1   **Severe acute respiratory disease in American mink (*Neovison vison*) experimentally**

2   **infected with SARS-CoV-2**

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29   **One Sentence Summary:** SARS-CoV-2 infected mink develop severe respiratory disease that

30   recapitulates some components of severe acute respiratory disease, including ARDS.

31

32   **Abstract**:

33   An animal model that fully recapitulates severe COVID-19 presentation in humans has been a

34   top priority since the discovery of SARS-CoV-2 in 2019. Although multiple animal models are

35   available for mild to moderate clinical disease, a non-transgenic model that develops severe

36   acute respiratory disease has not been described. Mink experimentally infected with SARS-CoV-

37   2 developed severe acute respiratory disease, as evident by clinical respiratory disease,

38   radiological, and histological changes. Virus was detected in nasal, oral, rectal, and fur swabs.

39   Deep sequencing of SARS-CoV-2 from oral swabs and lung tissue samples showed repeated

40   enrichment for a mutation in the gene encoding for nonstructural protein 6 in open reading frame

41   1a/1ab. Together, these data indicate that American mink develop clinical features characteristic

42   of severe COVID19 and as such, are uniquely suited to test viral countermeasures.

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46   **Keywords**: SARS-CoV-2, mustelid, mink, ARDS, COVID-19

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48   **INTRODUCTION**

49   Development of animal models has been a critical need since the emergence of SARS-

50   CoV-2 in order to test vaccines and viral countermeasures (*1*). Multiple SARS-CoV-2 models of

51   traditional laboratory species, including mice, hamsters, ferrets, and nonhuman primates, have

52   been developed (*2-6*). However, these models either result in mild to moderate disease or

53   experience distinctive viral dissemination due to altered angiotensin-converting enzyme 2

54   (ACE2) expression, specifically in the brain (*2,3,6*). An animal model that duplicates the severe

55   acute disease spectrum of COVID-19 is still needed. Such a model would allow experimental

56   research into the pathogenesis of severe COVID-19 and would facilitate the evaluation of

57   therapeutic countermeasures in the context of severe disease.

58   Experimental infections of ferrets resulted in viral replication and transmission to naïve

59   individuals, but very minimal to mild clinical signs of disease (*4,5*). However, this does not

60   appear to be the case for all members of the Mustelidae family (*4,5,7-9*). Reports of farmed mink

61   (*Neovison vison*) infected with SARS-CoV-2 emerged in the Netherlands in April 2020 (*10*). To

62   date, at least 12 countries have reported outbreaks in farmed mink, as well as two reports

63   describing positive feral or escaped mink (*11,12*). While many naturally infected mink exhibited

64   mild to moderate clinical disease, a subset of these animals experienced an acute interstitial

65   pneumonia that manifested with severe respiratory distress (*10,13*). Genomic surveillance in

66   samples originating from mink in Denmark identified a series of changes in SARS-CoV-2 spike

67   protein, known as the Cluster 5 variant (*14*). This mink-associated variant resulted in reduced

68   neutralization with human convalescent sera *in vitro* (*14*). A high susceptibility to infection

69   coupled with the public health risk of intra-host viral evolution prompted massive culls of an

70   estimated 17 million mink on Danish farms (*15*).

71   Here, we show that experimentally infected mink develop a severe acute respiratory

72   infection. After infection, progressive respiratory disease can be observed clinically,

73   radiographically, and by histopathology. High amounts of viral RNA and infectious virus can be

74   detected from the respiratory tract. Deep sequencing of SARS-CoV-2 genomes from oral swabs

75   and lung tissue samples collected 3 days post-inoculation demonstrate rapid enrichment for a

76   nonsynonymous mutation in the gene encoding for the nonstructural protein 6 (*nsp6*) in ORF1a

77   in lung tissue samples. These data indicate the potential for rapid viral evolution in mink at the

78   human-animal interface in a short timeframe. Together, these data suggest that the mink animal

79   model recapitulates severe disease observed in hospitalized and fatal human cases of COVID-19

80   and could be useful to test countermeasures against severe COVID-19.

81   **RESULTS**

82   **Mink ACE2 supports efficient entry of SARS-CoV-2**

83   To evaluate the utility of the mink model for COVID-19, we compared human and mink

84   ACE2 functional receptor entry using structural analysis and a vesicular stomatitis virus

85   pseudotype entry assay (*16*). The sequence of mink (*Neovison vison*) ACE2 was obtained by

86   sequencing from lung tissue. The obtained mink ACE2 sequence is 99.8% identical to the

87   previously published *Neovison vison* ACE2 sequence (GenBank QPL1221.1). Two substitutions

88   are observed at residues 231 and 613, in which the threonine and tyrosine in the previous

89   sequence are replaced by a lysine and a cysteine, respectively. These residues are not located in

90   the SARS-CoV-2 receptor binding domain (RBD)-ACE2 interface. Both American mink ACE2

91   sequences are 99% identical to the published European mink ACE2 sequence (*Mustela lutreola*

92   *biedermanni*, GenBank QNC68911.1), and ~83% identical to human ACE2 (*Homo sapiens*,

93   GenBank BAB40370.1) (Figure 1A).

94   To compare differences within the ACE2 interface with the SARS-CoV-2 spike receptor

95   binding domain (RBD), the residues participating in the interaction, as described by Lan, *et al*.
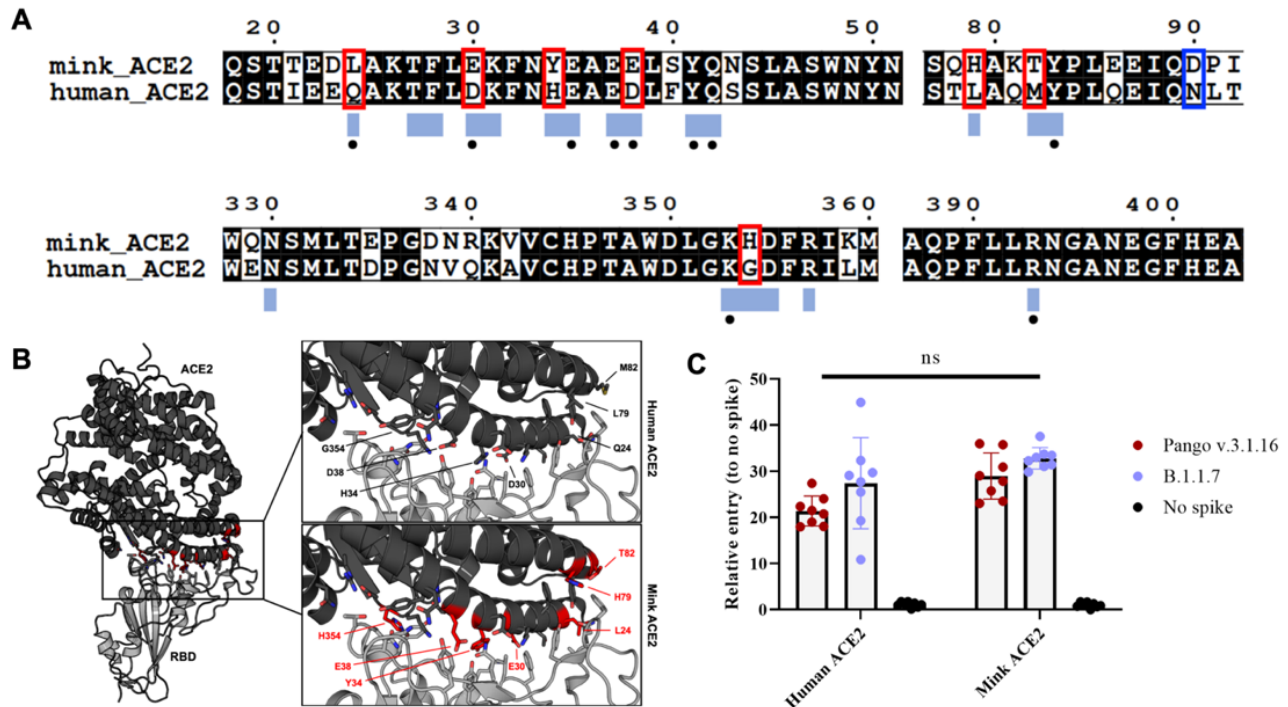
Figure 1. Functional SARS-CoV-2 entry analysis with human and mink ACE2. (A) An amino acid sequence alignment of ACE2 from the mink and human. Residues that participate in the SARS-CoV-2 RBD – ACE2 interaction are noted below the alignment by a blue box. Residues that participate in intermolecular hydrogen bonding or salt bridges are marked with a black dot. ACE2 residues that differ between mink and human within the interface are outlined with a red box. The substitution at residue 90 affecting an N-linked glycosylation site is noted with a blue box. (B) Differences between mink and human ACE2 are highlighted on the structure of the complex of SARS-CoV-2 RBD in gray bound to human ACE2 in black. Sidechains of the ACE2 and RBD residues that participate in the binding interaction are shown as sticks. The mutated residues are indicated by red. (C) SARS-CoV-2 spike pseudotype assay showing relative entry compared to no spike control in BHK cells expressing human or mink ACE2. Bars depict standard deviation.

96   (*17*), were mapped onto an amino acid sequence alignment of ACE2 from American mink

97   (*Neovison vison*), European mink (*Mustela lutreola biedermanni*, GenBank QNC68911.1), and

98   humans (*Homo sapiens*, GenBank BAB40370.1)(*18*). The binding residues are 65% identical

99   between mink and human ACE2, with seven of the 20 total interface residues differing in mink

100   (Figure 1A). These residues are highlighted on the structure of SARS-CoV-2 RBD bound to

101   human ACE2 to visualize these differences (Figure 1B). Consistent with a previous analysis,

102   critical residues for interaction with the spike RBD, K31, Y41, and Y353 are conserved (*18*).

103   To investigate if the observed discrepancies between human and mink ACE2 translates to

104   significant differences in spike entry, we directly compared the viral entry of VSV SARS-CoV-2

105   spike pseudotype particles on Baby Hamster Kidney fibroblasts (BHK cells) transfected with

106   either human or mink ACE2. We observed significantly increased entry in mink ACE2 expressing

107   cells compared to those expressing human ACE2 for the prototype WA1 lineage A SARS-CoV-2

108   spike (Figure 1C, alignment, entry data, $p = 0.0307$, 2-way ANOVA followed by Šídák's multiple).

109   However, the B.1.1.7 (Alpha) variant showed no difference ($p = 0.1505$, 2way ANOVA followed

110   by Šídák's multiple). Overall, the Alpha variant showed increased entry in both human and mink

111   ACE2. Considering both variants together, there was no statistical difference in entry of the spikes

112   to human and mink ACE2 ($p = 0.5633$, Two-tailed t-test) (Figure 1C).

113   We next determined the ACE2 expression in the respiratory tract of mink. ACE2 was

114   multifocally detected in the respiratory olfactory epithelium and there were multifocal SARS-

115   CoV-2 immunoreactive respiratory and olfactory epithelial cells (Supplemental Figure 1). ACE2

116   immunoreactivity was also detected in the lower respiratory bronchiolar epithelium and type I

117   and type II pneumocytes (Supplemental Figure 1, D, E).

118   **Experimentally infected mink develop severe respiratory disease by 2 days post inoculation**

119   Eleven adult farmed mink were inoculated intranasally and intratracheally with 10^5

120    $TCID_{50}$ of Alpha Variant, B.1.1.7 (hCoV-319 19/England/204820464/2020, EPI_ISL_683466).

121   Due to the severity of clinical disease and respiratory distress, two animals reached end-point

122   criteria and were euthanized the evening of 2 days post-inoculation (DPI). Eight animals reached

123   end-point criteria on 3 DPI, and one animal recovered from severe disease and was euthanized

124   on the predetermined experimental endpoint of 28 DPI.

125   Marked weight loss (up to 15%) was observed in all animals by 3 DPI (Figure 2A). In

126   the animal that survived infection, bodyweight returned to baseline values by 14 DPI

127   (Supplemental Figure 2A). Clinical signs were first detectable on 1 DPI in 5 of 11 (45 %)

128   animals, with clinical signs observed in 9 of 11 (82 %) animals by 2 DPI and all remaining

129   animals by 3 DPI. Signs of clinical disease included dull mentation, shivering, hunched or balled

130   posture, lethargy, anorexia, increased respiratory effort, tachypnea, with occasional nasal

131   discharge that included both epistaxis and serous discharge (Figure 2). Animals were examined

132   on 1,3, 5, 7, 10, 14, 17, 21, and 28 DPI under anesthesia; 10 animals were clinically dehydrated

133   by 3 DPI.

134   Complete blood count (CBC) and complete chemistry panels were performed on blood

135   samples collected at least one week prior to infection and at 0, 1, 3, 5, 7, 10, 14, 17, 21, and 28

136   DPI. At all time-points post infection, the CBC was unremarkable apart from a decreased white

137   blood cell (WBC) count characterized by a mild lymphopenia that was most pronounced in the 9

138   remaining animals on 3 DPI (Figure 2C). The neutrophil-to-lymphocyte ratio was significantly

139   increased at the terminal endpoint for clinically ill animals (Figure 2D). The single surviving

140   animal had an elevated neutrophil-to-lymphocyte ratio (NLR) that peaked on 5 DPI as compared

141   to baseline then quickly decreased (Supplemental Figure 2B). The blood chemistry panel was

142   clinically unremarkable for all values except for a mild hypoproteinemia and hypoalbuminemia

143   (Figure 2E).

