1   **Manuscript Number**:

2   **Article Summary Line:** Raccoon dogs are susceptible to and efficiently transmit SARS-CoV2

3   and may serve as intermediate host

4   **Running Title:** Susceptibility of raccoon dogs for SARS-CoV-2

5   **Keywords:** Raccoon dog, COVID-19, SARS-CoV-2, susceptibility, transmission, intermediate

6   host

7   **Title:** Susceptibility of raccoon dogs for experimental SARS-CoV-2 infection

8   Conrad M. Freuling[1], Angele Breithaupt[1], Thomas Müller, Julia Sehl, Anne Balkema-

9   Buschmann, Melanie Rissmann, Antonia Klein, Claudia Wylezich, Dirk Höper, Kerstin

10   Wernike, Andrea Aebischer, Donata Hoffmann, Virginia Friedrichs, Anca Dorhoi, Martin H.

11   Groschup, Martin Beer[2], Thomas C. Mettenleiter[2]

12   **Affiliations:**

13   Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany

14   [1] Authors contributed equally to this work

15   [2] Corresponding authors

16   Address for correspondence:

17   Martin Beer, Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493

18   Greifswald - Insel Riems, Germany, Email: Martin.Beer@fli.de, Telephone: +49 38351 7-1200,

19   Fax: +49 38351 7-1226 and Thomas C. Mettenleiter, President, Friedrich-Loeffler-Institut,

20   Südufer 10, 17493 Greifswald - Insel Riems, Germany, Email: ThomasC.Mettenleiter@fli.de,

21   Telephone: +49 38351 7-1250, Fax: +49 38351 7-1151

22   **Abstract**

23   Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in China at the end of

24   2019, and became pandemic. The zoonotic virus most likely originated from bats, but definite

25   intermediate hosts have not yet been identified. Raccoon dogs (*Nyctereutes procyonoides*) are

26   kept for fur production, in particular in China, and were suspected as potential intermediate host

27   for both SARS-CoV6 and SARS-CoV2. Here we demonstrate susceptibility of raccoon dogs for

28   SARS-CoV-2 infection after intranasal inoculation and transmission to direct contact animals.

29   Rapid, high level virus shedding, in combination with minor clinical signs and pathohistological

30   changes, seroconversion and absence of viral adaptation highlight the role of raccoon dogs as a

31   potential intermediate host. The results are highly relevant for control strategies and emphasize

32   the risk that raccoon dogs may represent a potential SARS-CoV-2 reservoir. Our results support

33   the establishment of adequate surveillance and risk mitigation strategies for kept and wild

34   raccoon dogs.

35   **Text**

36   Coronaviruses can infect a wide variety of animals, and are responsible for human

37   diseases including severe acute respiratory syndromes (SARS). Both SARS coronavirus (SARS-

38   CoV) (*1, 2*) and Middle East Respiratory Syndrome coronavirus (MERS-CoV) (*3, 4*), are ß-

39   coronaviruses and presumably originate from bats (*5*). They likely adapted to other reservoir

40   hosts like Asian palm civets (*Paradoxurus hermaphroditus*) (*6*) and dromedary camels (*Camelus*

41   *dromedarius*) (*7*). Natural SARS-CoV infections were also detected in raccoon dogs

42   (*Nyctereutes procyonoides*) which, among other candidate species, have been discussed as a

43   possible intermediate host for the first SARS-pandemic of 2002/2003 (*8*).

44   The current SARS-CoV-2 pandemic started from Wuhan, China, at the end of 2019.

45   Close relatives to SARS-CoV-2 were found in bats (*9*), and pangolins (*Pholidota spp*) (*10, 11*).

46   Furthermore, spill-over infections to different carnivores (dogs, cats, lions, tigers and minks)

47   were reported (*12, 13*). However, whether the pandemic started by a direct transmission of the

48   SARS-CoV-2 ancestor from bats to humans or via an intermediate mammalian host with further

49   adaptation, is still under debate (*14*). For both, SARS-CoV and MERS-CoV, intermediate hosts

50   played a crucial role in transmission to humans. However, no definite intermediate host for

51   SARS-CoV-2 has been identified up to now (*14*), but animal species like pangolins, palm civets,

52   or raccoon dogs are discussed (*15–17*). Although the pandemic is driven by direct human-to-

53   human transmission, case studies demonstrate that anthropo-zoonotic infections occurred by

54   contact of infected humans with companion animals and farmed minks kept for fur production in

55   the Netherlands, Denmark and Spain (*13, 18, 19*). There is also evidence for zoo-anthroponotic

56   infection of humans (*13*).

57   Natural infections of raccoon dogs with SARS-CoV were reported (*8*), indicating a

58   potential role in the previous SARS-CoV epidemic. In fact, 14.14 million captive raccoon dogs

59   held in China for fur production (*20*) represent 99% of the global share (Figure. S3A). However,

60   experimental infections of these animals with SARS-CoV or SARS-CoV-2 under controlled

61   conditions and serologic surveillance of kept or wild raccoon dogs have not been documented.

62   Since SARS-CoV and SARS-CoV-2 employ the same receptor molecule ACE2 for

63   contact with the receptor-binding-domain (RBD) of the spike (S) protein (*21*), a similar range of

64   susceptible host species can be assumed. Molecular studies indicate that the ACE2 proteins of

65   raccoon dogs can also serve as an efficient receptor for SARS-CoV (*22)* and SARS-CoV-2 (*15).*

66   Following a previously established study design (*23)* (Figure. 1A), we tested the

67   susceptibility of raccoon dogs to SARS-CoV-2. Nine animals were challenged by intranasal

68   inoculation of 10^5 TCID50 SARS-CoV-2 2019_nCoV Muc-IMB-1, and 3 additional animals

69   were introduced at one day post-infection (dpi) to evaluate direct viral transmission.

70   **Methods**

71   **Study design**

72   Fourteen adult, male (n=4) and female (n=10) raccoon dogs originating from a

73   commercial farm were used. All animals were tested negative by RT-qPCR and antibody tests

74   (ELISA, indirect immunofluorescence assay iIFAT, virus neutralization test VNT) for SARS-

75   CoV-2 prior to the experiment. All raccoon dogs had been vaccinated against distemper, adeno-

76   and parvovirus (Eurican® SHP, Merial, France). Animals were kept in individual stainless-steel

77   cages (1.5m x 0.95m x 2.0m) in four separate segments at 20°C room temperature, 60-80%

78   humidity and a 12hr/12hr (35% dimming during night modus) lighting control within a fan

79   forced draught ventilation equipped BSL3** animal facility at the Friedrich-Loeffler-Institut

80   (FLI). Water was offered ad libitum. Animals were fed daily with 400 gr commercially produced

81   feed for farmed foxes and raccoon dogs (Schirmer und Partner GmbH Co KG, Germany;

82   Michael Hassel GmbH, Langenargen, Germany). The diet was supplemented with vitamins,

83   minerals and items like one-day old chickens as described before (*24).* The general health status

84   of all animals, feed uptake and defecation were recorded daily. The body weight and temperature

85   of all animals were measured prior to inoculation and at days 2, 4, 8, 12, 16, 21, and 28 pi.

86   The outline of the experiments with an observation period of 28 days is depicted in

87   Figure 1. Nine raccoon dogs (3 males, 6 females) were infected intranasally with 10^5 TCID$_{50}$

88   SARS-CoV-2 2019_nCoV Muc-IMB-1. The inoculum of 2x1ml was administered to both

89   nostrils using a pipette. To test viral transmission by direct contact, three naïve sentinel animals

90   (all female) were added 24 hours post inoculation. Nasal, oropharyngeal and rectal swabs were

91   taken at 2, 4, 8, 12, 16, 21 and 28 days post infection (dpi), blood was taken at 4, 8, 12, 16, 21

92   and 28 dpi. Two animals each were sacrificed at day 0 (control #1, #2) day 4 (animals #1, #2),

93   day 8 (animals #3, #4) and day 12 pi (animals #5, #6). The remaining inoculated animals

94   (animals #7-9) and the contacts (animals #10-12) were euthanized 28 dpi. All animals were

95   subjected to autopsy for macroscopic evaluation and tissue sampling.

