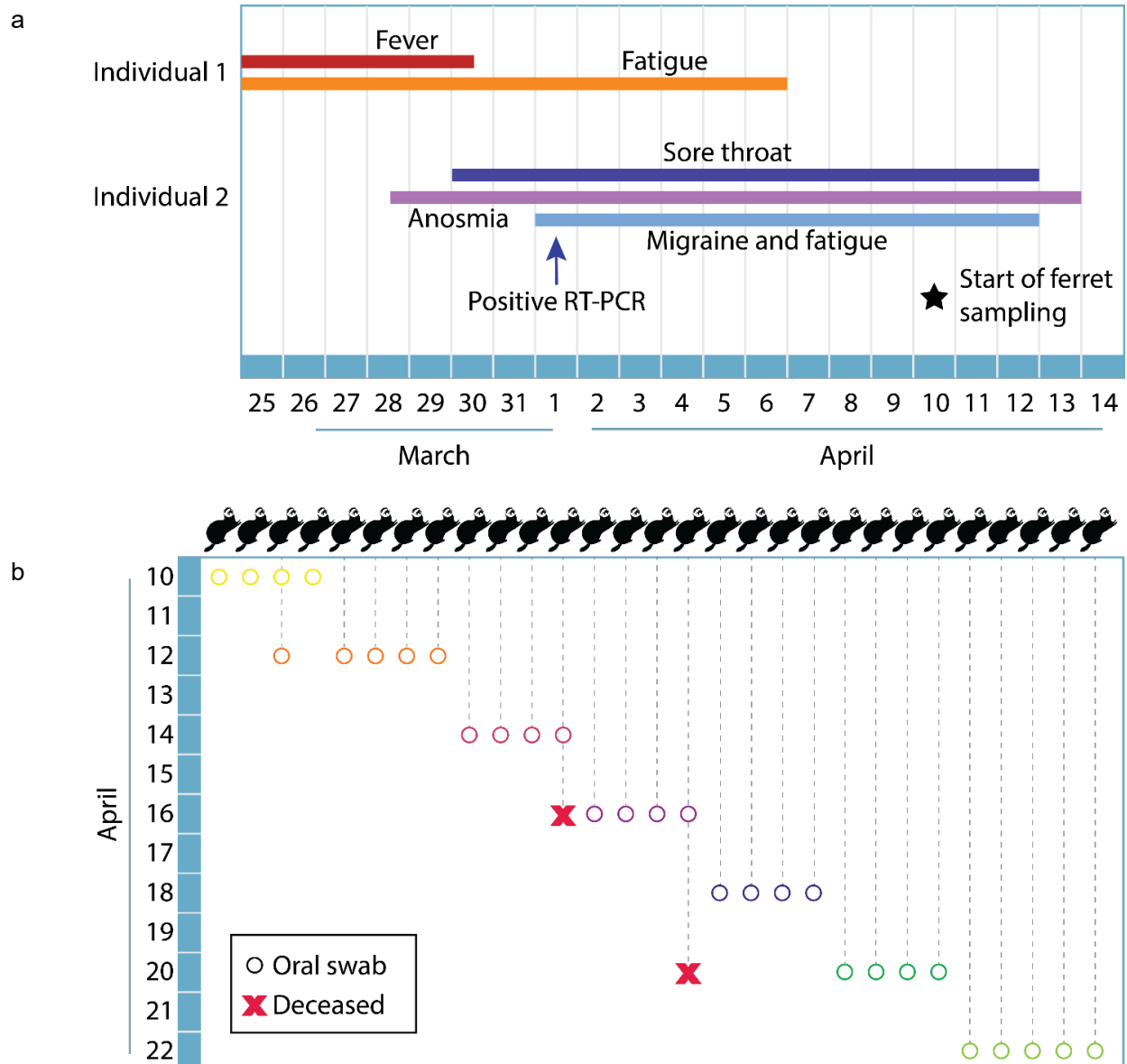


Figure 1. COVID-19 disease course and ferret sample collection timeline

A household with two adults and 29 free-roaming ferrets was enrolled in the CoVERS study. Both adults exhibited symptoms of SARS-CoV-2 infection in late March to early April of 2020, and one tested positive by RT-PCR on April 1st (a). Oral swabs were collected from all ferrets in the home over a two-week period, beginning April 10th, concurrent with symptomatic disease in Individual 2 (b). One ferret (3) was sampled twice. Two 7-year-old ferrets (12 and 16) died during the study period, one by euthanasia due to chronic disease, the other cause is unknown.