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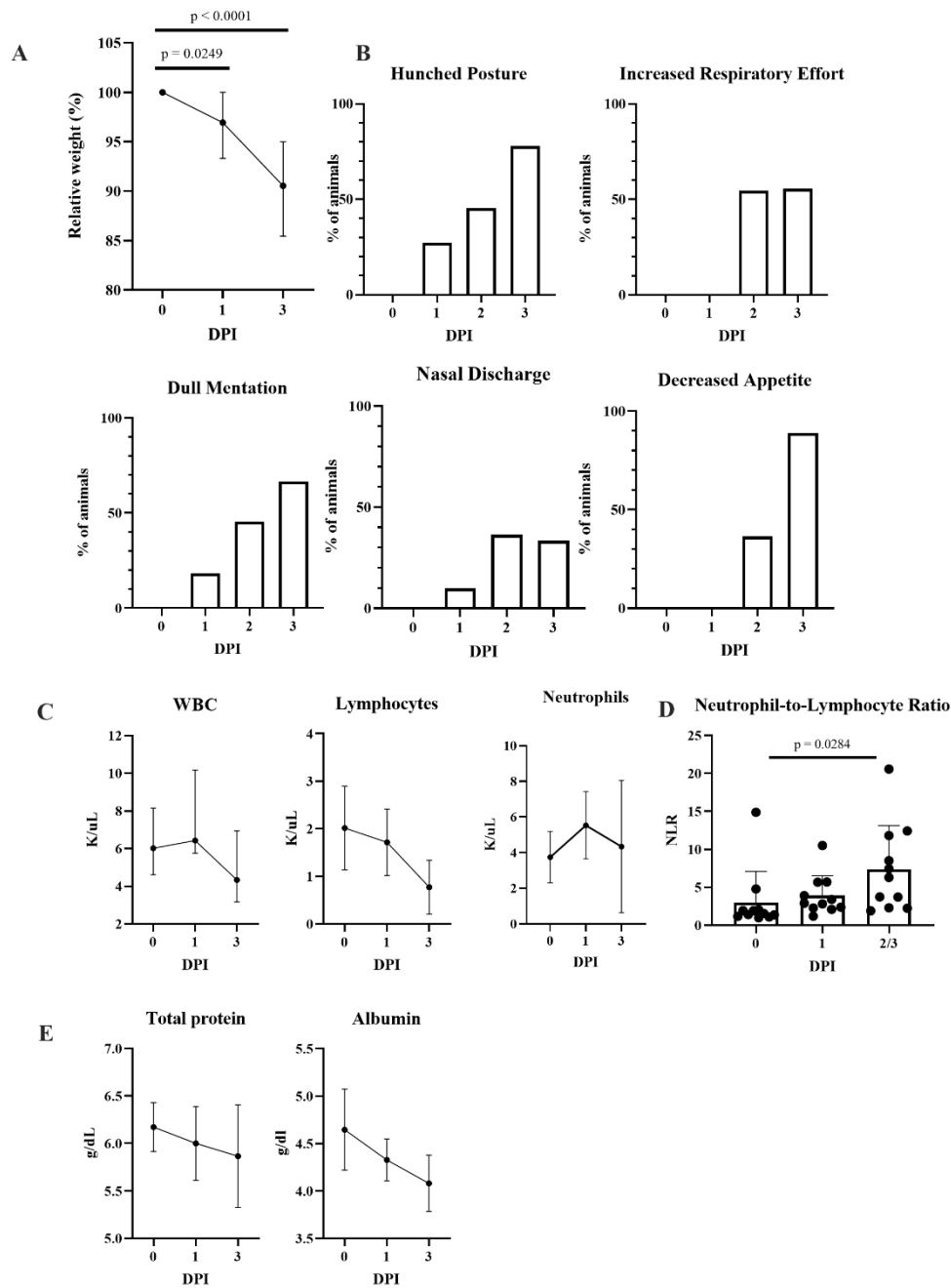
Figure 2. Features of acute respiratory disease in mink. (A) Percent of original body weight was collected during clinical exams on 1 and 3 DPI. Mink lost a significant amount of body weight on both 1 and 3 DPI (One-way ANOVA with Tukey's multiple comparisons test). (B) Mink were assessed at least twice daily and evaluated for hunched posture, respiratory effort, mentation, nasal discharge, and appetite. (C) Complete blood count values collected after infection. The median with the 95% confidence interval (CI) are depicted. (D) Increased Neutrophil-to-Lymphocyte Ratio as determined from the complete blood count. Mean with standard deviation depicted, 2-way ANOVA with Tukey's multiple comparisons test. (E) Selected blood chemistry values, median with 95% CI depicted.

147 **Progressive pulmonary infiltrates evident in pulmonary radiographs**

148   Radiographic scores on 1 and 3 DPI were increased as compared to baseline values

149   (Figure 3A) and indicated the presence of progressive pulmonary infiltrates consistent with viral

150   pneumonia likely with concurrent non-cardiogenic pulmonary edema secondary to acute

151   respiratory disease syndrome (ARDS)(Figure 3B). On 1 DPI, radiographic changes consistent

152   with viral pneumonia were present in the thoracic radiographs of 5 (45%) of 11 mink. Of these 5,

153   4 had evidence of a mild-to-moderate ground glass/unstructured interstitial pattern and the

154   remaining animal had a moderate-to-marked alveolar pattern affecting multiple lung lobes.

155   Interestingly, this animal had progressive multifocal grade 3 and 4 pulmonary infiltrates at 2 DPI

156   prior to euthanasia (Figure 3). At 3 DPI, 8 of the 9 remaining animals displayed disease
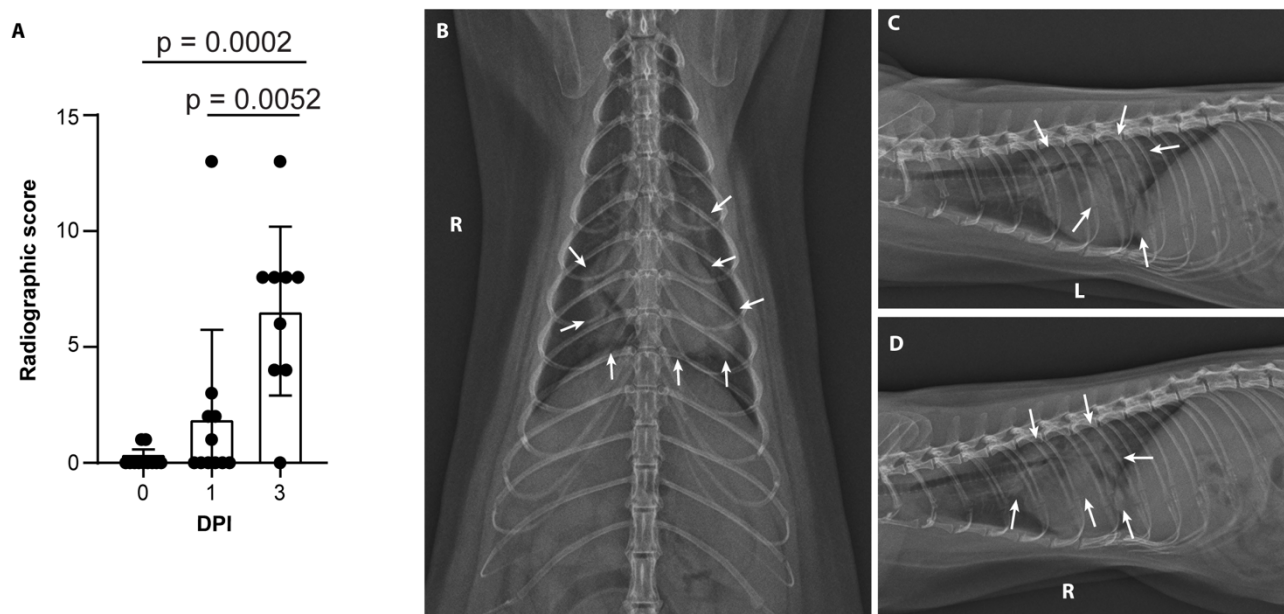
Figure 3. Severe radiological changes after infection with SARS-CoV-2. (A) Compiled radiographic scores. Bar graph depicts the mean with standard deviation and individuals, ordinary one-way ANOVA with Tukey's multiple comparisons test. Radiographs demonstrate multifocal pulmonary infiltrates, most severe in the left and right caudal lung lobes depicted in the (B) dorsoventral radiograph (C) left lateral and (D) right lateral radiograph on evening of 2 DPI. Arrows depict grade 4 pulmonary disease in the left and right caudal lung lobes with grade 3 pulmonary disease in the right middle lung lobe and cranial subsegment of the left cranial lung lobe.

157 progression that was characterized

158 by increased severity and more

159 extensive distribution of identified

160 multifocal alveolar pattern (grade

161 3 to 4).

162 The remaining animal was

163 monitored for resolution of disease

164 over 28 days. Changes consistent

165 with viral pneumonitis were first

166 detected on 3 DPI, with the most

167 severe changes noted on 5 DPI

168 characterized by alveolar pattern in

169 both caudal lung lobes. These

170 changes began to resolve on 7 DPI

171 with complete resolution noted on

172 14 DPI. (Supplemental Figure 3).

173 **Pathological changes in mink**

174 **resemble severe human COVID-**

175 **19 pulmonary damage and**

176 **coagulopathy**

177 Necropsy of all animals

178 was performed immediately after

179 euthanasia. Lung weight to body
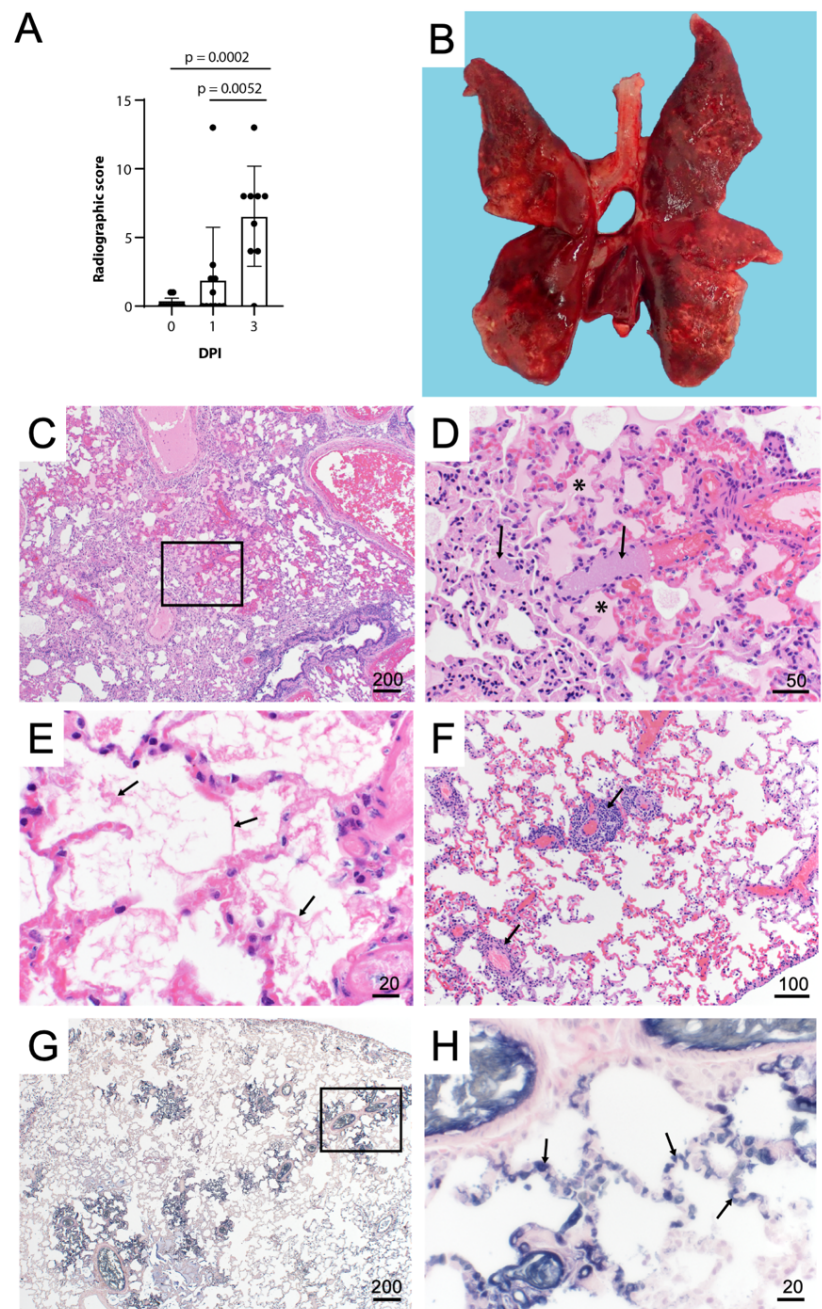
Figure 4. Pulmonary histopathology and immunohistochemistry. (A) Lung to body weight ratio, Mann-Whitney test. Graph depicts median with individuals. (B) Diffusely consolidated dark-mottled red lungs (C) Multifocal pulmonary congestion (Box) (D) Enlarged section of image C, vascular thrombi (arrows) alveolar edema (asterisks) (E) Alveolar fibrin (arrows) (F) Lymphoplasmacytic perivascular cuffing (arrows) (G) Pulmonary PTAH staining (black) (H) Enlarged section of image G, microthrombi (arrows). Scale bar expressed in μm in lower right corner of each image.

180 weight ratio was assessed to estimate the extent of pulmonary edema, and the ratio was

181 significantly increased in infected animals compared to uninfected controls (Figure 4A). There

182 were varying degrees of gross pulmonary pathology evident in all 10 animals euthanized on 2 or

183 3 DPI, with 100% of some lungs affected (Figure 4B). Grossly, lungs were hyperemic, and

184 several animals had undergone pulmonary hepatization (Figure 4B).