96   The animal experiments were evaluated and approved by the ethics committee of the State Office

97   of Agriculture, Food Safety, and Fishery in Mecklenburg – Western Pomerania (LALLF M-V:

98   LVL MV/TSD/7221.3-2-010/18-12).

99   **Virus and cells**

100   The virus was propagated once in Vero E6 cells in a mixture of equal volumes of Eagle

101   MEM (Hanks' balanced salts solution) and Eagle MEM (Earle's balanced salts solution)

102   supplemented with 2mM L-Glutamine, nonessential amino acids, adjusted to 850 mg/L,

103   NaHCO3, 120 mg/L sodium pyruvate, 10% fetal bovine serum (FBS), pH 7.2. No contaminants

104   were detected within the virus stock preparation by metagenomic analysis employing previously

105   published high throughput sequencing procedures Ion Torrent S5XL instrument *(25, 26)* and the

106   sequence identity of the passaged virus (study accession number: PRJEB39640) was confirmed.

107   The virus was harvested after 72 h, titrated on Vero E6 cells and stored at -80°C until further use.

RNA extraction and detection of SARS-CoV-2

108

109   Total RNA from nasal, oropharyngeal and rectal swab samples, fecal samples as well as

110   from tissues taken at autopsy were extracted using the NucleoMagVet kit (Macherey&Nagel,

111   Düren, Germany) according to manufacturer's instructions. Tissues were homogenized in 1 ml

112   cell culture medium (see above) and a 5 mm steel bead in a TissueLyser (Qiagen, Hilden,

113   Germany). Fecal samples were vortexed in sterile NaCl and the supernatant was sterile filtered

114   (22µm) after centrifugation. Swab samples were transferred into 1 ml of serum-free tissue

115   culture media and further processed after 30 min shaking. SARS-CoV-2 RNA was detected by

116   an E-gene based RT-qPCR (*27*) using the AgPath-ID-One-Step RT-PCR kit (Thermo Fisher

117   Scientific, Waltham, Massachusetts, USA) in a volume of 12.5 µl including 1 µl of ß-Actin-

118   mix2-HEX as internal control and 2.5 µl of extracted RNA. The reaction was performed as

119   described before (*23*). Nasal swab samples from raccoon dog #2 (2 dpi) and from contact animal

120   #10 (8 dpi) were subjected to high-throughput sequencing and compared to the inoculum (study

121   accession number: PRJEB39640) by employing previously published high throughput

122   sequencing procedures using Ion Torrent S5XL instrument (*25, 26*).

Detection of SARS-CoV-2 reactive antibodies

123

124   Serum samples collected throughout the study were tested for the presence of SARS-CoV-2

125   reactive antibodies by indirect immunofluorescence assay (iIFA), virus neutralization test (VNT)

126   as described before (*23*). For ELISA, medium-binding ELISA plates (Greiner Bio-One GmbH,

127   Germany) were coated with SARS-CoV-2 RBD (RBD-SD1 domain, amino acids 319 – 519 of the

128   SARS-CoV2 Spike ectodomain, for details see Appendix). The sera were diluted 1:100 in TBST

129   and incubated on the coated and uncoated wells for 1h at room temperature followed by three

130   washes using TBST. The saliva samples were used undiluted. Reactivity was shown by adding a

131   multi species conjugate (SBVMILK; IDvet, France) diluted 1:80 (serum) or 1:10 (saliva). After an

132   incubation period of 1 h at room temperature, the plates were washed again and

133   Tetramethylbenzidine (TMB) substrate (IDEXX, Switzerland) was added. The ELISA readings

134   were taken at a wavelength of 450 nm on a Tecan Spectra Mini instrument (Tecan Group Ltd,

135   Switzerland). The measurements were normalized to the respective samples tested on wells treated

136   only with the coating buffer.

137   For comparison, sera were also tested in a newly developed commercial SARS-CoV-2 sVNT (*28)*.

138   Briefly, 1:10 serum dilutions were incubated for 30 min at 37°C with HRP-coupled RBD before

139   transferring the samples to the capture plate pre-coated with the human ACE2 protein. After 15

140   min incubation at 37°C, plates were washed four times. TMB substrate was added and the plate

141   was incubated at room temperature for 15 min before stopping the reaction and reading the optical

142   density (OD) at 450 nm. Percent inhibition was calculated as (1- OD sample / OD negative control)

143   x 100.

144   **Identification of SARS-CoV-2 RBD-specific immunoglobulins**

145   SARS-CoV-2 specific immunoglobulins (Ig) were comparatively investigated in sera and saliva

146   of raccoon dogs by ELISA using exactly the same SARS-CoV-2 RBD-SD1 antigen-coated plates,

147   serum dilutions, washing and dilution buffers, TMB substrate, incubation periods and ELISA-

148   Reader as described above. After the incubation of the sera or saliva and the following washing,

149   dog-specific, horseradish-peroxidase (HRP) labelled Ig antibodies (goat-α-dog-IgA 1:1,000 for

150   saliva and 1:5,000 for serum; goat-α-dog-IgM1 1:15,000; goat-α-dog-IgG, goat-α-dog-IgG1, goat-

151 α-dog-IgG2 all 1:20,000; Bethyl Laboratories INC) were added and incubated for 1h at RT.

152 Antibodies were diluted in TBST.

Virus titration

154 Virus titer used for infection experiments was confirmed by titration on Vero E6 cells (Biobank

155 Friedrich-Loeffler-Institut, catalogue N° 0929) and evaluation of CPE after 5 days. RT-qPCR

156 positive swabs and tissue samples were titrated on Vero E6 cells as well

Autopsy, histopathology, immunohistochemistry

158 Full autopsy was performed on all animals under BSL3 conditions. A broad spectrum of tissues

159 was collected and fixed in 10% neutral-buffered formalin and trimmed for paraffin embedding,

160 including the upper and lower respiratory tract, the gastro-intestinal tract, the urinary tract, brain,

161 and main parenchyma (see appendix for details). Tissues were embedded in paraffin, and 3 μm

162 sections were stained with hematoxylin and eosin for light microscopical examination. For SARS-

163 CoV-2 antigen detection was performed as described (*25*). Evaluation and interpretation of

164 pathology data were performed by a board-certified pathologist (DiplECVP). The severity of

165 lesions and the distribution of SARS-CoV-2 antigen was recorded on an ordinal scoring scale with

166 scores 0 = no lesion/antigen, 1 = rare, affected cells/tissue <5% per slide; 2 = multifocal, 6-40 %

167 affected; 3 = coalescing, 41-80% affected; 4 = diffuse, >80%affected.

Statistical information

169 All data were analyzed and visualized using GraphPad Prism Version 7.0 (GraphPad Software,

170 San Diego, CA, USA). No statistical methods were used.

171

Results

172

173   Inoculation led to productive infection in six out of nine exposed animals. Based on the

174 lack of viral RNA detection throughout the observation period of 28 days, we concluded that

175 infection of animals #4, #8 and #9 failed (Figure 1, panel B). While several animals showed

176 reduced overall activity at 4 dpi (animal #4, #5, #10), none of the exposed and contact animals

177 showed any obvious clinical sign of infection until the termination of the experiment. In particular,

178 neither increase in body temperature nor weight loss were observed.

