

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

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## 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). American mink (*Neovison vison*) 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  $\beta$ -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 American  
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 mink farms in Europe, 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,  $4.2 \times 10^{-4}$ ,  
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. American minks and ferrets  
189 share 24 of 25 ACE2 residues with known viral S protein interactions, and 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 G354, and this  
193 site is also the only distinct residue between ferret (G354R) and American mink (G354H) (7).  
194 Mink have been naturally infected by virus without the N501T mutation and there have now  
195 been dozens of independent human-to-mink spillover events, therefore we do not expect that the  
196 ACE2 G354H mutation significantly limits infection. However, the appearance of N501T in all  
197 infected ferrets suggest ACE2 G354R may provide a host barrier to SARS-CoV-2 entry in  
198 ferrets. Additional work is needed to determine if N501T is a required adaptation for ferret  
199 transmission and, if so, if it affects transmission dynamics.

200

201 SARS-CoV-2 S protein S686G is another intriguing mutation as it lies directly adjacent to a  
202 motif that is likely to enhance virulence (25). To date, S686 is perfectly conserved in 9189/9189  
203 human sequences, indicating strong purifying selection. S686G changes a neutral polar residue to  
204 a non-polar one, which we estimated to decrease furin efficiency. Furthermore, S686 completes a  
205 novel glycosaminoglycan (GAG)-binding motif (XBBXB $\underline{X}$ /PRRARS) that enhances binding and  
206 the two flanking serines in the S1/S2 site (SPRRAR $\underline{L}$ SV) have been shown to be permissive to  
207 host phosphorylation and consequent down regulation of furin activity, (26, 34). We were  
208 surprised to see evidence of positive selection over time for this potentially unfavorable mutation  
209 in ferrets as described by Richard *et al* for these reasons (19). If there is further evidence of  
210 S686G selection in experimentally or naturally infected ferrets, it is essential to fully investigate  
211 changes in viral fusion activity, kinetics and pathology to determine if ferrets are an appropriate  
212 model for human disease.

213  
214 Our results suggest that virus and host genetic barriers significantly limit natural infection in  
215 ferrets, and these are only likely to be overcome by a concentrated and/or diverse inoculum of  
216 human-derived virus. To date, experimental ferret infections have been successful  $6 \times 10^5$  and  
217  $10^{5.5}$  TCID<sub>50</sub>, and at least one inoculum contained a minority of virus with the N501T and S686G  
218 variants (18, 19). These limitations and putative host-adaptations may negatively affect ferrets as  
219 a disease and/or transmission model and should be further investigated. We are, however,  
220 optimistic that the lack of spillover in this household supports that there is a very low risk of  
221 human-to-ferret SARS-CoV-2 transmission in domestic settings.

222

## 223 **Materials and methods**

### 224 225 **Study enrollment and sample collection**

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

### 233 234 **RNA extraction and RT-PCR**

235 Samples were vortexed and 50ul aliquoted for MagPlate OMEGA extraction following  
236 manufacturer protocols. RNA was tested by semi-quantitative real time reverse transcription  
237 polymerase chain reaction (RT-PCR) on the StepOnePlus platform (ABI, Beverly, MA) with  
238 qScript XLT 1-Step RT-PCR ToughMix, using five primer sets: one for internal controls  
239 (ACTB) and three for SARS-CoV-2 (ORF1b, N1, E, RdRP). CoVERS-ACTB, F:  
240 GATGCAGAAGGAGATCAC, R: CTAGAAGCATTGCGGTG, Probe: HEX-

241 CTCCTGCTTGCTGATCCACA-TAM; HKU-ORF1, F: TGGGGYTTTACRGGTAACCT, R:  
242 AACRCGCTTAACAAAGCACTC, P: FAM-TAGTTGTGATGCWATCATGACTAG-TAM;  
243 2019-nCoV\_N1 [CDC], F: GACCCCAAATCAGCGAAT, R:  
244 TCTGGTACTGCAGTTGAATCTG, P: FAM-ACCCCGCATTACGTTTGGTGGACC-TAM;  
245 RdRP\_SARSr, F: GTGARATGGTCATGTGTGGCmGG, R:  
246 CARATGTAAASACACTATTAGCAmTA, P: FAM-  
247 CAGGTGGAACCTCATCAGGAGATGC-TAM. All plates were run with negative VTM  
248 controls and positive control (NR-52285, Genomic RNA from SARS-Related Coronavirus 2,  
249 Isolate USA-WA1/2020, BEI Resources, Manassas, VA).