185   Histopathologic lesions associated with SARS-CoV-2 were restricted to the nasal

186 turbinates and lungs of animals euthanized on 2 or 3 DPI. Nasal turbinates were characterized by

187 a marked neutrophilic rhinitis with multifocal respiratory epithelial degeneration, necrosis, and

188 loss. Nasal cavities were filled with an exudate composed of abundant neutrophilic and necrotic

189 debris. There was rare neurotrophilic infiltration of olfactory epithelium (Figure 5). Pulmonary

190 pathology was more severe in 3 of 10 animals euthanized on 2 or 3 DPI. Lesions consisted of

191 moderate to marked vascular congestion (Figure 5, C, D) with thickening of the alveolar septa by

192 edema, fibrin, and cellular infiltrate. Multifocal fibrin thrombi were identified in the vasculature

193 of regions of congestion (Figure 5, E, F). Alveolar lumina often contained abundant edema fluid,

194 fibrin, and increased number of alveolar macrophages. There was a moderate lymphoplasmacytic

195 perivascular cuffing. Bronchial and bronchiolar epithelium was generally unaffected. The

196 remaining seven animals had mild congestion, moderate lymphoplasmacytic perivascular cuffing

197 with a mild increase in alveolar macrophages. SARS-CoV-2 antigen was observed

198 predominately in pulmonary macrophages, although it was unclear if this was the result of

199 replication or phagocytosis of viral antigen (Supplemental Figure 4, A, B). Multifocal SARS-

200 CoV-2 antigen positivity was identified in bronchial epithelium and type I and II pneumocytes

201 and bronchiolar epithelium (Supplemental Figure 5, C, D).

Viral shedding is detected as early as 1 DPI in experimentally infected mink
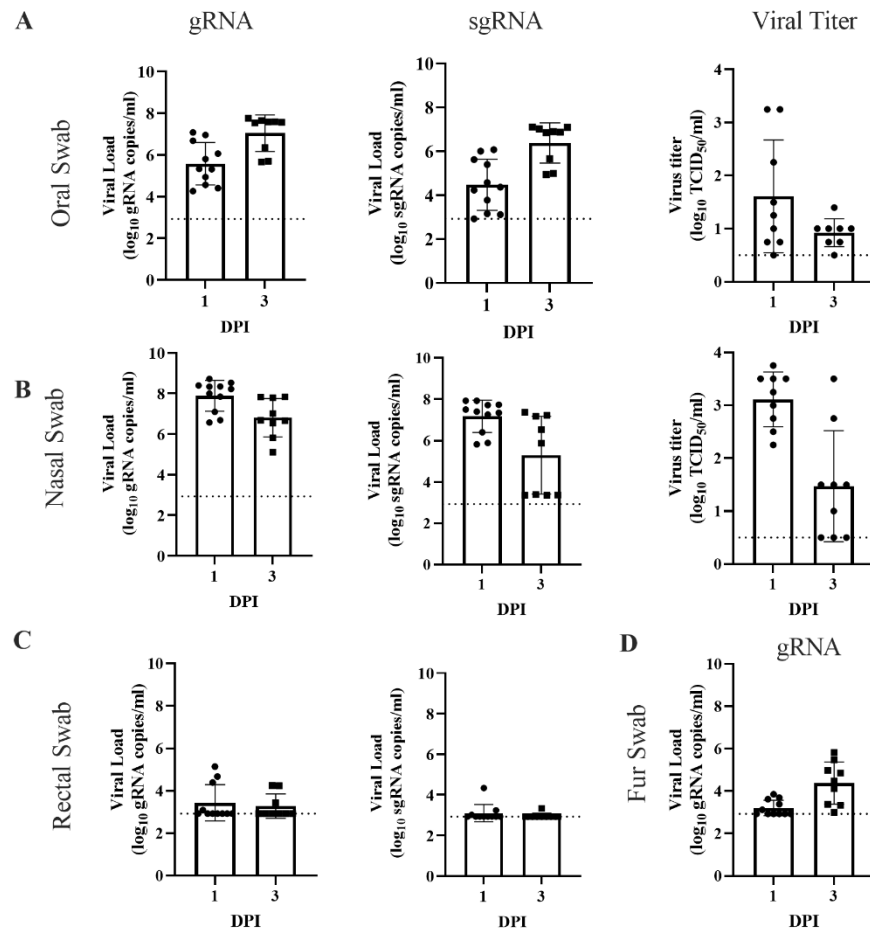
Viral RNA was detected from all animals beginning on 1 DPI, with the highest viral RNA loads detected in oral and nasal swabs. In nasal, oral, and rectal swabs, both genomic (gRNA) RNA and sub-genomic RNA (sgRNA) were detected, with sgRNA as a marker
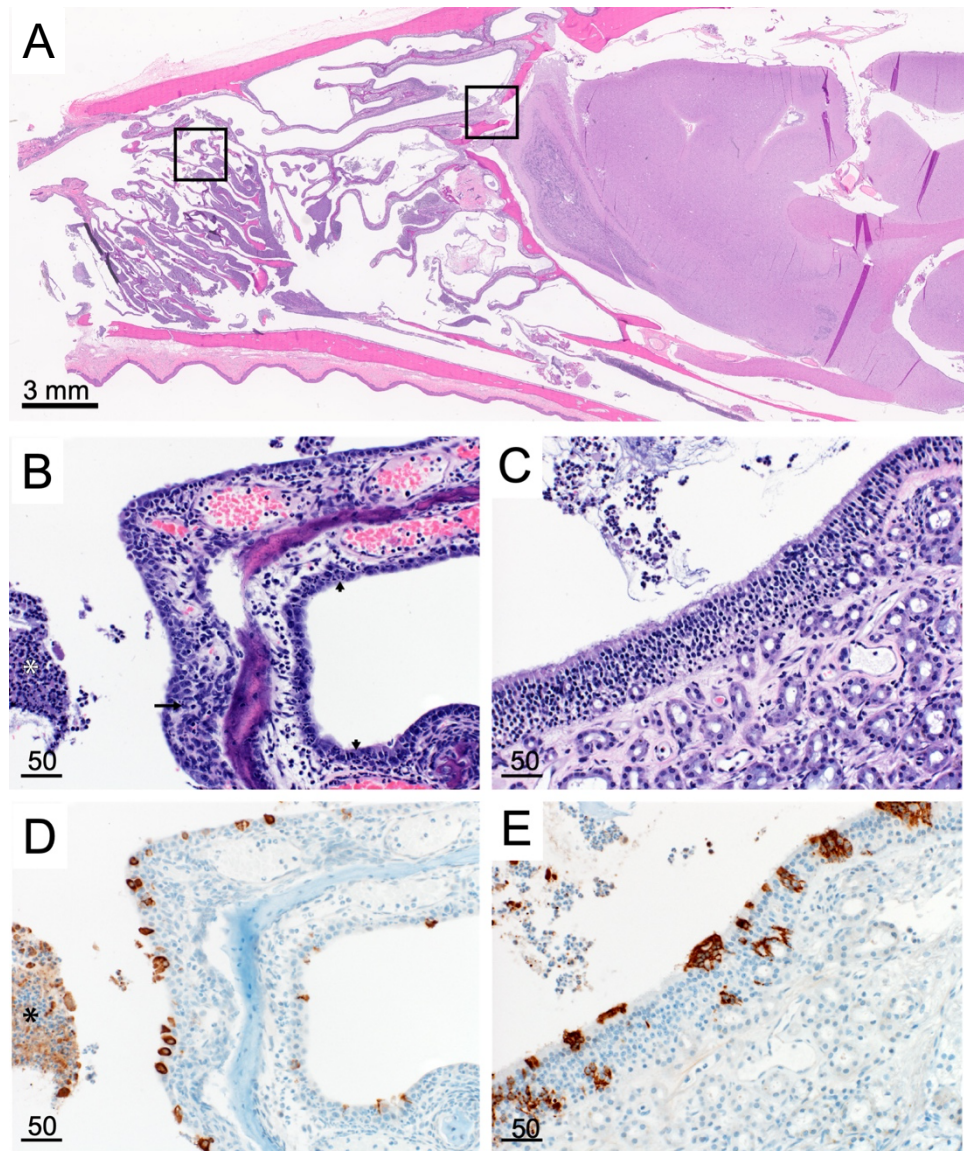
Figure 5. Nasal turbinate pathology and histopathology. (A) Sagittal section of skull, H&E (B) Respiratory epithelium neutrophilic infiltrates (small arrows) necrotic epithelium (long arrow) cellular exudate (asterisk) (C) Olfactory epithelium with cellular exudate (D) Respiratory epithelium SARS-CoV-2 IHC immunoreactivity (brown), immunoreactive cellular exudate (asterisk) (E) Olfactory epithelium SARS-CoV-2 IHC immunoreactivity and cellular exudate (brown). Scale bars expressed in μm unless indicated.

for viral replication. Viral RNA was readily detected in both oral and nasal swabs (Figure 6, A, B). Viral RNA was also detected in rectal swabs collected from 4 of 11 (36 %) animals at 1 DPI and 3 of 9 (33%) animals at 3 DPI. (Figure 6C). Fur swabs were collected to estimate the risk of

225 handlers and

226 processers in the

227 fur industry;

228 genomic RNA

229 was detected

230 from most

231 animals (Figure

232 4D). In the sole

233 animal that

234 survived until 28

235 DPI, viral RNA

236 was detected in

237 nasal swabs until

238 7 DPI and oral

239 swabs until 10

240 DPI

241 (Supplemental

242 Figure 2C).

Figure 6. Viral shedding in infected mink. Oral (A), nasal (B), rectal (C), and fur (D) swabs were collected on 1 and 3 DPI and analyzed for genomic RNA, sub-genomic RNA, and infectious virus. Bar graphs depict the mean and standard deviation and individuals. Dotted line indicates the limit of detection.

243   Infectious virus was detected in most oral and nasal swabs at 1 (8 of 9, 89%) and 3 (7 of

244 8; 88%) DPI. Low amounts of infectious virus were detected in one rectal swab on 1 DPI (1.5

245 log10 $TCID_{50}$/ml) and one fur swab on 3 DPI (0.75 log10 $TCID_{50}$/ml). Infectious virus was

246 detected in the surviving animal until 7 DPI in nasal swabs, and 10 DPI in oral swabs (Figure 6,

247 A, B).

High viral load in respiratory tract of SARS-CoV-2-infected mink

At necropsy, 37 tissues were collected from each animal and analyzed for the presence of both gRNA and sgRNA. gRNA and sgRNA was detected in the tissues from all animals necropsied on 2 or 3 DPI (Figure 7, Supplemental Figure 6). While viral RNA was


Figure 7. High levels of viral replication in the respiratory tract of infected mink. Tissues from animal euthanized on 3 DPI were analyzed for genomic (A), sub-genomic (B), and infectious virus (C). Bar graphs depict individuals, mean, and standard deviation. The dotted line depicts the limit of detection.

detected in multiple organ systems, the highest viral loads were detected in respiratory tissues (Figure 7A, B). Within the respiratory tract, the highest viral loads were detected in the upper (nasal turbinate) and lower (all lung lobes) as compared to the mid-respiratory tract (trachea, right and left bronchus). In addition, high levels of viral RNA were detected in the frontal lobe,

271   cerebellum, and brainstem (Supplemental Figure 6). Despite high levels of viral RNA, SARS-

272   CoV-2 antigen was not observed in the olfactory bulb, cerebral cortex, or brainstem

273   (Supplemental Figure 5). The respiratory tract was tested for the presence of infectious virus,

274   with the majority of infectious virus found in the upper and lower respiratory tract (Figure 7C).

275   **Within-host evolution of SARS-CoV-2 in mink indicates potential for rapid adaptation**

276   SARS-CoV-2 genomes were deep sequenced for 23 oral swabs and 10 lung tissue

277   samples and the SARS-CoV-2 inoculum diluent used in the experimental challenge

278   (Supplemental Table 1). The deep sequencing runs yielded an average of 88,686 reads mapped

279   for each sample (Supplemental Table 1). One sample for which fewer than 50,000 reads were

280   recovered was not used in

281   subsequent analyses. Direct

282   comparison of intrahost

283   single nucleotide variants

284   (iSNVs) detected at minor

285   allele frequency thresholds of

286   3% and 5% showed a lack of

287   concordance between
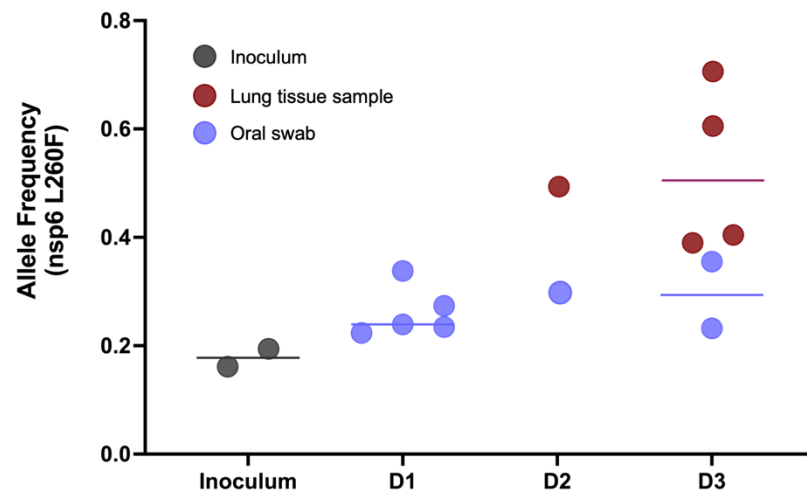
288   technical replicates

289   (Supplemental Figure 7),

290   regardless of the SARS-CoV-

291   2 genome copy number

292   (Supplemental Figure 8A) or

293   the number of sequencing

Figure 8. Enrichment of a nonsynonymous mutation (L260F) in the gene encoding for *nonstructural protein 6* in ORF1a of SARS-CoV-2 in oral swabs and lung tissue samples of five experimentally challenged mink. Deep sequencing of SARS-CoV-2 positive samples show rapid enrichment for L260F in *nsp6* in lung tissue samples but not the oral swabs for five of ten experimentally inoculated mink with SARS-CoV-2 genomic material detected in the lungs. Oral swab samples indicated by blue and lung tissue samples indicated by red, the line indicates group mean. Allele frequency for the L260F mutation is plotted on the y-axis.