179   Next, we examined the presence of viral RNA and infectious virus in nasal, oropharyngeal

180 and rectal swab samples as well as in feces by quantitative reverse transcription PCR (RT–qPCR)

181 and titration on Vero E6 cells. Raccoon dogs started to shed virus already at 2 dpi in nasal and

182 oropharyngeal swabs (Figure 2, panels A, B). While infectious virus was isolated from individual

183 animals up to 4 dpi (Figure. 2B), viral RNA was present in nasal swabs up to 16 dpi (animal #7,

184 Figure 2, panel C). Viral genome loads were highest in nasal swabs (mean genome copies

185 Log10/ml: 3.2, min: 1.0, max: 6.45), followed by oropharyngeal swabs (2.9; 0.54-4.39) and rectal

186 swabs (0.71; 0.31-1.38, Figure 2, panel A). Virus titrations revealed the same trend, with the

187 highest viral titres of up to 4.125 Log10 TCID50/ml in nasal swabs at 2 dpi. Infectious virus was

188 never isolated from rectal swabs. In general, virus isolation failed above quantification cycle (Cq)-

189 values of around 27 (Appendix Figure 1).

190   Virus was transmitted to two of three contact animals (#10, #11) (Figure 1, panel B, Figure

191 2, panel C). One contact raccoon dog (#12) remained negative due to the fact that both cage

192 neighbors (#8, #9, Figure 1, panel B), did not shed virus after inoculation. In contact animals, viral

193 RNA indicative of infection was first detected at 8 dpi (7 days post contact (dpc), #10). As in the

194   inoculated animals, excretion in contact animals was mainly via nasal secretions and lasted until

195   16 dpi (15 dpc) and virus isolation yielded viral titers of 1.625 Log10 $TCID_{50}$/ml in nasal swabs

196   of one contact animal (#10) at 8 dpi (7 dpc).

197   Tissues and body fluids of euthanized animals were tested for the presence of SARS-CoV-

198   2 RNA and replicating virus at day 4, 8, 12, and 28 pi (Figure 2, panel D). Highest viral genome

199   loads of up to 4.87 Log10 genome copies per ml were observed in samples from the oro-nasal

200   cavity, whereas only minute amounts were sporadically identified in other organs. The caudal,

201   olfactory region of the oro-nasal cavity in general yielded higher viral genome loads than the

202   cranial, respiratory region. Infectious virus could be cultivated from the nasal conchae of animals

203   #1 (2.86 Log10 $TCID_{50}$/ml) and #2 (1.63 Log10 $TCID_{50}$/ml). Of note, none of the lung samples

204   was positive for viral RNA, nor did any of the animals demonstrate viremia. Both animals

205   investigated at 4 dpi had viral RNA in samples of the CNS with low genome loads (max 2.95

206   Log10 genome copies/ml), but cerebrospinal fluid was negative in all tested animals.

207   At autopsy, no gross lesions were recorded that could be assigned to the SARS-CoV-2-

208   infection. However, histopathology identified mild rhinitis at 4, 8, and 12 dpi (animals #1-3, #5,

209   #6). The olfactory, caudal region of the nasal cavity was more consistently affected compared to

210   the respiratory, cranial region and included degeneration, necrosis and loss of the respiratory and

211   olfactory epithelium, presence of intraluminal cellular debris, degeneration and necrosis of the

212   submucosal glands, mucosal edema, endothelial swelling and acute, submucosal hemorrhage

213   (Figure 2, panels E-G). At 4 dpi, mainly neutrophils with fewer macrophages and lymphocytes

214   were found, later lesions showed predominantly lymphocytes and fewer neutrophils and

215   macrophages. Mucosal coagulative necrosis with early re-epithelization and granulation tissue

216   formation was present in one case (Figure 2, panel G, 8 dpi). At 28 dpi, one infected (#7) and one

217 contact animal (#10) showed lesions indicative for previous viral replication sites in the nasal

218 conchae. Viral RNA was still present, but no viral antigen was found (Appendix Figure 2).

219   Immunohistochemistry verified the presence of viral antigen only in the nasal conchae.

220 Lesion associated antigen was found to be oligofocal at day 4 (animal #1, #2) in the respiratory

221 and olfactory epithelium and to a lesser extent at day 8 (animal #3) only in the olfactory epithelium

222 (Figure 2, panels H-J). No viral antigen could be found at 12 dpi and 28 dpi, and neither

223 histopathologic lesions nor viral antigen were detected in animal #4 (8 dpi) in the nasal cavity. All

224 other tissues tested negative for SARS-CoV-2 antigen.

225   SARS-CoV-2-specific antibodies were detected in all infected animals at 8 dpi as shown

226 by ELISA (Figure 3, panels A-G) and iIFAT (> 1:64, Table 1). Titers increased up to 1:1024,

227 detected at 28 dpi via iIFAT (animal #7). Neutralizing antibodies (VNA) were observed in two of

228 the inoculated animals (#6, #7) as early as 8 dpi (#6, 1:5.04, Tab. 1). The highest VNA titer was

229 1:12.7 (#6, day 12; #7 day, 28). Interestingly, animal #7 showed fluctuating iIFAT titers, but

230 demonstrated a consistent increase in VNA titers until termination of the experiment (1:12.7, 28

231 dpi). A similar pattern was observed in a surrogate assay mimicking virus neutralization (sVNT).

232 Using the RBD of the SARS-CoV-2 spike-protein we further characterized antibody responses in

233 an in-house ELISA (Figure 3, panels B-G, panel I). Anti-RBD IgM and IgG levels peaked at 8 and

234 12 dpi, respectively. A similar kinetics was observed in the infected contact animals #10 and #11.

235 RBD-specific IgG2 patterns were highly similar to those of total IgG and total RBD antibodies.

236 IgG2 antibodies with high neutralizing capacity had also been reported in dogs and their abundance

237 correlates with neutralizing capacity (26) (Table 1, animals #5 and 6, 8 dpi). Although the amount

238 of IgA in serum was limited (Figure 3, panel F), a similar trend as detected for the overall serum

239 antibody levels was observed (Figure 3, panel G), e.g. with animal #6 having the highest values,

240   and contact animals #10 and #11 reaching peak levels at later time points. RBD-specific antibodies

241   were also detected in saliva samples 8 and 12 dpi (Figure 3, panel H-I) from animals that developed

242   serum antibodies.

243   To test whether viral adaptions occurred during infection of raccoon dogs with this human

244   SARS-CoV-2 isolate, we performed high throughput sequencing of SARS-CoV-2 re-isolated from

245   nasal swabs of infected raccoon dog #2 at 2 dpi and contact animal #10 at 8 dpi yielding 100%

246   identity to the inoculum (2019_nCoV Muc-IMB-1).

247   **Discussion**

248   The present experimental study demonstrates that raccoon dogs are susceptible to SARS-

249   CoV-2 infection and transmit the virus to contact animals.   Six out of nine animals were

250   successfully infected by intranasal inoculation. The susceptibility of raccoon dogs thus appears

251   similar to Rousettus bats (*Rousettus aegyptiacus*) and slightly lower than ferrets (*Mustela putorius*

252   *furo*) (*23*) (*23*).   Virus shedding in nasal and oropharyngeal swabs of raccoon dogs resulted in

253   successful onward transmission of SARS-CoV-2 to two out of three contact animals as has been

254   observed for other animal species with direct cage neighbors (*23, 29–31*).

255   Increasing evidence supports the potential of several carnivore species to become infected

256   by SARS-CoV-2 as a result of anthropo-zoonotic transmission (*13*), possibly leading to re-

257   infections of humans (*13*). Therefore, wild carnivore species whether free-living or held in

258   captivity should also be considered as intermediate hosts. With China's substantial contribution to

259   the global fur production of > 50 million animals per annum (*20*) (Appendix Figure3, panel A), it

260   is conceivable that raccoon dogs may have played a hitherto unexplored role in the development

261   of the pandemic, particularly considering the very mild signs of infection, efficient replication and

262 transmission, and genetic stability. These environments with close contact between animals and

263 an obvious interface with humans support SARS-CoV-2 transmission as was seen in several large

264 mink farm outbreaks in The Netherlands, Denmark and Spain (*13, 19, 32, 33*).