Ferret	ACTB	ORF1b	N	RdRP	Total IgG	α RBD IgG
1	33.036	LOD	LOD	LOD	P	N
2	28.120	LOD	LOD	LOD	P	N
3a	27.954	LOD	LOD	LOD	P	N
3b	28.945	LOD	LOD	LOD	P	N
4	26.230	LOD	LOD	LOD	P	N
5	29.067	LOD	LOD	LOD	P	N
6	29.729	LOD	LOD	LOD	P	N
7	29.360	LOD	LOD	LOD	P	N
8	26.755	LOD	LOD	LOD	P	N
9	33.049	LOD	LOD	LOD	P	N
10	32.820	LOD	LOD	LOD	N	NA
11	29.781	LOD	LOD	LOD	P	N
12	29.010	LOD	LOD	LOD	P	N
13	27.730	LOD	LOD	LOD	N	NA
14	32.163	LOD	LOD	LOD	P	N
15	30.230	LOD	LOD	LOD	P	N
16	27.861	LOD	LOD	LOD	P	N
17	27.701	LOD	LOD	LOD	P	N
18	27.687	LOD	LOD	LOD	N	NA
19	30.832	LOD	LOD	LOD	N	NA
20	31.758	LOD	LOD	LOD	P	N
21	31.758	LOD	LOD	LOD	N	NA
22	32.635	LOD	LOD	LOD	P	N
23	27.098	LOD	LOD	LOD	P	N
24	29.290	LOD	LOD	LOD	P	N
25	29.806	LOD	LOD	LOD	N	NA
26	35.042	LOD	LOD	LOD	N	NA
27	30.032	LOD	LOD	LOD	P	N
28	31.464	LOD	LOD	LOD	P	N
29	29.476	LOD	LOD	LOD	P	N

Table 1. No evidence of SARS-CoV-2 infection in ferrets

Thirty samples from 29 ferret oral swabs were tested by semi-quantitative real time RT-PCR and ELISA. RT-PCR was performed on a StepOnePlus (ABI, Beverly, MA) with qScript XLT 1-Step RT-PCR ToughMix. Sample and RNA viability was confirmed by β -actin (ACTB). Three separate primers sets were used to test for SARS-CoV-2: ORF1b, N and RdRP; All SARS-CoV-2 results were under the limit of detection (LOD). Oral swabs were further tested for antibodies at a 1:5 dilution ELISA against recombinant protein A/G (Total IgG) or purified SARS-CoV-2 RBD (α RBD IgG). Plates were read on a BioTek Synergy 4 Multidetector plate reader (Winooski, VT). Positive cutoff was set at ($\mu + 3\sigma$) of the negative controls (n=24).