294   reads mapped for the sample (Supplemental 8B). These results are consistent with findings in

295   other deep viral genome sequencing efforts (*19, 20*) and the need for tempered conclusions with

296   the detection of low-level variants and small datasets. Therefore, we focus on changes in

297   consensus sequence relative to the inoculum. Consensus sequences were largely unchanged from

298   the SARS-CoV-2 inoculum sequence in all samples with the exception of a nonsynonymous

299   mutation in the gene encoding nonstructural protein 6 (*nsp6*, L260F) which appeared enriched in

300   the lung tissue samples of 5 mink relative to the inoculum and the oral swab samples (Figure 8).

301   This mutation was identified eight times in 1064 available mink-associated SARS-CoV-2

302   genome sequences recovered from GISAID including a farmed mink in the USA

303   (EPI_ISL_1014945) in Oct 2020, three mink the Netherlands (EPI_ISL_523102, 577749, and

304   523102) in May and Aug 2020, and four mink in Latvia (EPI_ISL_8514994, 8514995, 8514997,

305   and 4548647, Supplemental Table 2) in July and Sept 2021.

306   **Seroconversion in surviving mink by 14 DPI**

307   We analyzed serum for the development of a neutralizing antibody response. All animals

308   had a titer of <20 prior to challenge and only the surviving animal developed a measurable

309   neutralizing response. This animal seroconverted by 14 DPI with a peak neutralizing titer of 960,

310   which decreased to a titer of 640 at euthanasia at 28 DPI.

311   **DISCUSSION**

312   The continued emergence of SARS-CoV-2 variants of interest and variants of concern

313   highlight the urgent need for animal models who consistently recapitulate the spectrum of

314   disease in COVID-19 patients. Overall, the pseudotype entry data from this study demonstrates

315   comparable spike entry between human and mink ACE2 confirming the suitability of mink for

316   modeling SARS-CoV-2 infection and COVID-19 diseases.

317   Humans infected with SARS-CoV-2 present with a spectrum of clinical disease that

318   ranges from asymptomatic infection to severe disease characterized by respiratory distress,

319   sepsis, or multiorgan failure. Currently, an animal model for severe COVID-19 disease is not

320   available (1). Most human infections are confined to the upper respiratory tract. Mild or early

321   disease manifests with nonspecific symptoms that can include fatigue, fever, headache, loss of

322   smell and taste, congestion, and fever (21). Progression into severe disease is typically presented

323   as worsening respiratory disease, hypoxemia, and radiographic lesions, with end-point markers

324   that can include coagulopathies, thromboembolism, acute kidney injury, and ARDS (21-24).

325   Infected mink displayed clinical disease consistent with worsening human COVID-19 disease.

326   Infected mink consistently demonstrated a greater degree of weight loss than that reported in

327   nonhuman primates or hamsters in the days following infection. Increased respiratory effort and

328   tachypnea in mink mark progression into severe COVID-19 disease. This study did not look at

329   odor discrimination; however, neutrophilic infiltrate in olfactory epithelium could suggest a loss

330   of smell that resulted in decreased appetite. Interestingly, while multiple field reports of fur farm

331   outbreaks commonly report nasal discharge, this was not a consistent finding in these mink.

332   Features of complete blood counts of COVID-19 disease patients include leukopenia,

333   lymphopenia, thrombocytopenia, and an increased NLR (13,21,25,26). While we were unable to

334   rule out a stress leukogram resulting in increased NLR, this finding has been reported in ferrets

335   infected with H5N1(27) and approximates critically ill human COVID-19 disease patients, where

336   NLR can be used as a prognostic indicator (25). Interestingly, the NLR ratio for the single

337   surviving animal was highest on 5 DPI, when the most severe changes were observed on thoracic

338   radiographs. Mink displayed minimal to mild hypoproteinemia and hypoalbuminemia in the face

339   of clinical dehydration, indicating a true hypoproteinemia. During acute disease, albumin can act

340   as a negative phase protein and hypoalbuminemia has been associated with poor outcomes in

341   COVID-19 patients (*28,29*).

342   One important hallmark of severe human COVID-19 disease is progression to ARDS.

343   The Berlin Definition of ARDS addresses timing, thoracic imaging, the origin of thoracic edema,

344   and the degree of hypoxemia (*23*). Our high-dose intratracheal inoculation likely contributed to

345   the acute presentation of disease, and additional studies are necessary to better understand the

346   course of SARS-CoV-2 infection in mink in relationship to dose, route of inoculation, and other

347   emerging variants. Similar to the radiologic features described in humans, mink displayed

348   bilateral ground glass opacities (*30*). However, unlike humans, these radiological features were

349   not most severe in the gravitationally dependent regions (*30*). One theory explaining this atypical

350   distribution is that the method of viral inoculation may have resulted in greater distribution in the

351   caudal lung lobes as the virus was administered intratracheally in anesthetized subjects as

352   opposed to a more passive inhalation of viral fomites. Additionally, a component of non-

353   cardiogenic pulmonary edema, secondary to ARDS, may contribute to pulmonary infiltrates and

354   is more commonly distributed in the caudal lung lobes. The hearts were radiographically and

355   grossly normal, indicating the pulmonary changes were not likely due to cardiogenic pulmonary

356   edema. Finally, this study was not able to evaluate the degree of hypoxemia, a critical step in

357   diagnosing ARDS (*23*). Additional studies using advanced tools such as the flexiVent (SCIREQ,

358   Emka Technologies Co., Sterling, VA, USA) and blood gas analysis are required to fully

359   evaluate this model for clinical ARDS (*2,29,31,32*).

360   Histologically, diffuse alveolar disease (DAD) is an important finding in patients with

361   severe COVID-19 disease and has not been regularly described in currently available animal

362   models. Although not every mink had severe pulmonary pathology, all 10 animals euthanized on

363   2 or 3 DPI displayed pathology consistent with human COVID-19 disease (*24*). Animals with

364   less severe histologic disease likely represent earlier stages in disease progression and follow up

365   studies are required to better understand disease progression. The multifocal fibrin thrombi,

366   cellular infiltrate, and resulting edema in the 3 mink with histologically severe disease likely

367   reflects a coagulopathy, described in human patients with severe disease (*29,33*). Additional

368   studies focusing on D-dimer, fibrinogen, and PT/aPTT are required to further tease out the

369   pathogenesis in this model (*29*).

370   Outbreaks of COVID-19 on mink farms suggest the potential for novel SARS-CoV-2

371   variants to emerge in mink, with a high probability of spillback (*7-11,17*). This study provides

372   experimental evidence of rapid enrichment in 5 of 10 mink for L260F in the gene encoding for

373   nonstructural protein 6 in SARS-CoV-2 (Figure 8) which is hypothesized to affect viral

374   autophagy and suppress the type I interferon response (*18,19*). This mutation has been identified

375   in multiple COVID-19 outbreaks on mink farms in the Netherlands, Latvia, and the US.

376   Interestingly, enrichment for the L260F mutation is most prominent in the lung tissue samples

377   rather than the oral swabs, suggesting some tissue-specific tropism and likely a reduced

378   probability of onward transmission. The repeated detection of the L260F mutation among

379   COVID-19 outbreaks on mink farms through time and space supports that this mutation confers

380   a selective advantage in mink and merits further study.

381   A pre-clinical model of severe COVID-19 disease is desperately needed to better evaluate

382   SARS-CoV-2 vaccines and therapeutics. Current models demonstrate a reduction in viral titer

383   and reduction of mild pathology, but no current model can recapitulate severe disease. In this

384   study, we showed the utility of experimentally infected mink as a model for severe human

385   COVID-19 disease. After infection, mink develop severe clinical disease associated with

386   histological changes consistent with worsening human disease, making this new model the most

387   translatable animal model available for severe COVID-19 disease.

388   **Materials and Methods**

389   *Experimental Design*

390   The objective of this study is to evaluate American mink as an animal model of severe

391   COVID-19. SARS-CoV-2 infection in the American mink were determined through virological,

392   histopathological, clinical, and radiographical analyses. Comparative analyses of functional

393   SARS-CoV-2 entry using human and mink ACE2 were determined through a vesicular stomatitis

394   virus pseudotyping assay. Deep viral genome sequencing was employed to study the intrahost

395   evolutionary dynamics in the experimentally infected mink.

396   *Ethics statement*

397   All animal experiments were approved by the Institutional Animal Care and Use

398   Committee of Rocky Mountain Laboratories, NIH and carried out in an Association for

399   Assessment and Accreditation of Laboratory Animal Care (AALAC) International accredited

400   facility, according to the institution's guidelines for animal use, following the guidelines and

401   basic principles in the Guide for the Care and Use of Laboratory Animals, the Animal Welfare

402   Act, United States Department of Agriculture and the United States Public Health Service Policy

403   on Humane Care and Use of Laboratory Animals. The Institutional Biosafety Committee (IBC)

404   approved work with infectious SARS-CoV-2 strains under BSL3 conditions. Sample inactivation

405   was performed according to IBC-approved standard operating procedures for removal of

406   specimens from high containment.

407   *Mink ACE2 Sequence and modeling*

408   DNA was extracted from mink lung tissue using QIAamp DNA Tissue Kit according to

409   the manufacturer. Mink ACE2 full length gene was amplified using long range PCR (LRPCR)

410   amplification assay in two overlapping fragments using high-fidelity PrimeSTAR GXL DNA

411   Polymerase (Takara Bio USA) as previously described (*34,35*). Briefly, 50 µL LRPCR master

412   mix contained 0.2 µM of each primer (Supplementary Table 3), 1X PrimeSTAR GXL Buffer,

413   200 µM each deoxyribonucleotide triphosphate, 5 µL cDNA template, and 1.25 units of

414   PrimeSTAR GXL DNA Polymerase (Takara Bio USA, Inc., San Jose, CA). The LRPCR mixture

415   was incubated at 98°C for 2 minutes for the initial denaturation, followed by 4 cycles at 98°C for

416   10 seconds, 68°C for 15 seconds (−2°C per cycle), and 72°C for 10 minutes before an additional

417   26 cycles of 98°C for 10 seconds, 56°C for 15 seconds, and 72°C for 10 minutes. Sequencing

418   libraries were generated using the TruSeq DNA PCR-Free library prep kit (Illumina Inc., San

419   Diego, CA, USA) and sequenced on an Illumina MiSeq instrument at 2 x 151 paired-end reads.

420   Reads were *de novo* assembled using SPAdes v. 3.13 (*36*). Sequence alignments between

421   American mink (*Neovison vison*, sequence generated in this study) ACE2, European mink ACE2

422   (*Mustela lutreola biedermanni*, GenBank QNC68911.1), and human ACE2 (*Homo sapiens*,

423   GenBank BAB40370.1) were generated using Multalin (*37*) and plotted using ESPript (*38*).

424   Residues that participate in the SARS-CoV-2 RBD–ACE2 interaction, as described by Lan et al.

425   (*17*), are noted below the alignment. The percent identity between the ACE2 sequences was

426   calculated by Clustal Omega (*39*).

427   Structure analysis utilized the human ACE2 and SARS-CoV-2 RBD crystal structure,

428   PDB ID 6M0J (*17*). Mutagenesis to show residues that differ in mink ACE2, and the alpha and

429   delta variant RBD, was performed in COOT (*40*). The figures were generated using The Pymol

430   Molecular Graphics System (https://www.schrodinger.com/pymol).

431 *Plasmids*

432   The spike coding sequences for SARS-CoV-2 lineage B (hCoV-19/Denmark/DCGC-

433   3024/2020, EPI_ISL_616802) were truncated by deleting 19 aa at the C-terminus. The S proteins

434   with the 19 AA deletion of coronaviruses were previously reported to show increased efficiency

435   regarding incorporation into virions of VSV (*41,42*). These sequences were codon optimized for

436   human cells, then appended with a 5′ kozak expression sequence (GCCACC) and 3′ tetra-glycine

437   linker followed by nucleotides encoding a FLAG-tag sequence (DYKDDDDK). These spike

438   sequences were synthesized and cloned into pcDNA3.1$^+$ (GenScript Biotech, Piscataway, NJ,

439   USA). Mink ACE2 were synthesized and cloned into pcDNA3.1$^+$ (GenScript Biotech,

440   Piscataway, NJ, USA). All DNA constructs were verified by Sanger sequencing (ACGT).

441   *Pseudotype production and luciferase-based cell entry assay*

442   Pseudotype production was carried out as described previously (*16*). Briefly, plates pre-

443   coated with poly-L-lysine (Sigma-Aldrich, St. Louis, MO, USA) were seeded with 293T cells

444   and transfected the following day with 1,200 ng of empty plasmid and 400 ng of plasmid

445   encoding coronavirus spike or no-spike plasmid control (green fluorescent protein (GFP)). BHK

446   cells were seeded in black 96-well plates and transfected the next day with 100 ng plasmid DNA

447   encoding human or mink ACE2, using polyethylenimine (Polysciences, Inc., Warrington, PA,

448   USA). After 24 hours, transfected cells were infected with VSVΔG seed particles pseudotyped

449   with VSV-G, as previously described (*16,43*). After one hour of incubating with intermittent

450   shaking at 37 °C, cells were washed four times and incubated in 2mL DMEM supplemented with

451   2% fetal bovine serum (FBS), penicillin/streptomycin and L-glutamine for 48 hours.

452   Supernatants were collected, centrifuged at 500xg for 5 minutes, aliquoted, and stored at −80 °C.

453   BHK cells previously transfected with ACE2 plasmid of interest were inoculated with equivalent

454   volumes of pseudotype stocks. Plates were then centrifuged at 1200xg at 4 °C for one hour and

455   incubated overnight at 37 °C. Approximately 18-20 hours post-infection, Bright-Glo luciferase

456   reagent (Promega Corp., Madison, WI, USA) was added to each well, 1:1, and luciferase was

457   measured. Relative entry was calculated normalizing the relative light unit for each pseudotyped

458   spike to the relative light unit average for the no-spike control.

459   *Animals*

460   Seventeen apparently healthy adult farmed mink (*Neovison vison*) were used in this

461   study: 11 were used for experimental infection and 6 were used as controls. All mink were pre-

462   screened and negative for SARS-CoV-2 using a qRT-PCR, a pan-coronavirus assay (*44*), viral

463   neutralization assay, and Aleutian disease using a lateral flow immunoassay (Scintilla

464   Development Company LLC, Bath, Pennsylvania).

465   The animals used in the infection study consisted of 9 females and two males; intake female

466   body weight range 1.04 kg – 1.47 kg, mean = 1.18 kg, male weights were 2.06 kg and 2.73. The

467   females were approximately two years of age, the males were approximately one year of age.