265 No obvious clinical signs could be observed, which is in line with experimental studies in

266 other carnivores, i.e. adult cats (Felis catus) and ferrets that showed productive SARS-CoV-2

267 infection with no, or only mild clinical signs (*23, 31*). By prominent nasal virus shedding in the

268 absence of symptoms, raccoon dogs present a picture of infection resembling asymptomatic

269 infections in other animals reflected by restriction of virus replication to the upper respiratory

270 tract, substantiating the role of the nasal cavity in infection as shown for other animal species (*23,*

271 *31)*, as well as the majority of human cases (*34*). Except for a mild rhinitis associated with the

272 presence of viral antigen in the nasal mucosa, no other infection-related histopathological changes

273 were observed. However, the absence of viral genome, pathohistological changes or viral antigen

274 in the lungs of infected animals argue against raccoon dogs as a model for pulmonary manifestation

275 of COVID-19.

276 The serological results suggest that the induction of SARS-CoV-2 specific VNA in raccoon

277 dogs is reduced compared to ferrets but comparable to Egyptian fruit bats (*23*). A delayed

278 production of VNAs cannot be excluded, but appears unlikely against the dynamic increase of the

279 measured ELISA antibodies. A mucosal immune response to SARS-CoV-2, i.e. antibodies in

280 saliva were detected in raccoon dogs already 12 dpi (Figure 3), supporting the use of saliva as an

281 early and non-invasive sample for epidemiological studies (*35*). The limited presence of viral

282 antigen in infected raccoon dogs at 4 dpi and the rapid decrease in viral loads prior to the

283 development of measurable humoral immunity indicates that innate immune responses including

284   interferon, mucus movement and epithelial cell turnover may play a prominent role in reducing

285   infection.

286   High throughput sequencing of SARS-CoV-2 re-isolated from nasal swabs of infected

287   raccoon dogs and contact animals yielded 100% identity to the inoculum (2019_nCoV Muc-IMB-

288   1), demonstrating that no mutations occurred during virus replication in raccoon dogs. This is in

289   contrast to findings in infected ferrets where two nonsynonymous single nucleotide exchanges

290   after the ferret passage were identified (25). This may indicate that the virus is already sufficiently

291   adapted to this putative intermediate host.

292   In conclusion, further evidence is required from research about the origin of this pandemic.

293   Large-scale sero-epidemiological studies in susceptible animals are needed. Historical samples

294   collected prior to the epidemic are of particular importance and should preferentially also include

295   a time series of archived samples. Further, affected fur farms may serve as reservoirs for SARS-

296   CoV-2 and this risk should be mitigated by efficient and continuous surveillance. While SARS-

297   CoV-2 might be controlled in holdings by very strict measures (*13, 32)*, a spill-over into

298   susceptible wildlife species, in particular free living raccoon dogs representing one of the most

299   successful invasive carnivore species in Europe (*36)* (Appendix Figure 3, panel B), would be even

300   a greater challenge for elimination as long as preventive options are limited.

Acknowledgments

306   Ministry of Food and Agriculture was provided to the Friedrich-Loeffler-Institut. The funder of

307   the study had no role in study design, data collection, data analysis, data interpretation, or writing

308   of the report. T.C.M and M.B had full access to all the data in the study and had final responsibility

309   for the decision to submit for publication.

310   **Disclaimers**

311   The authors declare no competing interests.

312   <u>**References**</u>

313   1. Tsang KW, Ho PL, Ooi GC, Yee WK, Wang T, Chan-Yeung M, et al. *A cluster of cases of severe*
314   *acute respiratory syndrome in Hong Kong.* N Engl J Med. 2003; 348:1977–1985. doi:
315   10.1056/NEJMoa030666. PMID: 12671062.

316   2. Peiris JSM, Lai ST, Poon LLM, Guan Y, Yam LYC, Lim W, et al. *Coronavirus as a possible cause of*
317   *severe acute respiratory syndrome.* Lancet. 2003; 361:1319–1325. doi: 10.1016/s0140-6736(03)13077-2.
318   PMID: 12711465.

319   3. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus ADME, Fouchier RAM. *Isolation of a novel*
320   *coronavirus from a man with pneumonia in Saudi Arabia.* N Engl J Med. 2012; 367:1814–1820. doi:
321   10.1056/NEJMoa1211721. PMID: 23075143.

322   4. Haagmans BL, Al Dhahiry SHS, Reusken CBEM, Raj VS, Galiano M, Myers R, et al. *Middle East*
323   *respiratory syndrome coronavirus in dromedary camels: An outbreak investigation.* Lancet Infect Dis.
324   2014; 14:140–145. doi: 10.1016/S1473-3099(13)70690-X. PMID: 24355866.

325   5. Drexler JF, Corman VM, Drosten C. *Ecology, evolution and classification of bat coronaviruses in the*
326   *aftermath of SARS.* Antiviral Res. 2014; 101:45–56. doi: 10.1016/j.antiviral.2013.10.013. PMID:
327   24184128.

328   6. Song H-D, Tu C-C, Zhang G-W, Wang S-Y, Zheng K, Lei L-C, et al. *Cross-host evolution of severe*
329   *acute respiratory syndrome coronavirus in palm civet and human.* Proc Natl Acad Sci U S A. 2005;
330   102:2430–2435. doi: 10.1073/pnas.0409608102. PMID: 15695582.

331   7. Reusken CB, Haagmans BL, Müller MA, Gutierrez C, Godeke G-J, Meyer B, et al. *Middle East*
332   *respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative*
333   *serological study.* Lancet Infect Dis. 2013; 13:859–866. doi: 10.1016/S1473-3099(13)70164-6.

334   8. Guan Y, Zheng BJ, He YQ, Liu XL, Zhuang ZX, Cheung CL, et al. *Isolation and characterization of*
335   *viruses related to the SARS coronavirus from animals in southern China.* Science. 2003; 302:276–278.
336   doi: 10.1126/science.1087139. PMID: 12958366.

337   9. Zhou P, Yang X-L, Wang X-G, Hu B, Zhang L, Zhang W, et al. *A pneumonia outbreak associated with*
338   *a new coronavirus of probable bat origin.* Nature. 2020; 579:270–273. doi: 10.1038/s41586-020-2012-7.
339   PMID: 32015507.

340   10. Zhang T, Wu Q, Zhang Z. *Probable Pangolin Origin of SARS-CoV-2 Associated with the COVID-19*
341   *Outbreak.* Curr Biol. 2020; 30:1346-1351.e2. doi: 10.1016/j.cub.2020.03.022. PMID: 32197085.

342   11. Xiao K, Zhai J, Feng Y, Zhou N, Zhang X, Zou J-J, et al. *Isolation of SARS-CoV-2-related*
343   *coronavirus from Malayan pangolins.* Nature. 2020; 583:286–289. doi: 10.1038/s41586-020-2313-x.
344   PMID: 32380510.

345   12. Leroy EM, Ar Gouilh M, Brugère-Picoux J. *The risk of SARS-CoV-2 transmission to pets and other*
346   *wild and domestic animals strongly mandates a one-health strategy to control the COVID-19 pandemic.*
347   One Health. 2020:100133. doi: 10.1016/j.onehlt.2020.100133. PMID: 32363229.

348   13. Oreshkova N, Molenaar RJ, Vreman S, Harders F, Oude Munnink BB, Hakze-van der Honing RW, et
349   al. *SARS-CoV-2 infection in farmed minks, the Netherlands, April and May 2020.* Euro Surveill. 2020; 25.
350   doi: 10.2807/1560-7917.ES.2020.25.23.2001005. PMID: 32553059.

351   14. Andersen KG, Rambaut A, Lipkin WI, Holmes EC, Garry RF. *The proximal origin of SARS-CoV-2.*
352   Nat Med. 2020; 26:450–452. doi: 10.1038/s41591-020-0820-9.