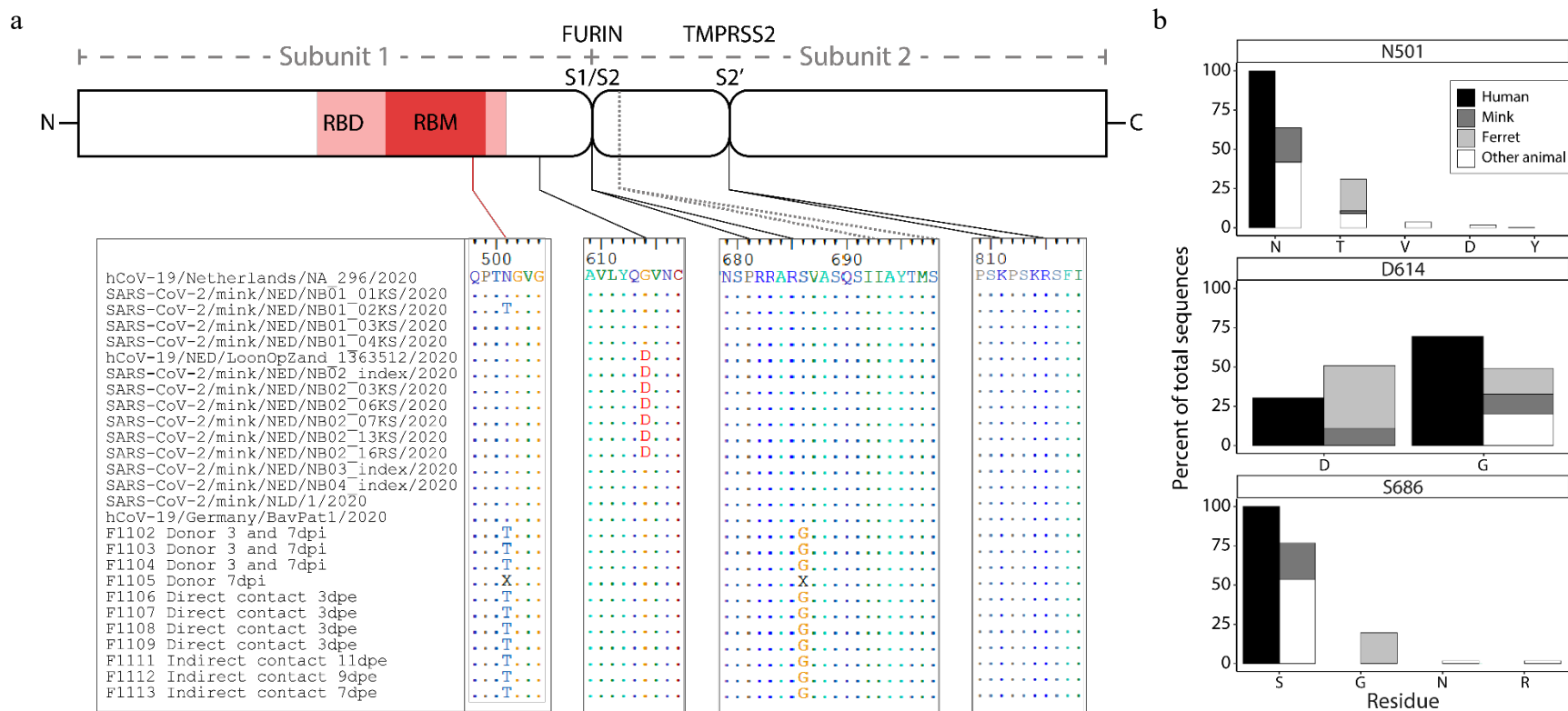


Figure 2. Mustelid-associated mutations in SARS-CoV-2 surface glycoprotein

All available SARS-CoV-2 surface glycoprotein (S) sequences from natural (mink) and experimental (ferret) infections were compared and three mutations identified. a) A schematic diagram (not to scale) of the S protein with Subunit 1, which is involved in host receptor protein attachment and Subunit 2, which is involved in host cell fusion. Mutation N501T is located in the receptor binding domain (RBD) and receptor binding motif (RBM), shown in red. Mutation D614G is located in Subunit 1 downstream of the RBD, and mutation S686G is located directly adjacent to the novel S1/S2 cleavage motif (PPAR↓S) processed by furin. A second S1/S2 cleavage site (IAY↓TMS) seen in SARS-CoV is conserved. The S2' cleavage site (KPSKR↓S) processed by TMPRSS2 is also conserved. Viral amino acid sequences from regions of interest are shown below the schematic, and dots represent conserved residues

using the top sequence as a reference (hCoV-19/Netherlands/NA_296/2020). Viruses from European mink are separated into two clades from distinct farms (NB01 and NB02-4, respectively), and are preceded by the closest observed human sequence (hCoV-19/Netherlands) for reference. Experimentally infected ferrets are in the bottom half (F1102-1113). The sequence from the human inoculum (hCoV-19/Germany) is included for reference. Ferrets are separated into three groups: donors, which received direct inoculum; direct contact, which were housed with donors; and indirect contact, which were housed adjacent to donors without physical contact. Identical sequences were found from samples taken at 3 and 7 days post inoculation (dpi) in 3 of 4 donors. Donor F1105 exhibited two equivalent single nucleotide variants (A1502C and A2056G) resulting in N501/N501T and S686/S686G, respectively, and are not consensus-called (“X”) in those locations. b) 9,253 human-derived SARS-CoV-2 S protein sequences and 57 animal-derived SARS-CoV-2 or SARS-CoV-like virus S protein sequences were aligned to calculate percent amino acid representation at three positions: N501 (top), D614 (middle) and S686 (bottom).