468   Upon arrival whole blood from all mink were screened for antibodies against SARS-CoV-2.

469   Animals were single-housed in a climate-controlled room with a fixed light-dark cycle (12-hour

470   light and 12-hour dark) for the duration of the experiment with access to food and water *ad*

471   *libitum* with enrichment that included human interaction, commercial toys, music, and treats. All

472   manipulations were done on anesthetized animals using Telazol (10-20 mg/kg administered

473   subcutaneously).

474   *Animal study*

475   Eleven animals were inoculated intratracheally (1.7 mL) and intranasally (0.15 mL per

476   naris delivered using a MAD Nasal™ Mucosal Atomization Device (Teleflex, US) for a total

477   dose of 10^5 TCID$_{50}$ delivered in 2 total mL. Animals were evaluated at least twice daily

478   throughout the study. Clinical exams (including thoracic radiographs) were performed on 0, 1, 3,

479   5, 7, 10, 14, 17, 21, 28 DPI on anesthetized animals, during which the following parameters were

480   assessed: bodyweight, body temperature, heart rate, respiratory rate, and radiographs. Clinical

481   samples collected included nasal, oral, rectal, and fur swabs, and blood. Fur swabs were

482   collected down the dorsal midline of the animal. Swabs were collected in 1mL of DMEM

483   supplemented with 2% FBS, 1 mM L-glutamine, 50 U/ml penicillin, and 50 g/ml streptomycin.

484   *Radiographs*

485   Ventrodorsal, left lateral, and right lateral thoracic radiographs were taken prior to

486   clinical exams on 0, 1, 3, 5, 7, 10, 14, 17, 21, and 28 DPI with 0 DPI being performed prior to

487   inoculation and serving as a baseline. Thoracic radiographs were taken immediately after

488   animals were anesthetized and each lung lobe was evaluated by a board-certified veterinary

489   radiologist as follows: 0 = normal lung, 1 = mild interstitial infiltrate, 2 = moderate to marked

490   unstructured interstitial pattern, 3 = <25% alveolar pattern, 4 = >25% alveolar pattern.

491   *Clinical pathology*

492   Hematology analysis was completed on a ProCyte Dx® (IDEXX Laboratories,

493   Westbrook, ME, USA) and the following parameters were evaluated: red blood cells (RBC);

494   hemoglobin (Hb); hematocrit (HCT); mean corpuscular volume (MCV); mean corpuscular

495   hemoglobin (MCH); mean corpuscular hemoglobin concentration (MCHC); red cell distribution

496   width (RDW); platelets; mean platelet volume (MPV); white blood cells (WBC); neutrophil

497   count (absolute and percentage); lymphocyte count (absolute and percentage); monocyte count

498   (absolute and percentage); eosinophil count (absolute and percentage); and basophil count

499   (absolute and percentage). Serum chemistry analysis was completed on a VetScan VS2®

500 Chemistry Analyzer (Abaxis, Union City CA) and the following parameters were evaluated:

501 glucose; blood urea nitrogen (BUN); creatinine; calcium; albumin; total protein; alanine

502 aminotransferase (ALT); aspartate aminotransferase (AST); alkaline phosphatase (ALP); total

503 bilirubin; globulin; sodium; potassium; chloride and total carbon dioxide. Clinical pathology

504 samples were evaluated by a board-certified clinical veterinarian.

505 *Histopathology*

506 Histopathology and immunohistochemistry were performed on mink tissues. Tissues

507 were fixed for a minimum of 7 days in 10% neutral-buffered formalin with 2 changes. Tissues

508 were placed in cassettes and processed with a Sakura VIP-6 Tissue Tek, on a 12-hour automated

509 schedule, using a graded series of ethanol, xylene, and PureAffin. Embedded tissues were

510 sectioned at 5um and dried overnight at 42 degrees C prior to staining. The skulls were placed in

511 Cancer Diagnostic acid free EDTA for 4 weeks and the solution was changed weekly.

512 Tissue sections were stained with hematoxylin and eosin (HE). The tissues were then

513 processed for immunohistochemistry using the Discovery Ultra automated stainer (Roche

514 Tissues Diagnostics) with a ChromoMap DAB kit (Roche Tissue Diagnostics cat #760-159).

515 Specific anti-CoV immunoreactivity was detected using SARS-CoV-2 nucleocapsid antibody

516 (GenScript Biotech, Piscataway, NJ, USA) at a 1:1000 dilution. The secondary antibody was the

517 Vector Laboratories ImPress VR anti-rabbit IgG polymer (cat# MP-6401). To detect ACE-2,

518 ACE-2 Antibody R&D Systems (catalog #AF933) was used at a 1:100 dilution with Vector

519 Laboratories ImPress anti-goat IgG polymer (Cat #MP-7405) as a secondary antibody.

520 *Virus and cells*

521 SARS-CoV-2 variant B.1.1.7 (hCoV-19/England/204820464/2020, EPI_ISL_683466;

522 designated B.1.1.7 through the manuscript) was obtained from Public Health Agency England

523   via BEI Resources. The obtained passage 2 material was propagated once in VeroE6 cells in

524   DMEM supplemented with 2% FBS, 1mM L-glutamine, 50 U/ml penicillin, and 50 g/ml

525   streptomycin. Mycoplasma testing was performed at regular intervals and no mycoplasma was

526   detected. For sequencing from viral stocks, sequencing libraries were prepared using Stranded

527   Total RNA Prep Ligation with Ribo-Zero Plus kit per manufacturer's protocol (Illumina Inc.,

528   San Diego, CA, USA) and sequenced on an Illumina MiSeq at 2 x 150 base pair reads. Low level

529   sequence variation in the stock of B.1.1.7 (nsp6/D165G/14%, nsp6/L257F/18% and

530   nsp7/V11L/13%).

531   *RNA extraction and quantitative reverse-transcription polymerase chain reaction*

532   RNA was extracted from nasal, oral, rectal, and fur swabs using the QiaAmp Viral RNA kit

533   (Qiagen Sciences, Inc., Germantown, MD, USA) according to the manufacturer's instructions

534   and following high containment laboratory protocols. Tissue samples were homogenized and

535   extracted using the RNeasy kit (Qiagen Sciences, Inc., Germantown, MD, USA) according to the

536   manufacturer's instructions and following high containment laboratory protocols. A viral sgRNA

537   specific assay was used for the detection of viral RNA (*46*). Five µL of extracted RNA was

538   tested with the Quantstudio 3 system (Thermofisher Scientific, Waltham, MA, USA) according

539   to instructions from the manufacturer. A standard curve was generated during each run using

540   SARS-CoV-2 standards containing a known number of genome copies.

541   *Viral titration*

542   Tissue sections were weighed and homogenized in 1mL of DMEM. Virus titrations were

543   performed by end point titration of 10-fold dilutions of swab media or tissue homogenates on

544   VeroE6 cells in 96-well plates. When titrating tissue homogenate, the top 3 rows of cells were

545   washed 2 times with DMEM prior to the addition of a final 100μL of DMEM. Cells were

546   incubated at 37°C and 5% CO_2. Cytopathic effect was read 6 days later.

547   *SARS-CoV-2 genome sequencing and within-host evolution*

548   Oral swabs and lung tissue samples were collected into Trizol for RNA extraction. 200

549   μL of 1-Bromo-3-chloropropane (MilliporeSigma, St. Louis, MO, USA) was added to the

550   Trizol/sample lysate, mixed, and centrifuged at 16,000 x *g* for 15 min at 4°C. RNA containing

551   aqueous phase of 600 μl was collected from each sample and aqueous phase was combined with

552   600uL of RLT lysis buffer (Qiagen, Valencia, CA, USA) with 1% beta mercaptoethanol

553   (MilliporeSigma, St. Louis, MO, USA). RNA was extracted using Qiagen AllPrep DNA/RNA

554   96-well system (Valencia, CA, USA). An additional on-column Dnase 1 treatment was

555   performed during RNA extraction. All sample processing was performed using amplicon-free

556   reagents and tools in aerosol resistant vials. RNA quality was analyzed using Agilent 2100

557   Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA samples were quantitated by

558   qRT-PCR targeting NSP5 using the AgPath-ID One-Step RT-PCR Buffer and Enzyme Mix (Life

559   Technologies, Carlsbad, CA, USA). The reactions were carried out in 20 μL reactions using

560   NSP5 forward primer (5'-CTGGCACAGACTTAGAAGGTAACTT-3'), reverse primer

561   (5'TCGATTGAGAAACCACCTGTCT-3'), fluorescent probe (5'-6FAM-

562   TTGACAGGCAAACAGCACAAGCAG-BHQ1-3') (Biosearch Technologies, Novato, CA,

563   USA). The QPCR reactions were carried out at 50 °C for 10 minutes, 95 °C for 10 minutes, 55

564   cycles of 95 °C for 15 seconds and 60 °C for 45 seconds. Data was analyzed using ABI 7900HT

565   version 2.4 sequence detection system software (Thermofisher Scientific, Waltham, MA, USA)

566   and SARS-CoV-2 genome copy (equivalent/mL) numbers were determined by absolute

567   quantitation method. Next generation libraries were generated using the TruSeq DNA PCR Free

568   Nano kit (Illumina, Inc., San Diego, CA, USA) and the ARTIC multiplex PCR genome

569   amplification protocol with the V3 primer scheme (www.protocols.io/view/ncov-2019-

570   sequencing-protocol-bbmuik6w) and libraries were sequenced on an Illumina MiSeq at 2 x 250

571   paired-end reads. The ARTIC multiplex PCR SARS-CoV-2 genome amplification protocol has

572   been widely used in viral genome sequencing during the COVID-19 pandemic and to study

573   within-host dynamics of SARS-CoV-2 (*21*).

574   To determine reproducibility of our assay, a subset of 12 samples determined to have

575   high (10^4), medium (10^3), and low (10^2) SARS-CoV-2 genome copy (equivalent/mL) numbers

576   by NSP5 qRT-PCR were selected as technical replicates. ARTIC primers and Illumina adapters

577   were trimmed, low quality bases and duplicate reads were filtered out, and mapping and variant

578   calling were completed as described in the iVar and PrimalSeq pipeline described by Grubaugh

579   *et al.*(*47*). Intrahost single nucleotide variants (iSNVs) were included in further analysis if they

580   passed the Fisher's exact test for variation above the mean error rate at that locus and had a depth

581   of coverage at or above 100X. iSNVs were called with minor allele frequency (MAF) thresholds

582   at 3% and 5% and compared against technical replicates (Supplemental SNS2). iSNVs detected

583   at 3% MAF were plotted against the SARS-CoV-2 genome copy number for each sample

584   (Supplemental SNS3A) and the number of reads mapped for each sample (Supplemental

585   SNS3B).

586   To compare variation arising in the experimentally challenged mink to variation

587   previously detected in SARS-CoV-2 circulating at mink farms, all available mink-associated

588   SARS-CoV-2 genomes were downloaded from GISAID from 01-Jan-2020 through 22-Nov-

589   2021. The resulting alignment of 1002 SARS-CoV-2 genome sequences included 999 with

590   *Neovison vison* as the host species and 3 SARS-CoV-2 sequences from the genus *Mustela*

591   (GISAID Acknowledgements, Supplemental Table SNS4).

592   *Serology*

593   Sera were heat-inactivated (30 min, 56°C). After an initial 1:10 dilution of the sera, two-

594   fold serial dilutions were prepared in DMEM. 100 $TCID_{50}$ of SARS-CoV02 variant B.1.1.7 was

595   added to the diluted sera. After a 1-hour incubation at 37°C and 5% CO_2, the virus-serum

596   mixture was added to VeroE6 cells. The cells were incubated for 6 days at 37°C and 5% CO_2 at

597   which time they were evaluated for CPE. The virus neutralization titer was expressed at the

598   reciprocal value of the highest dilution of the serum that still inhibited virus replication.

599   *Statistical analysis*

600   Statistical analysis was performed using GraphPad Version 8.4.3. Significance tests were

601   performed as indicated where appropriate with reported p-values.

602

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746   **Author contributions:**

747   Conceptualization: DRA, VJM

748   Methodology: DRA, JL, DY, JRS, CT, CC, CE, JE, SNS, VJM

749   Investigation: DRA, JL, JES, VAA, EH, MH, KCY, JRP, SNS, PWH, GS, DS, JVM

750   Visualization: DRA, JL, VAA, PWH, GS, JRS, CT, CC, NMW, SNS

751   Formal analysis: DRA, JL, DY, VAA, SNS, CM

752   Funding acquisition: VJM

753   Resources: JE

754   Supervision: VJM

755   Writing – Original Draft: DRA

756   Writing – Review and editing: DRA, SNS, JRS, VJM

757   **Competing interests:** The authors declare no competing interests.