353   15. Zhai X, Sun J, Yan Z, Zhang J, Zhao J, Zhao Z, et al. *Comparison of SARS-CoV-2 spike protein*
354   *binding to ACE2 receptors from human, pets, farm animals, and putative intermediate hosts.* J Virol.
355   2020:JVI.00831-20. doi: 10.1128/JVI.00831-20.

356   16. Liu P, Jiang J-Z, Wan X-F, Hua Y, Li L, Zhou J, et al. *Are pangolins the intermediate host of the*
357   *2019 novel coronavirus (SARS-CoV-2)?* PLoS Pathog. 2020; 16:e1008421. doi:
358   10.1371/journal.ppat.1008421. PMID: 32407364.

359   17. Lam TT-Y, Jia N, Zhang Y-W, Shum MH-H, Jiang J-F, Zhu H-C, et al. *Identifying SARS-CoV-2-*
360   *related coronaviruses in Malayan pangolins.* Nature. 2020; 583:282–285. doi: 10.1038/s41586-020-2169-
361   0. PMID: 32218527.

362   18. Newman A, Smith D, Ghai RR, Wallace RM, Torchetti MK, Loiacono C, et al. *First Reported Cases*
363   *of SARS-CoV-2 Infection in Companion Animals - New York, March-April 2020.* MMWR Morb Mortal
364   Wkly Rep. 2020; 69:710–713. doi: 10.15585/mmwr.mm6923e3. PMID: 32525853.

365   19. *Promed Post – ProMED-mail;* 2020 Jul 29 [accessed 2020 Jul 29]. https://promedmail.org/promed-
366   post/?id=20200717.7584560.

367   20. ACTAsia.org. *China's fur trade and its position in the global fur industry;* 2019.
368   https://www.actasia.org/wp-content/uploads/2019/10/China-Fur-Report-7.4-DIGITAL-2.pdf.

369   21. Hoffmann M, Kleine-Weber H, Schroeder S, Krüger N, Herrler T, Erichsen S, et al. *SARS-CoV-2 Cell*
370   *Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor.* Cell.
371   2020; 181:271-280.e8. doi: 10.1016/j.cell.2020.02.052. PMID: 32142651.

372   22. Xu L, Zhang Y, Liu Y, Chen Z, Deng H, Ma Z, et al. *Angiotensin-converting enzyme 2 (ACE2) from*
373   *raccoon dog can serve as an efficient receptor for the spike protein of severe acute respiratory syndrome*
374   *coronavirus.* J Gen Virol. 2009; 90:2695–2703. doi: 10.1099/vir.0.013490-0.

375   23. Schlottau K, Rissmann M, Graaf A, Schön J, Sehl J, Wylezich C, et al. *Experimental Transmission*
376   *Studies of SARS-CoV-2 in Fruit Bats, Ferrets, Pigs and Chickens.* SSRN. Epub ahead of print. doi:
377   10.2139/ssrn.3578792.

378   24. Freuling CM, Eggerbauer E, Finke S, Kaiser C, Kaiser C, Kretzschmar A, et al. *Efficacy of the oral*
379   *rabies virus vaccine strain SPBN GASGAS in foxes and raccoon dogs.* Vaccine. 2019; 37:4750–4757.
380   doi: 10.1016/j.vaccine.2017.09.093. PMID: 29042202.

381   25. Wylezich C, Papa A, Beer M, Höper D. *A Versatile Sample Processing Workflow for Metagenomic*
382   *Pathogen Detection.* Sci Rep. 2018; 8:13108. doi: 10.1038/s41598-018-31496-1. PMID: 30166611.

383   26. Wylezich C, Calvelage S, Schlottau K, Ziegler U, Pohlmann A, Höper D, et al. *Next-generation*
384   *diagnostics: virus capture facilitates a sensitive viral diagnosis for epizootic and zoonotic pathogens*
385   *including SARS-CoV-2*. Epub ahead of print. doi: 10.1101/2020.06.30.181446.

386   27. Etievant S, Bal A, Escuret V, Brengel-Pesce K, Bouscambert M, Cheynet V, et al. *Performance*
387   *Assessment of SARS-CoV-2 PCR Assays Developed by WHO Referral Laboratories.* J Clin Med. 2020; 9.
388   doi: 10.3390/jcm9061871. PMID: 32560044.

389   28. Tan CW, Chia WN, Chen MI-C, Hu Z, Young BE, Tan Y-J, et al. *A SARS-CoV-2 surrogate virus*
390   *neutralization test (sVNT) based on antibody-mediated blockage of ACE2-spike (RBD) protein-protein*
391   *interaction*. Epub ahead of print. doi: 10.21203/rs.3.rs-24574/v1.

392   29. Richard M, Kok A, Meulder D de, Bestebroer TM, Lamers MM, Okba NMA, et al. *SARS-CoV-2 is*
393   *transmitted via contact and via the air between ferrets;* 2020.

394   30. Kim Y-I, Kim S-G, Kim S-M, Kim E-H, Park S-J, Yu K-M, et al. *Infection and Rapid Transmission*
395   *of SARS-CoV-2 in Ferrets.* Cell Host Microbe. Epub ahead of print. doi: 10.1016/j.chom.2020.03.023.
396   PMID: 32259477.

397   31. Shi J, Wen Z, Zhong G, Yang H, Wang C, Huang B, et al. *Susceptibility of ferrets, cats, dogs, and*
398   *other domesticated animals to SARS-coronavirus 2*. Science. Epub ahead of print. doi:
399   10.1126/science.abb7015. PMID: 32269068.

400   32. *Promed Post – ProMED-mail;* 2020 Jul 9 [accessed 2020 Jul 9]. https://promedmail.org/promed-
401   post/?id=7533033.

402   33. Enserink M. *Coronavirus rips through Dutch mink farms, triggering culls.* Science. 2020; 368:1169.
403   doi: 10.1126/science.368.6496.1169. PMID: 32527808.

404   34. Wölfel R, Corman VM, Guggemos W, Seilmaier M, Zange S, Müller MA, et al. *Virological*
405   *assessment of hospitalized patients with COVID-2019.* Nature. 2020; 581:465–469. doi: 10.1038/s41586-
406   020-2196-x. PMID: 32235945.

407   35. Randad PR, Pisanic N, Kruczynski K, Manabe YC, Thomas D, Pekosz A, et al. *COVID-19 serology*
408   *at population scale: SARS-CoV-2-specific antibody responses in saliva.* medRxiv.
409   2020:2020.05.24.20112300. doi: 10.1101/2020.05.24.20112300. PMID: 32511537.

410   36. Kauhala K, Kowalczyk R. *Invasion of the raccoon dog Nyctereutes procyonoides in Europe: History*
411   *of colonization, features behind its success, and threats to native fauna.* Curr Zool. 2011; 57:584–598.
412   doi: 10.1093/czoolo/57.5.584.

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415 **Table 1**. Serological response of raccoon dogs to SARS-CoV-2 infection using the indirect

416 immunoflourescence assay (iIFA), the virus neutralization test (VNT) and a surrogate Virus

417 Neutralization test (sVNT). Positive results are highlighted in red (bold font) for inoculated (#1-

418 9) and contact (#10-12) animals. No serological response on day 0 and day 4 pi (data not shown).