250

## 251 **ELISA**

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

268

## 269 **Viral sequence collection and assembly**

270 High quality SARS-CoV-2 surface glycoprotein sequences were curated using NCBI Virus and  
271 GISAID EpiCoV databases as follows. 9,664 full length S nucleotide sequences were collected  
272 from NCBI Virus and aligned using ClustalΩ 1.2.4. Sequences were trimmed to coding region  
273 sequence (CDS), translated and realigned. Sequences with >10% unknown residues were  
274 excluded. All non-human animal-derived SARS-CoV-2 and SARS-CoV-2-like viral sequences  
275 were collected from GISAID EpiCoV. To collect viral genomes from experimental ferret  
276 infection, sequencing reads were downloaded from 23 Illumina and Minion sequencing runs  
277 uploaded to NCBI Sequence Read Archive (PRJNA641813). Reads were confirmed to be post-  
278 quality control by Prinseq and mapped to the human donor sequence (hCoV-  
279 19/Germany/BavPat1/2020|EPI\_ISL\_406862|2020-01-28) using BWA (Illumina) and Pomoxis

280 mini\_align (Minion). Consensus was called using Samtools and replicate Illumina/Minion  
281 libraries were compared to confirm consistency.

282

### 283 **Mammalian gene collection, assembly and phylogenetic analysis**

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

295

### 296 **Acknowledgements**

297

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

305

### 306 **Citations**

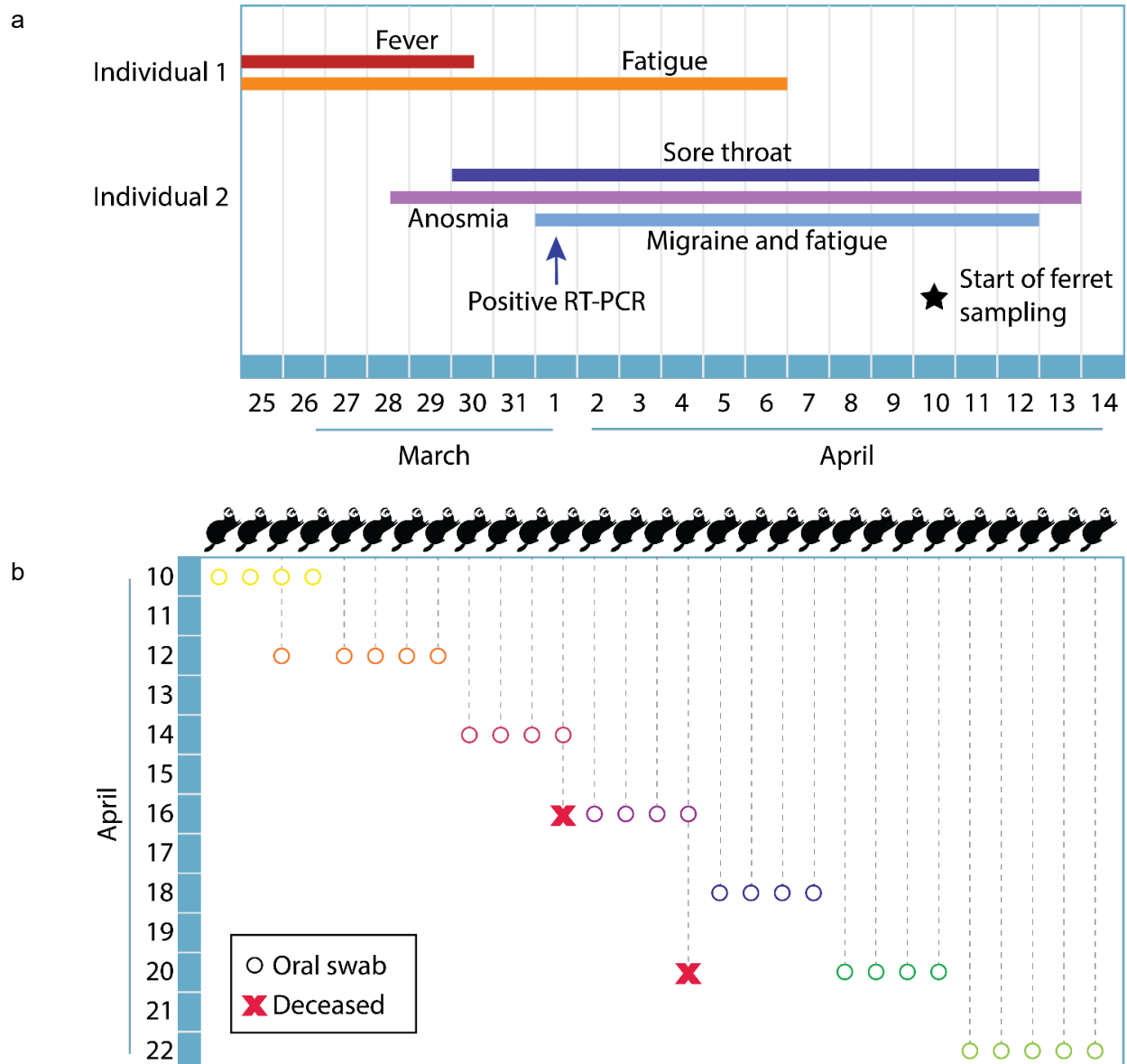
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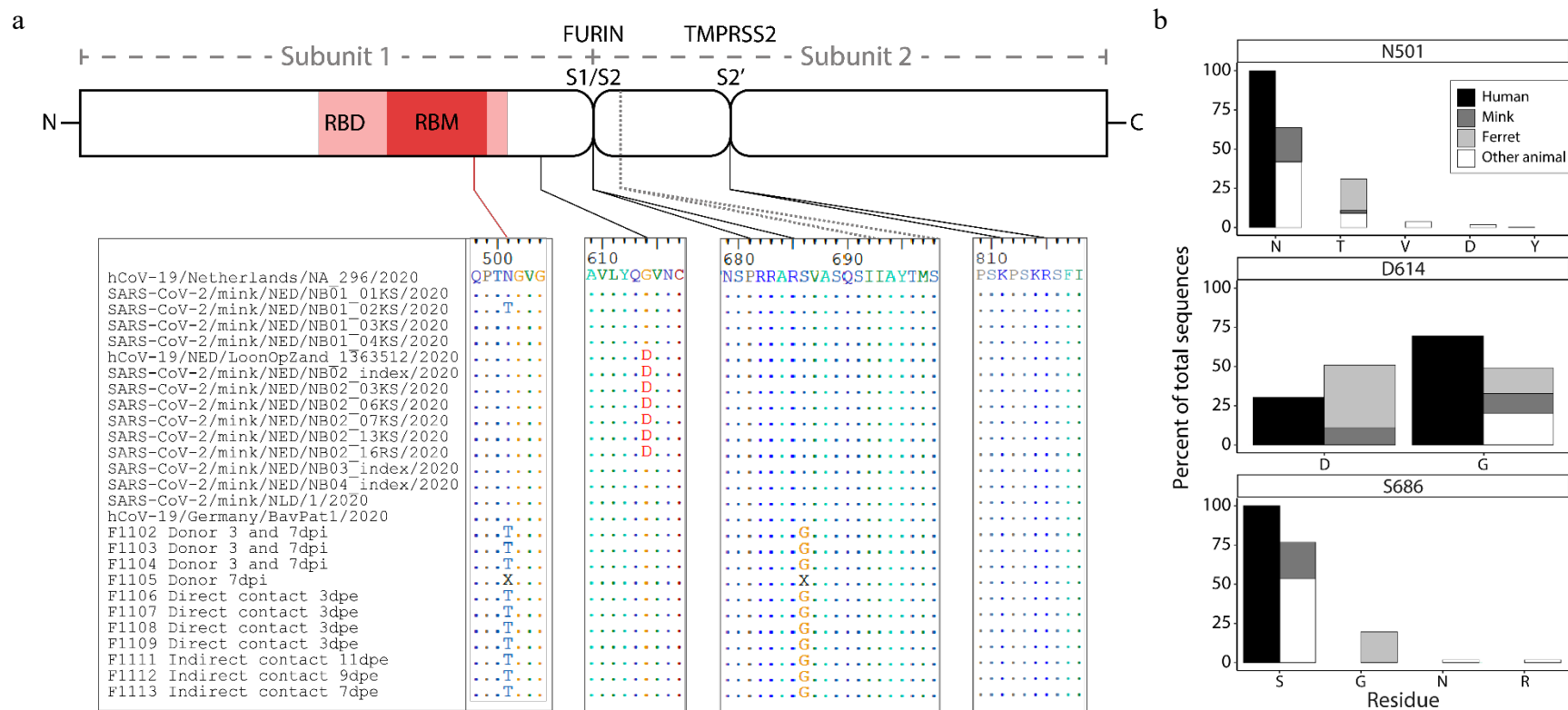
**Figure 1. COVID-19 disease course and ferret sample collection timeline**

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 1<sup>st</sup> (a). Oral swabs were collected from all ferrets in the home over a two-week period, beginning April 10<sup>th</sup>, 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	$\alpha$ 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  $\beta$ -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 ( $\alpha$ 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 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).