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761   **Supplementary Materials**
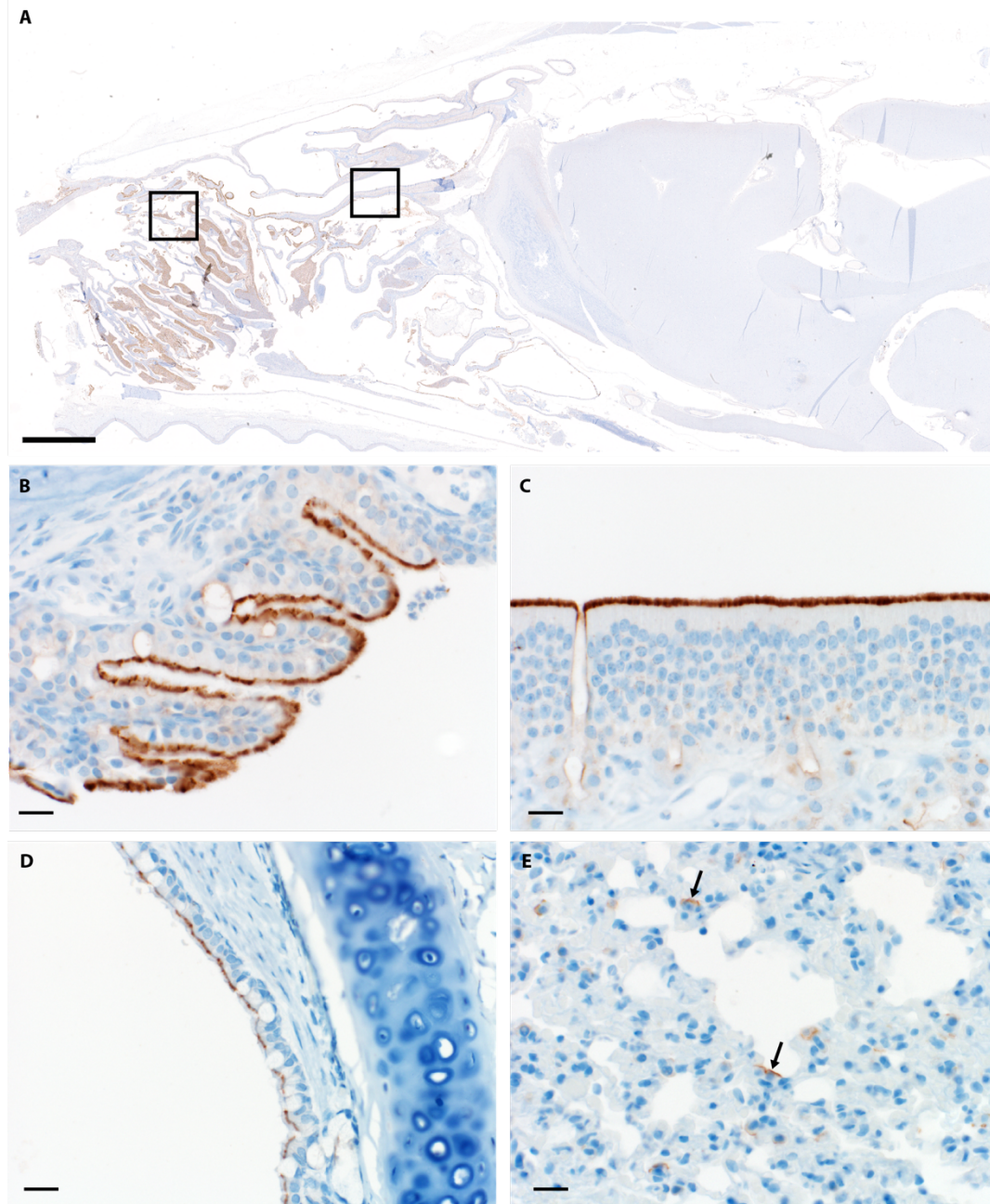
Supplemental Figure 1. ACE2 Immunoreactivity. (A) Sagittal section of skull ACE2 immunoreactivity (brown) bar = 3mm (B) Respiratory epithelium (C) Olfactory epithelium (D) Bronchiolar epithelium (E) Type 1 pneumocytes (arrows) Bars=20um

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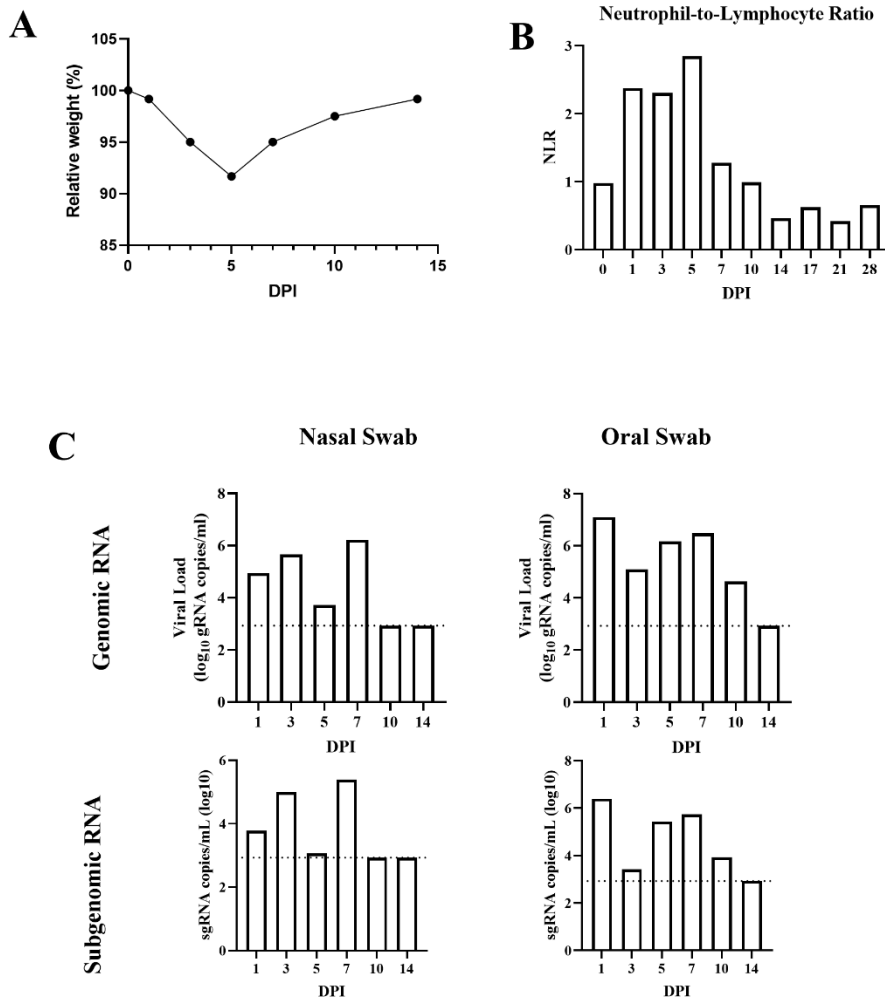
Supplemental Figure 2. Resolution of clinical disease in the surviving animal. The surviving animal was monitored for change in relative weight (A). Weight loss was most severe on 5 DPI, after which the animal began recovery. Neutrophil-to-lymphocyte ratio was monitored over time (B), with the most severe change appreciated on 5 DPI. Nasal and oral swabs were evaluated for resolution of viral shedding through genomic and subgenomic RT-PCR (C). All swabs on 14 DPI were below the limit of detectable virus.

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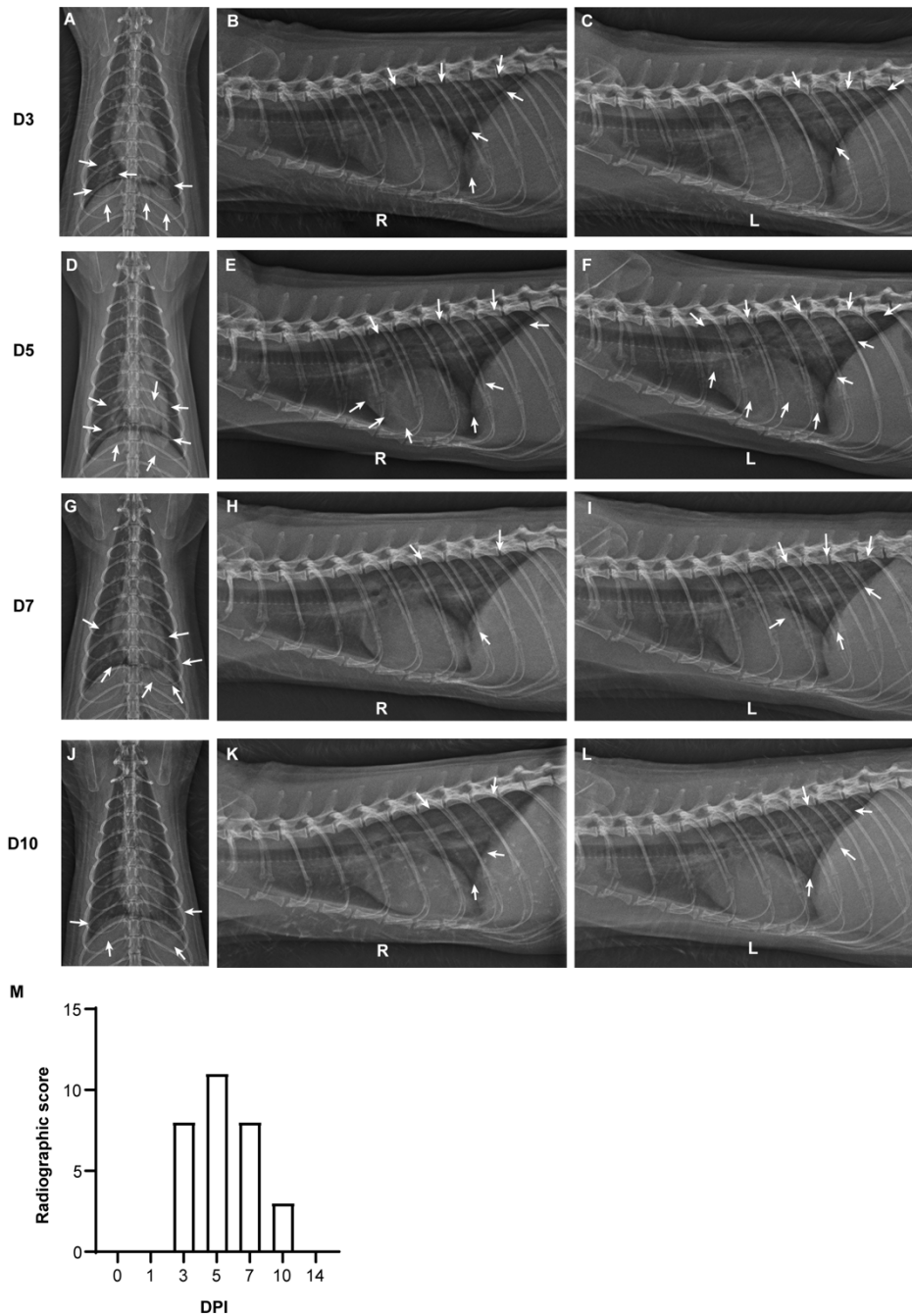
Supplemental Figure 3. Resolution of radiological disease in the surviving animal. (A-L) Dorsoventral, right lateral, and left lateral radiographs from surviving animal on 3, 5, 7, and 10 DPI. Arrows indicate pulmonary infiltrates, first visible in the left and right caudal lung lobes at 3 DPI (A-C) with additional involvement in the caudal subsegment of the left cranial lung lobe on 5 DPI (D-F). There is mild improvement in the alveolar pattern in the left and right caudal lung lobes on 7 DPI, with resolution in the caudal subsegment of the left cranial lung lobe (G-I). The pulmonary changes continued to improve by 10 DPI, with grade 2 pulmonary disease in the right caudal lung lobe with grade 1 pulmonary disease in the left caudal lung lobe consistent with improving viral pneumonia and pneumonitis (J-L).(M) Radiographic scores for surviving animal.
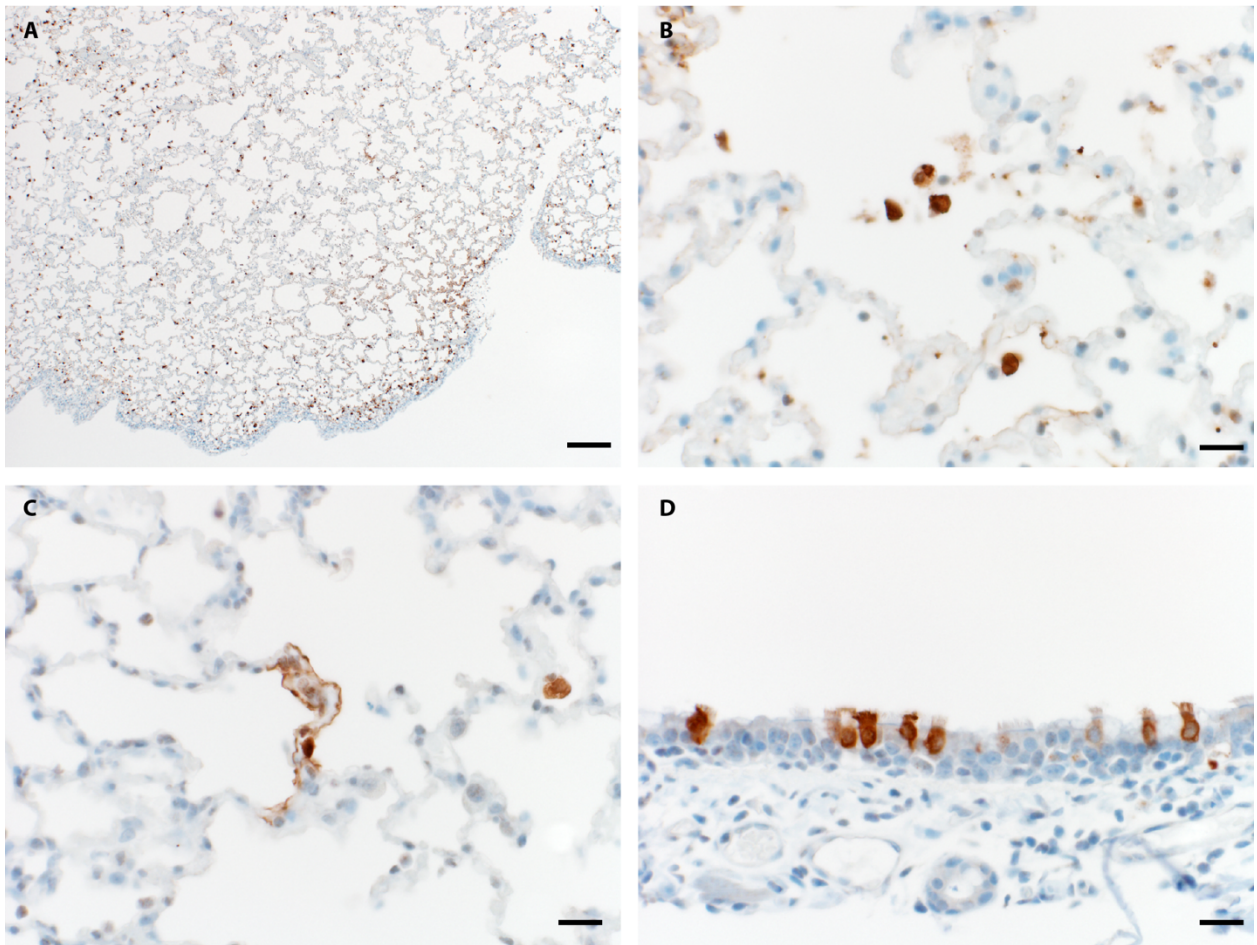
Supplemental Figure 4. SARS-CoV-2 Pulmonary immunohistochemistry. (A) Lung: Bar=200um (B) Alveolar macrophage immunoreactivity (C) Type I & II pneumocyte immunoreactivity (D) Bronchiolar epithelium immunoreactivity (brown=immunoreactive cells) C-E Bar=20um