No.	Day 8pi iIFAT	VNT	sVNT	Day 12pi iIFAT	VNT	sVNT	Day 16pi iIFAT	VNT	sVNT	Day 21pi iIFAT	VNT	sVNT	Day 28pi iIFAT	VNT	sVNT
#1															
#2															
#3	**1:128**	< 1:4	**56.39**												
#4	< 1:20	< 1:2	13.42												
#5	**1:64**	< 1:2	**52.26**	**1:64**	< 1:2	**71.62**									
#6	**1:128**	**1:5.04**	**52.19**	**1:64**	**1:12.7**	**83.99**									
#7	**1:128**	< 1:4	**38.94**	**1:64**	< 1:2	**72.56**	**1:64**	**1:4**	**76.22**	**1:128**	**1:10.08**	**84.99**	**1:1024**	**1:12.7**	**88.57**
#8	< 1:20	< 1:2	6.62	< 1:20	< 1:2	9.20	< 1:20	< 1:2	10.58	< 1:20	< 1:2	4.99	< 1:20	< 1:2	-8.80
#9	< 1:20	< 1:2	2.12	< 1:20	< 1:2	16.08	< 1:20	< 1:2	11.42	< 1:20	< 1:2	3.99	< 1:20	< 1:2	-2.88
#10	< 1:20	< 1:2	3.02	< 1:20	< 1:2	**24.62**	**1:64**	< 1:2	**62.34**	**1:128**	< 1:4	**82.31**	**1:512**	< 1:4	**82.98**
#11	< 1:20	< 1:2	-9.55	< 1:20	< 1:2	**47.88**	**1:64**	< 1:2	**71.94**	**1:128**	**1:5.04**	**69.10**	**1:256**	< 1:4	**81.82**
#12	< 1:20	< 1:2	-0.77	< 1:20	< 1:2	8.89	< 1:20	< 1:2	12.17	< 1:20	< 1:2	**22.91**	< 1:20	< 1:2	7.34

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420
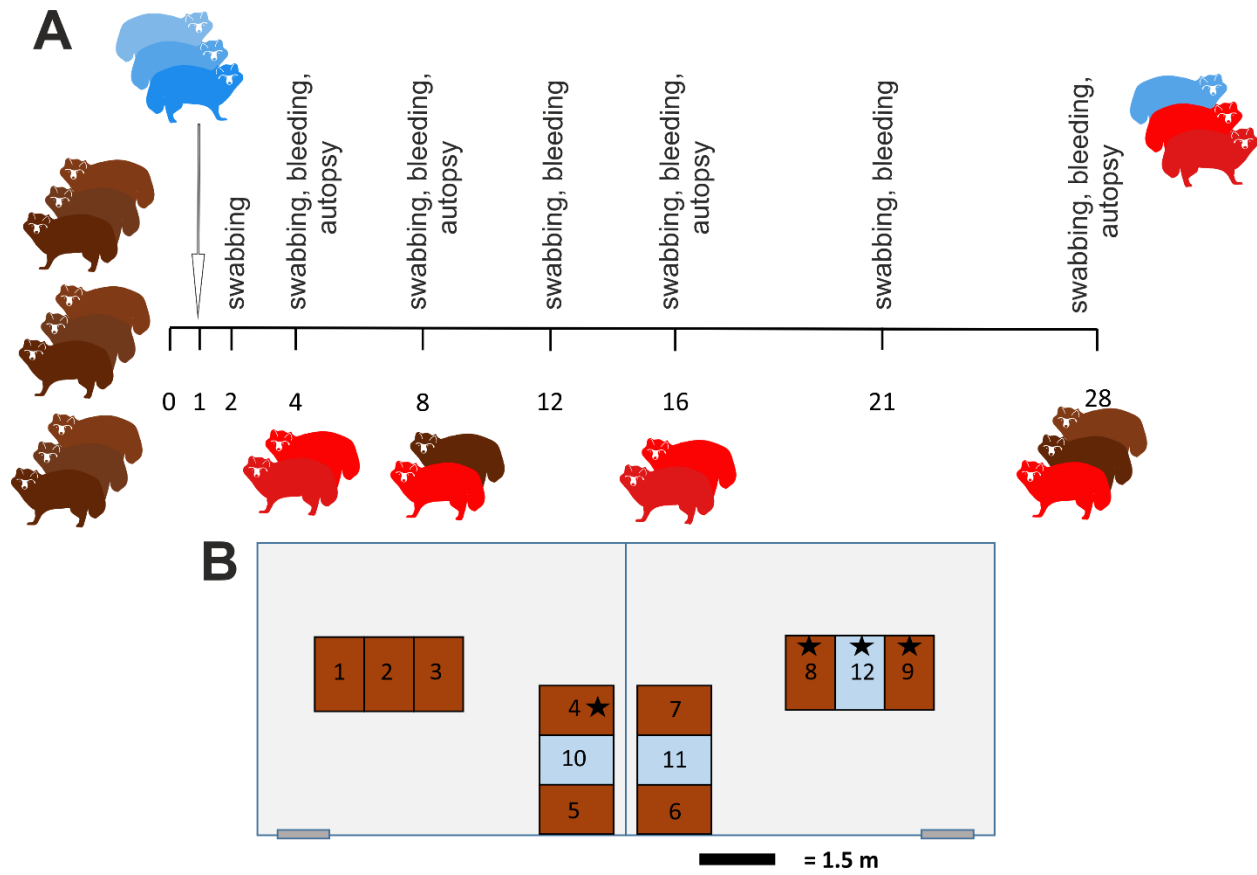
Figure 1. Study design (A) Outline of the in vivo experiments with an observation period of 28 days. Animals (n=9) were inoculated intranasally with 10^5 TCID$_{50}$/ml and three naïve direct contacts were added 1 dpi. On day 4 (animals #1, #2), day 8 (#3, #4), and 12 (#5, #6) two raccoon dogs each were sacrificed and subjected to autopsy. All remaining animals were euthanized on day 28 pi. Animals that became infected are highlighted in red. (B) Arrangement of the individual cages for the raccoon dogs in two separate rooms of the BSL 3 facility at the Friedrich-Loeffler-Institut. Inoculated animals (brown), contact animals (blue) and animals that remained uninfected (★) are indicated.

431
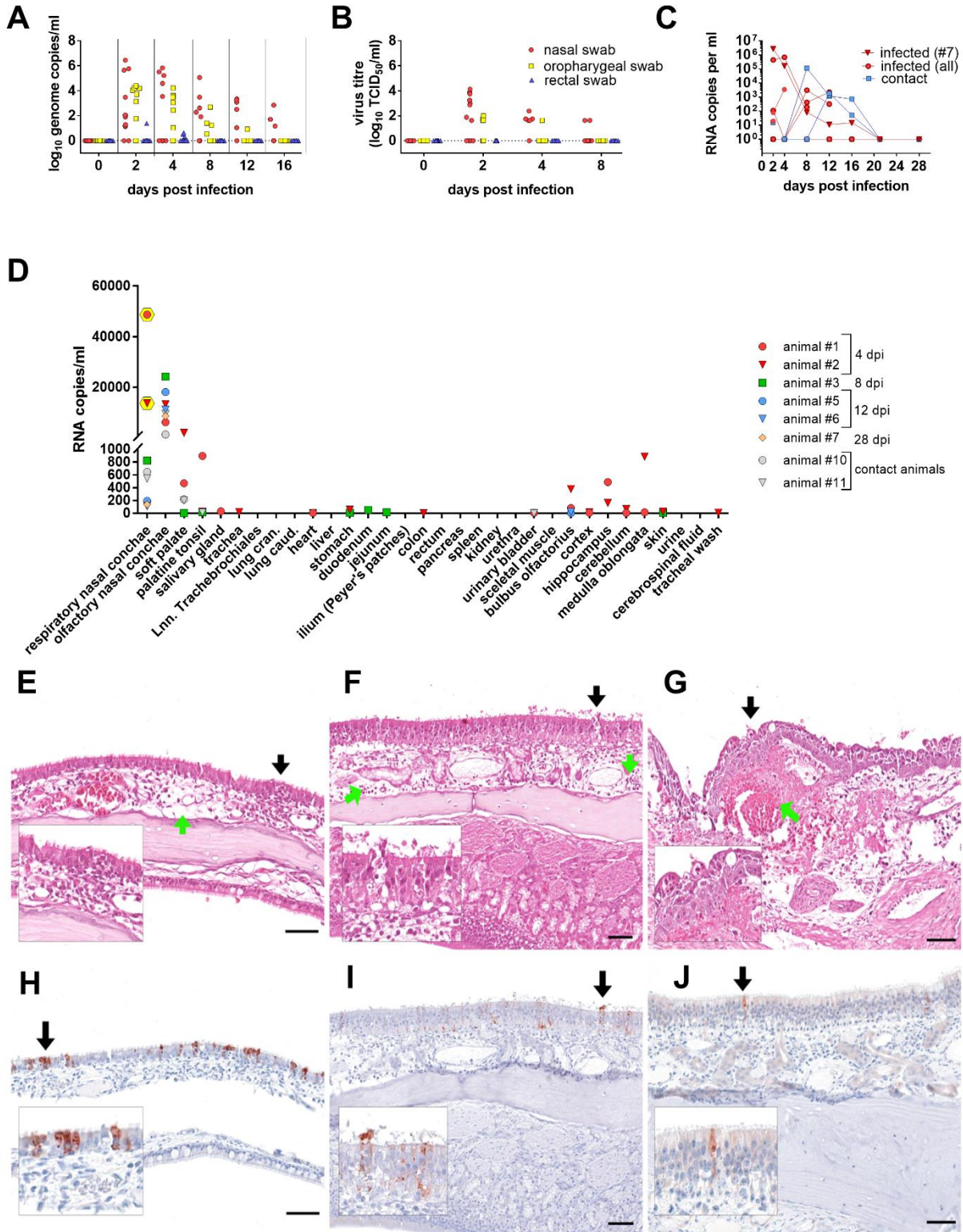
Figure 2. Virus detection in swab and tissue samples (A) SARS-CoV-2 viral genome loads in