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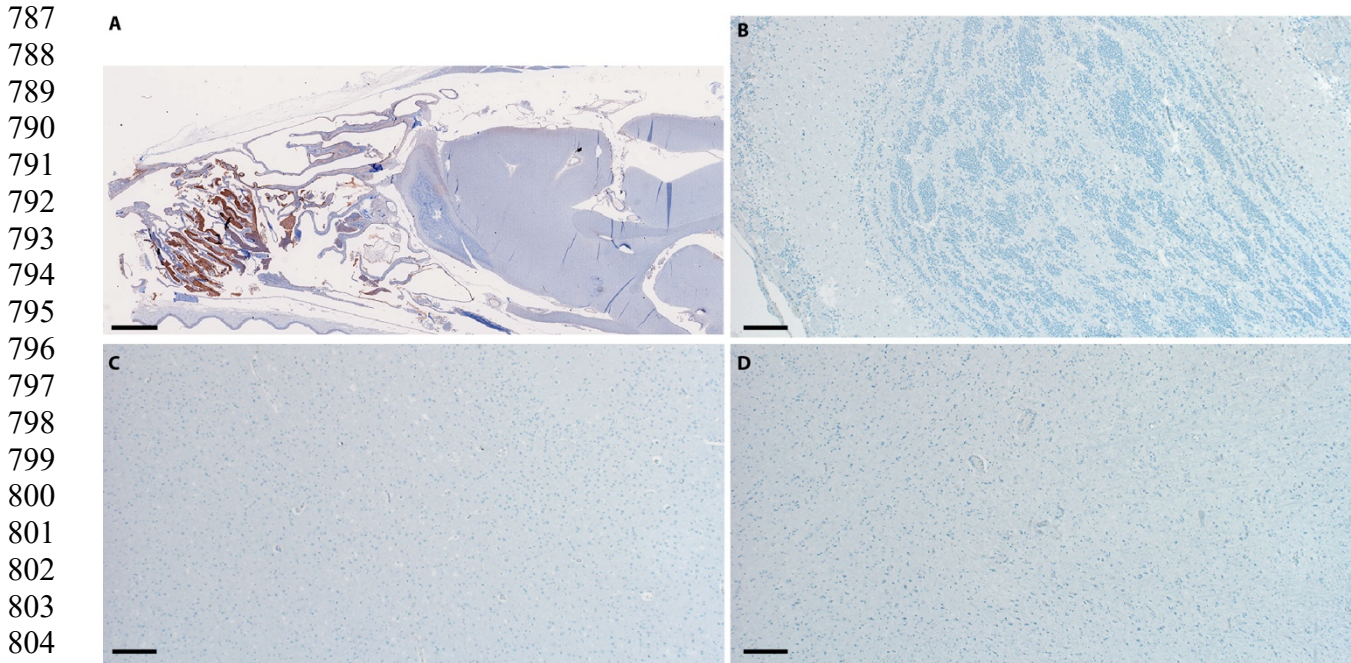
Supplemental Figure 5. Nasal turbinate and brain SARS-CoV-2 immunohistochemistry.
(A) Sagittal section of skull: Abundant nasal turbinate epithelium and exudate immunoreactivity (brown); bar = 3mm (B) Olfactory bulb: no immunoreactivity (C) Cerebral cortex: no immunoreactivity (D) Brainstem: no immunoreactivity. B-D bar = 200μm

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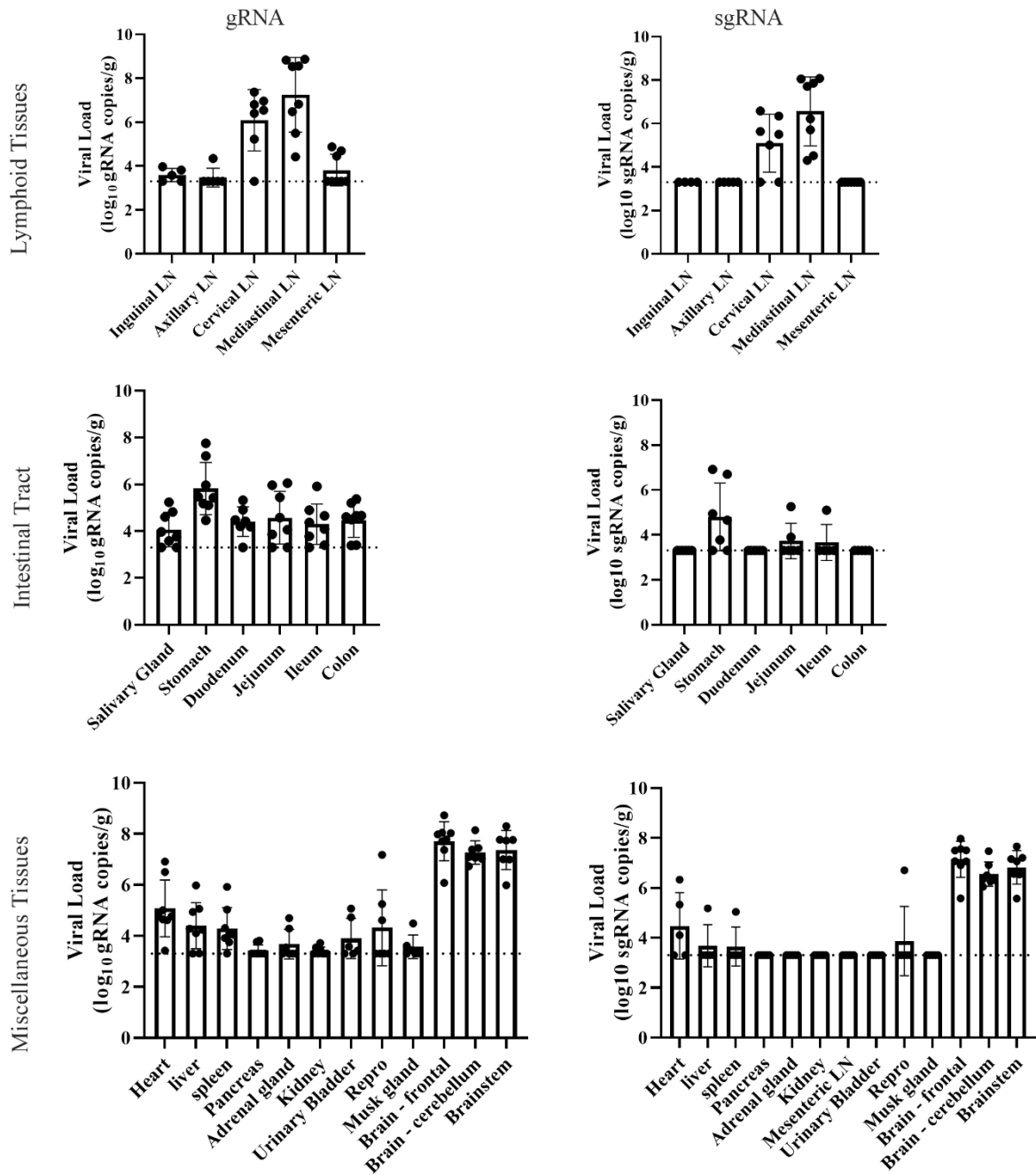
Supplemental Figure 6. Viral burden in non-respiratory tissues. Lymphoid (A), intestinal (B), and miscellaneous (C) tissues from animals euthanized on 3 DPI were evaluated for genomic and sub-genomic RNA. Graphs depict the mean and standard deviation.

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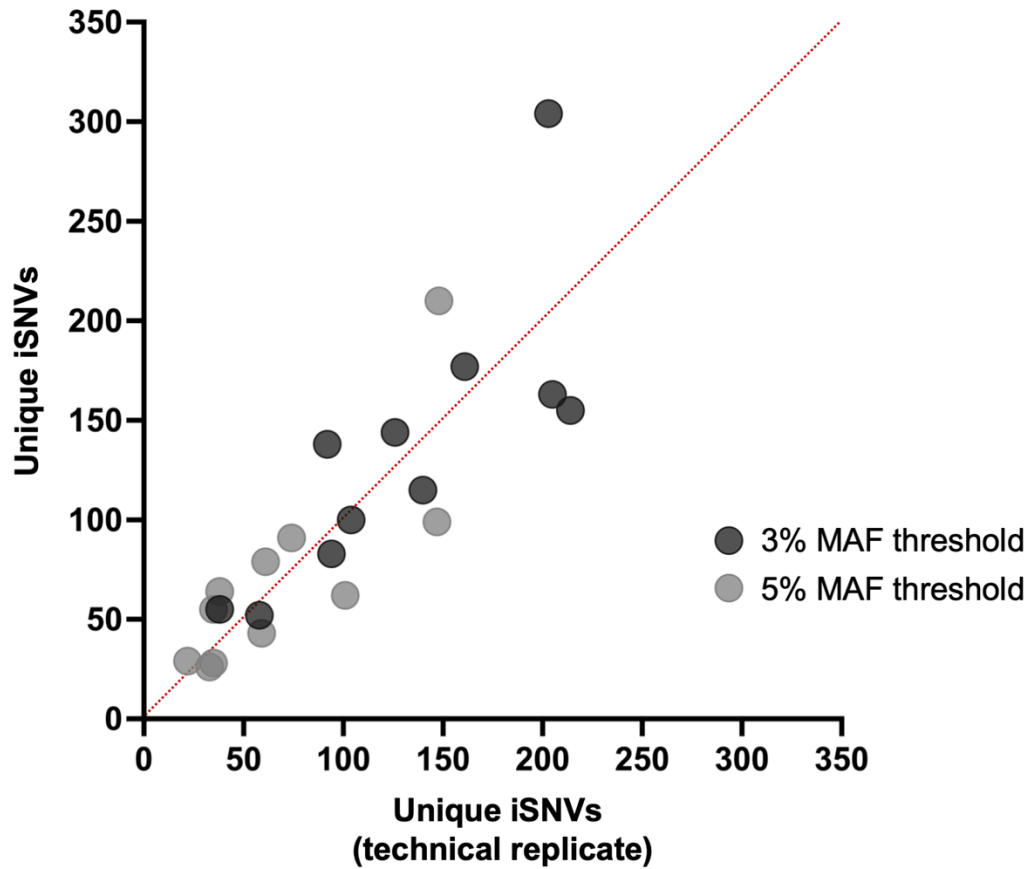
Supplemental Figure 7. **Comparison of intrahost single nucleotide variants (iSNVs) detected between technical replicates at minor allele frequencies (MAFs) of 3% and 5%.** Dots along the red line indicate perfect concordance between replicates, whereas dots farther from the red line indicate less concordance between replicates.

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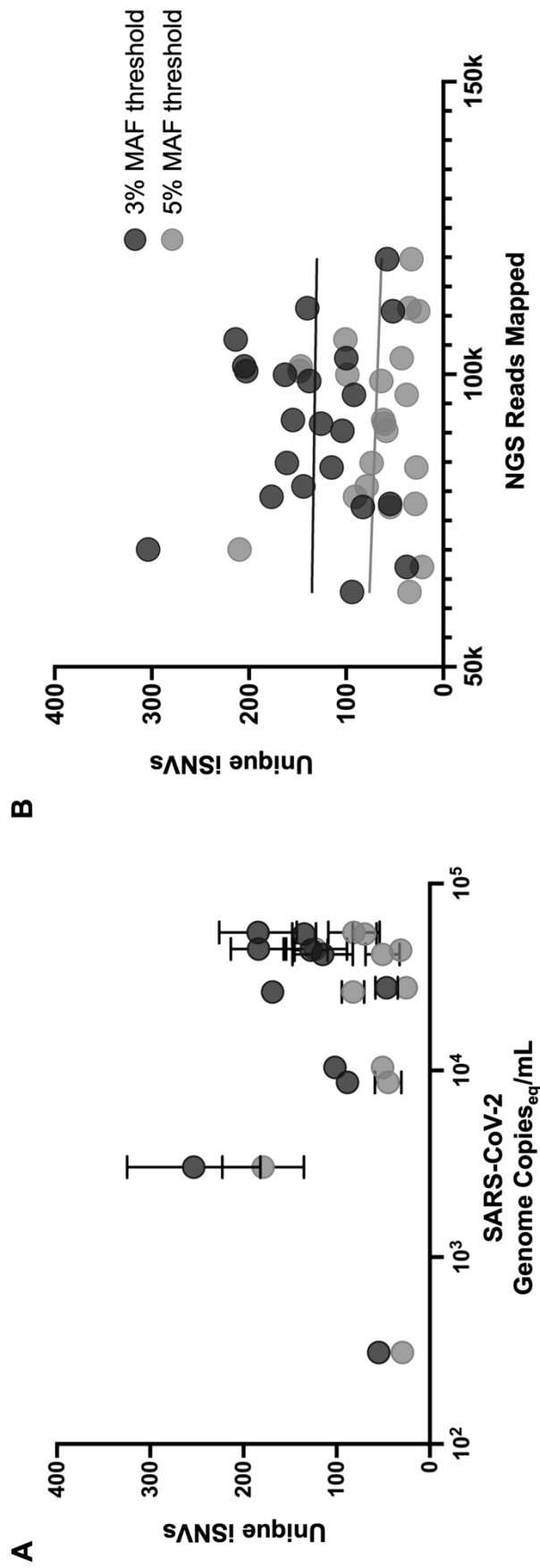
Supplemental Figure 8 (A) Unique intrahost single nucleotide variants (iSNVs) relative to SARS-CoV-2 genome copy number or (B) number of sequenced reads mapped for each sample. Data shown for all samples with minor allele frequency thresholds of 3% and 5%.