433   swab samples over time, and (B) virus titres using isolation on Vero E6 cells. Two replicates per

434   sample were analysed and both results are shown. (C) Individual viral loads of nasal swabs taken

435   from infected and contact animals. (D) Viral genome loads in organs, infectious virus was

436   isolated only from nasal conchae at 4 dpi (yellow hexagons) from animal #1 (2.86 Log_{10}

437    $TCID_{50}$/ml) and animal #2 (1.63 Log_{10} $TCID_{50}$/ml. (E) Rhinitis, respiratory region, with mucosal

438   edema (green arrow) and epithelial degeneration with inflammation (black arrow, see also inlay)

439   at 4 dpi. (F) Rhinitis, olfactory region, with mucosal edema and inflammation (green arrows) and

440   epithelial necrosis and loss with minimal intraluminal debris (black arrow, see also inlay), 4 dpi.

441   (G) Rhinitis, olfactory region, focal coagulative necrosis and hemorrhage (green arrow) and

442   epithelial necrosis with early re-epithelisation (black arrow, see also inlay), 8 dpi. (H)

443   Intralesional viral antigen oligofocal in the respiratory epithelium, 4 dpi, (I) Intralesional antigen

444   labelling oligofocal in the olfactory epithelium, 4 dpi. (J) Single antigen-positive olfactory cells,

445   8 dpi. (E-G) Histopathology, hematoxylin & eosin stain, (H-J) immunohistochemistry, ABC

446   method, AEC chromogen (red-brown), Mayer's hematoxylin counter stain (blue). All bars = 50
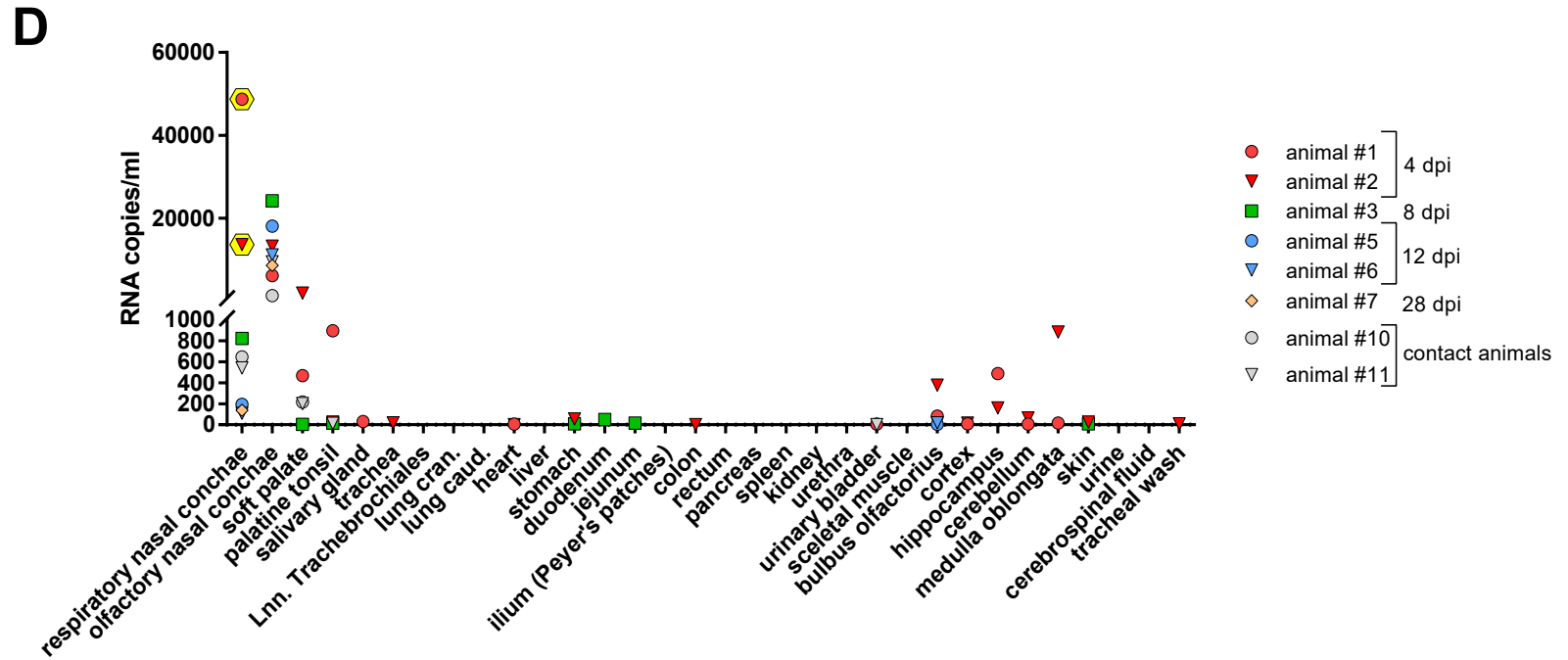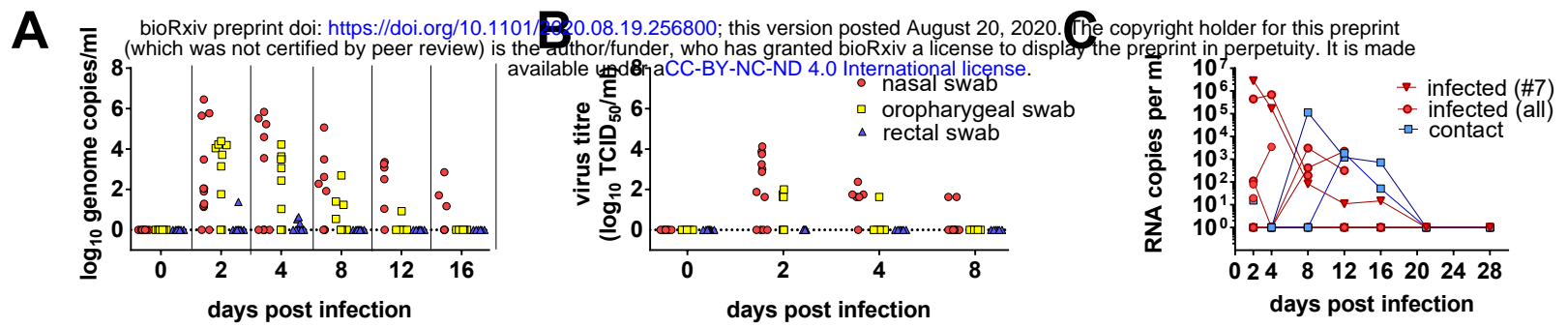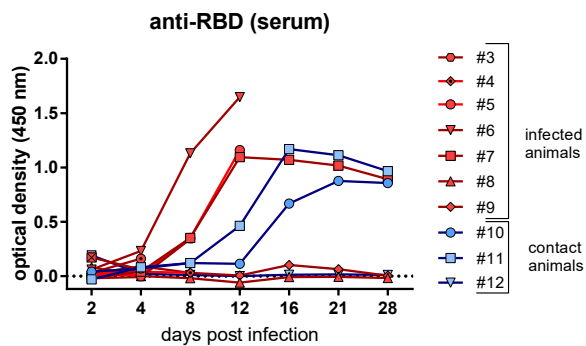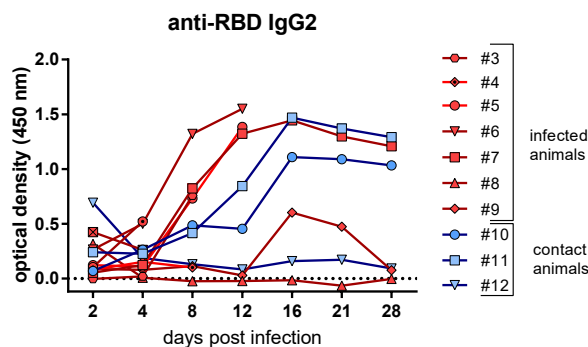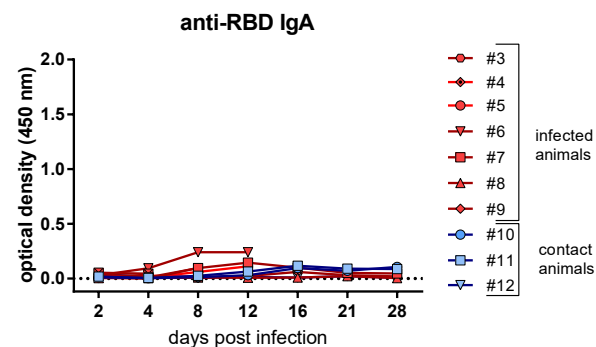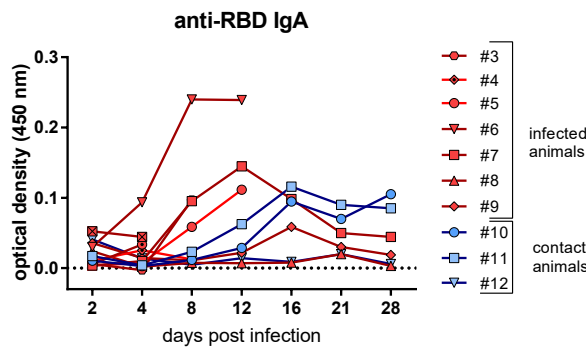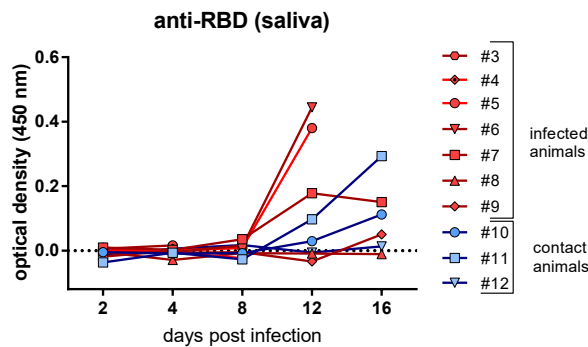
447   µm

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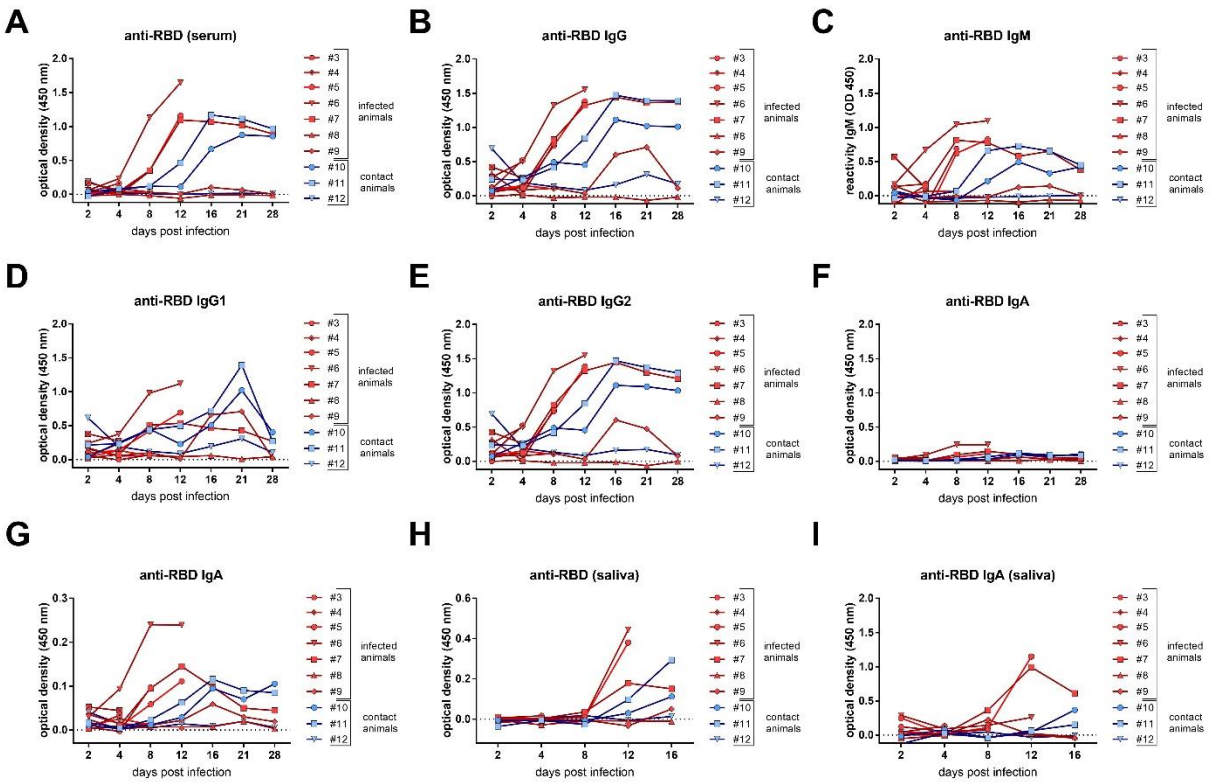
Figure 3. SARS-Co-V-2-specific antibody response (A) Individual immune response in sera as measured using an in-house RBD ELISA with a multi species conjugate (SBVMILK, kindly provided by ID-VET, Grabels, FRANCE); (B) with a secondary anti-dog IgG-conjugate; (C) with a secondary anti-dog IgM; (D) with a secondary anti-dog IgG1; (E) with a secondary anti-dog IgG2; (F) with RBD as antigen and a secondary anti-dog IgA, at the same scale, and (G) at a zoomed scale. (H) Total anti-RBD antibodies in saliva and (I) anti-RBD IgA in saliva

A

swabbing

swabbing, bleeding, autopsy

swabbing, bleeding, autopsy

swabbing, bleeding

swabbing, bleeding, autopsy

swabbing, bleeding

swabbing, bleeding, autopsy

0 1 2   4   8   12   16   21   28

B

| 1 | 2 | 3 |

| 8 ★ | 12 ★ | 9 ★ |

4 ★	7
10	11
5	6

= 1.5 m