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Supplemental Table 1. Samples from mink experimentally challenged with SARS-CoV-2 that were deep sequenced for within-host evolutionary analyses.

Name	Days Post Inoculation	Sample Type	NSP5 (Ct)	SARS-CoV-2 genome copies (eq)/mL	Reads Mapped	Reads Mapped (Technical Rep)
	1	oral swab	25.2	1.82E+05	82,994	
mink01	3	lung tissue	24.8	2.34E+05	96,688	
	3	oral swab	25.4	1.59E+05	73,964	
	1	oral swab	23.5	5.48E+05	94,620	
mink02	2	lung tissue	20.5	3.53E+06	83,812	
	2	oral swab	27.1	5.48E+04	92,177	105,980
	1	oral swab	27.1	5.37E+04	80,835	91,544
mink03	3	lung tissue	20.5	3.72E+06	68,368	
	3	oral swab	25	2.08E+05	87,343	
	1	oral swab	23.9	4.16E+05	89,083	
mink04	3	lung tissue	17.9	1.90E+07	120,414	
	3	oral swab	24.1	3.72E+05	90,275	
	1	oral swab	27.5	4.18E+04	98,844	96,509
mink05	3	lung tissue	17.6	2.28E+07	95,960	
	3	oral swab	25.1	1.91E+05	82,691	
	1	oral swab	24.9	2.12E+05	82,531	
mink06	3	lung tissue	16.9	3.59E+07	83,560	
	3	oral swab	31.6	3.04E+03	70,069	100,539
	1	oral swab	23.1	6.73E+05	82,750	
mink07	2	lung tissue	25.2	1.84E+05	83,869	
	2	oral swab	29.7	1.04E+04	102,762	90,328
mink08	1	oral swab	25.8	1.24E+05	96,249	
	3	lung tissue	16.6	4.44E+07	91,707	
	1	oral swab	26	1.10E+05	105,882	
mink09	3	lung tissue	16.1	5.93E+07	70,836	
	3	oral swab	27.4	4.47E+04	99,943	101,347
	1	oral swab	27.8	3.32E+04	77,189	13 (excluded)
mink10	3	lung tissue	28.1	2.77E+04	77,862	67,088
	3	oral swab	27.4	4.41E+04	84,064	111,319
	1	oral swab	24.9	2.14E+05	66,375	
mink11	3	oral swab	28.2	2.63E+04	79,066	84,833
(survivor)	5	oral swab	35.2	3.09E+02	110,791	119,696
	7	oral swab	30	8.64E+03	77,324	62,811
Group 1	0	inoculum	24.7	2.50E+05	75,081	
Group 2	0	inoculum	24.2	3.34E+05	91,597	

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Supplemental Table 2. Acknowledgements for mink-associated sequences downloaded from GISAID.

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All Submitters of data may be contacted directly via www.gisaid.org

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EPI_ISL_641396, EPI_ISL_641397, EPI_ISL_641398, EPI_ISL_641399, EPI_ISL_641400, EPI_ISL_641401, EPI_ISL_641402, EPI_ISL_641403, EPI_ISL_641404, EPI_ISL_641405, EPI_ISL_641406, EPI_ISL_641407, EPI_ISL_641408, EPI_ISL_641409, EPI_ISL_641410, EPI_ISL_641411, EPI_ISL_641412, EPI_ISL_641413, EPI_ISL_641414, EPI_ISL_641415, EPI_ISL_641416, EPI_ISL_641417, EPI_ISL_641418, EPI_ISL_641419, EPI_ISL_641420, EPI_ISL_641421, EPI_ISL_641422, EPI_ISL_641423, EPI_ISL_641424, EPI_ISL_641425, EPI_ISL_641426, EPI_ISL_641427, EPI_ISL_641428, EPI_ISL_641429, EPI_ISL_641430, EPI_ISL_641431, EPI_ISL_641432, EPI_ISL_641433, EPI_ISL_641434, EPI_ISL_641435, EPI_ISL_641436, EPI_ISL_641437, EPI_ISL_641438, EPI_ISL_641439, EPI_ISL_641440, EPI_ISL_641441, EPI_ISL_641442, EPI_ISL_641443, EPI_ISL_641444, EPI_ISL_641445, EPI_ISL_641446, EPI_ISL_641447, EPI_ISL_641448, EPI_ISL_641449, EPI_ISL_641450, EPI_ISL_641451, EPI_ISL_641452, EPI_ISL_641453, EPI_ISL_641454, EPI_ISL_641455, EPI_ISL_641456, EPI_ISL_641457, EPI_ISL_641458, EPI_ISL_641459, EPI_ISL_641460, EPI_ISL_641461, EPI_ISL_641462, EPI_ISL_641463, EPI_ISL_641464, EPI_ISL_641465, EPI_ISL_641466, EPI_ISL_641467, EPI_ISL_641468, EPI_ISL_641469, EPI_ISL_641470, EPI_ISL_641471, EPI_ISL_641472, EPI_ISL_641473, EPI_ISL_641474, EPI_ISL_641475, EPI_ISL_641476, EPI_ISL_641477, EPI_ISL_641478, EPI_ISL_641479, EPI_ISL_641480, EPI_ISL_641481, EPI_ISL_641482, EPI_ISL_641483, EPI_ISL_641484, EPI_ISL_641485, EPI_ISL_641486, EPI_ISL_641487, EPI_ISL_641488, EPI_ISL_641489, EPI_ISL_641490, EPI_ISL_641491, EPI_ISL_641492, EPI_ISL_641493, EPI_ISL_641494, EPI_ISL_641495, EPI_ISL_641496, EPI_ISL_641497, EPI_ISL_641498, EPI_ISL_641499, EPI_ISL_641500, EPI_ISL_641501, EPI_ISL_641502, EPI_ISL_641503, EPI_ISL_641504, EPI_ISL_641505, EPI_ISL_641506, EPI_ISL_641507, EPI_ISL_641508, EPI_ISL_641509, EPI_ISL_641510, EPI_ISL_641511, EPI_ISL_641512, EPI_ISL_641513, EPI_ISL_641514, EPI_ISL_641515, EPI_ISL_641516, EPI_ISL_3050753, EPI_ISL_3050754, EPI_ISL_3050755, EPI_ISL_3050756, EPI_ISL_3050757, EPI_ISL_3050758, EPI_ISL_3050759, EPI_ISL_3050760, EPI_ISL_3050761, EPI_ISL_3050762, EPI_ISL_3050763, EPI_ISL_3050764, EPI_ISL_3050765, EPI_ISL_3050766			
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see above	Dutch COVID-19 response team	Erasmus Medical Center	Anne van der Linden; Annemiek van der Eijk; Bas Oude Munnink; Corine GeurtsvanKessel; David Nieuwenhuijse; Emmanuelle Munger; Irina Chestakova; Marion Koopmans; Marjan Boter; OH consortium; Reina Sikkema; Richard Molenkamp; on behalf of the OH consortium.
EPI_ISL_1265354, EPI_ISL_1265355, EPI_ISL_1265356, EPI_ISL_1265357, EPI_ISL_1265358, EPI_ISL_1265359, EPI_ISL_1265360, EPI_ISL_1265361, EPI_ISL_1265362, EPI_ISL_1265363, EPI_ISL_1265364, EPI_ISL_1265365, EPI_ISL_1265366, EPI_ISL_1265367			
see above	Dutch COVID-19 response team	Wageningen Bioveterinary Research	OH consortium
EPI_ISL_8144460, EPI_ISL_8144461, EPI_ISL_8144462, EPI_ISL_8527354	E. Gulbja Laboratorija	Latvian Biomedical Research and Study Centre	Alise Jakovele; Daina Pule; Davids Fridmanis; Dmitrijs Perminovs; Elina Dimina; Guntars Zarins; Irena Meistere; Ivars Silamikelis; Janis Klovins; Janis Pjalkovskis; Jurijs Perevoscikovs; Kaspars Megnis; Laila Silamikele; Lauma Freimane; Laura Ansone; Liga Birzniece; Mikus Gavars; Monta Briviba; Nikita Zrelovs; Uga Dumpis; Una Krumina; Vita Rovite
EPI_ISL_1392906	Laboratoire de la rage et de la faune sauvage, ANSES, Malzévillle.	GRAM2.0, Université de Caen Normandie / Laboratoire de Virologie, CHU de Caen / National Reference Center for Respiratory Viruses France Nord, Institut Pasteur, Paris. / Laboratoire de la rage et de la faune sauvage ANSES, Malzéville.	Angela BRISEBARRE; Astrid VABRET; Camille Capel; Elodie MONCHATRE-LEROY.; Estelle LEPERCHOIS; Etienne SIMON-LORIERE; Evelyne PICARD-MEYER; Fabiana GAMBARO; Flora DONATI; Franck BOUE; Gilles SALVAT; Januel VOUTEAU; Justine HAMEL; Marine WASNIEWSKY; Maud Vanpeene; Meriadeg AR GOUILH; Mélanie ALBERT; Méline Bizard; Nicolas NESI; Sandrine CORBET; Sophie LEVALTIER; Sylvie BEHILLIL; Sylvie VAN DER WERF; Vincent ENOUF
EPI_ISL_925307, EPI_ISL_925308, EPI_ISL_925309, EPI_ISL_925310, EPI_ISL_925311, EPI_ISL_925312, EPI_ISL_925313, EPI_ISL_925314, EPI_ISL_925315, EPI_ISL_925316, EPI_ISL_925317, EPI_ISL_925318, EPI_ISL_925319, EPI_ISL_925320, EPI_ISL_925321, EPI_ISL_925322, EPI_ISL_925323, EPI_ISL_925324, EPI_ISL_925325, EPI_ISL_925326, EPI_ISL_925327, EPI_ISL_925328, EPI_ISL_925329, EPI_ISL_925330, EPI_ISL_925331, EPI_ISL_925332, EPI_ISL_925333, EPI_ISL_925334, EPI_ISL_925335, EPI_ISL_925336, EPI_ISL_925337, EPI_ISL_925338, EPI_ISL_925339, EPI_ISL_925340, EPI_ISL_925341, EPI_ISL_925342, EPI_ISL_925343, EPI_ISL_925344, EPI_ISL_925345, EPI_ISL_925346, EPI_ISL_925347, EPI_ISL_925348, EPI_ISL_925349, EPI_ISL_925350, EPI_ISL_925351, EPI_ISL_925352, EPI_ISL_925353, EPI_ISL_925354, EPI_ISL_925355, EPI_ISL_925356, EPI_ISL_925357, EPI_ISL_925358, EPI_ISL_925359, EPI_ISL_925360			
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EPI_ISL_2834696, EPI_ISL_2834697	Michigan State University Veterinary Diagnostic Laboratory	Diagnostic Virology Laboratory, National Veterinary Services Laboratories, USDA1920 Dayton Avenue, Ames, IA 50010, USA	Emily R. Love; Kerrie M. Franzen; Mary L. Killian; Mia Torchetti; Suelee Robbe-Austerman; Tod P. Stuber
EPI_ISL_851055, EPI_ISL_851056, EPI_ISL_851057, EPI_ISL_851058, EPI_ISL_851059, EPI_ISL_851060, EPI_ISL_851061			
see above	National Food and Veterinary Risk Assessment Institute	Lithuanian University of Health Sciences, Molecular cardiology lab.	Algirdas Malakauskas; Arnoldas Pautienius; Arunas Stankevicius; Astra Vitkauskiene; Dovydas Gecys; Ingrida Olendraitė; Kamile Tamusauskaite; Laura Pareckaite; Lukas Zemaitis; Vaiva Lesauskaite
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EPI_ISL_1490748	Pharmacy Department, Facultad de CC Salud, UCH-CEU University	Pharmacy Department, Facultad de CC Salud, UCH-CEU University	Miguel Padilla-Blanco
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see above	Statens Serum Institute	Statens Serum Institute	A.S.; Afaro-Nunez, A.; Belsham; C.S.; Fonager, J.; Foomsgaard, A.; G.J. and Botner, A.; Halasa, T.; Hammer; Jorgensen; Lohse, L.; M.L.; M.W.; Mundbjerg, K.; Quaade; Rasmussen; Rasmussen, M.; Rosenstierne; Strandbygaard, B.; T.B.
EPI_ISL_2467943	The Laboratory of Biosafety with Pathogens Collection The Republican Research and Practical Center for Epidemiology	Laboratory for HIV and opportunistic infections diagnosis The Republican Research and Practical Center for Epidemiology and Microbiology (RRPCEM)	Anatoli Krasko; Artur Akhremchuk; Elena Gasich; Ilarion Kupryianav; Iryna Subotsina; Kirill Bulda; Larysa Radzienava; Leonid Valentovich; Olga Klimovich; Olga Saleuskaya; Sergei Semenov

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Supplementary Table 3: Long range PCR primers for amplification of ACE2.

Primer	Sequence (5' - 3')
Amplicon 1-F	CCCAACCCAAGTTCAAAGGCTGATGAGAGAGAA
Amplicon 1-R	CTTCATGTTTAGCTATTTGACAAAGGGCTTCTT
Amplicon 2-F	GGACACATCCAGTATGACATGGCATATG
Amplicon 2-R	TGGGGGAGTACAAGATTTAAGAGATTGGGAA

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