

1 **Compellingly high SARS-CoV-2 susceptibility of Golden Syrian hamsters suggests multiple**
2 **zoonotic infections of pet hamsters during the COVID-19 pandemic**

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4 Short title: Exceptional SARS-CoV-2 susceptibility of Syrian hamsters

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20

21 **Abstract**

22 Golden Syrian hamsters (*Mesocricetus auratus*) are used as a research model for severe
23 acute respiratory syndrome coronavirus type 2 (SARS-CoV-2). Millions of Golden Syrian
24 hamsters are also kept as pets in close contact to humans. To determine the minimum
25 infective dose (MID) for assessing the zoonotic transmission risk, and to define the optimal
26 infection dose for experimental studies, we orotracheally inoculated hamsters with SARS-
27 CoV-2 doses from 1×10^5 to 1×10^{-4} tissue culture infectious dose 50 (TCID₅₀). Body weight
28 and virus shedding were monitored daily. 1×10^{-3} TCID₅₀ was defined as the MID, and this
29 was still sufficient to induce virus shedding at levels up to $10^{2.75}$ TCID₅₀/ml, equaling the
30 estimated MID for humans. Virological and histological data revealed 1×10^2 TCID₅₀ as the
31 optimal dose for experimental infections. This compellingly high susceptibility resulting in
32 productive infections in Golden Syrian hamsters needs to be considered also as a source of
33 SARS-CoV-2 infections in humans.

34

35 Introduction

36 To date, more than 499 million confirmed Coronavirus Disease 2019 (COVID-19) cases
37 and more than 6.1 million deaths have been reported globally since the initial detection of
38 severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in December 2019
39 (<https://coronavirus.jhu.edu>; 12.04.2022). COVID-19 has been studied extensively since,
40 and the incubation time in humans has been calculated as 6.4 days for the initially described
41 virus variants (Cheng et al., 2021; Elias, Sekri, Leblanc, Cucherat, & Vanhems, 2021), while
42 the estimated minimum infective dose (MID) for humans has been calculated to be
43 approximately 10^2 tissue culture infectious dose 50 (TCID₅₀) (Basu, 2021; Karimzadeh,
44 Bhopal, & Nguyen Tien, 2021).

45 Golden Syrian hamsters (*Mesocricetus auratus*) are widely used as a COVID-19 animal
46 model, as they efficiently support SARS-CoV-2 replication and display pathological
47 manifestations similar to human COVID-19 pneumonia, such as focal diffuse alveolar
48 destruction, hyaline membrane formation, and mononuclear cell infiltration (Chan et al.,
49 2020; Sia et al., 2020). Efficient transmission from inoculated to naïve hamsters by direct
50 contact and aerosol has also been reported (Dowall et al., 2021; Sia et al., 2020). Several
51 hamster species are kept as pets in millions of households worldwide. These include Golden
52 Syrian hamsters, Chinese hamsters (*Cricetulus griseus*), and three Dwarf hamster species
53 (*Phodopus roborowskii*, *P. campbelli* and *P. sungorus*). According to the German Pet Trade
54 & Industry Association (Zentralverband Zoologischer Fachbetriebe e.V. ZZF),
55 approximately 520.000 pet hamsters were kept in German households in 2020. Golden
56 Syrian hamsters comprised 43% (223.600 animals), and the Dwarf hamster species 54%
57 (280.800 animals) (source: Zentralverband Zoologischer Fachbetriebe (ZZF) and
58 Industrieverband Heimtierbedarf (IVH)). While Golden Syrian hamsters, Chinese hamsters
59 (*Cricetulus griseus*), and Roborovski Dwarf hamsters (*Phodopus roborovskii*) are highly
60 susceptible and develop clinical disease and lung pathology, both other Dwarf hamster
61 species (Campbell's Dwarf hamster and Djungarian Dwarf hamster) only developed
62 subclinical disease with mild tissue damage and a rapid onset of regeneration under
63 experimental conditions (Bertzbach et al., 2021; Trimpert et al., 2020).

64 Since the onset of the pandemic, several virus variants have emerged, five of which have
65 been classified as variants of concern (VOC) by the World Health Organization (WHO) for
66 their enhanced transmissibility and virulence or their reduced susceptibility to immune
67 reactions (WHO, 01.03.2022). These include variants B.1.1.7 (Alpha), B.1.351 (Beta), P.1
68 (Gamma), B.1.617.2 (Delta) and B.1.1.529 (Omicron). Especially the rapid worldwide

69 spread of the Delta and Omicron variants have raised concern about an acceleration of the
70 pandemic's dynamic, despite increasing proportions of fully vaccinated humans
71 (<https://github.com/owid/covid-19-data/tree/master/public/data/vaccinations>). Therefore,
72 the pathogenesis induced by VOCs has been addressed in a number of recent animal studies,
73 mostly performed in Golden Syrian hamsters (Abdelnabi et al., 2022; McMahan et al., 2022;
74 Sreelekshmy Mohandas et al., 2022; S. Mohandas et al., 2021; O'Donnell et al., 2021).
75 While Alpha, Beta and Delta variants caused clinical manifestation and pathology similar
76 to the ancestral virus (S. Mohandas et al., 2021; O'Donnell et al., 2021), infection with the
77 Omicron variant caused significantly milder clinical signs and lower levels of pulmonary
78 affection (Abdelnabi et al., 2022; McMahan et al., 2022). However, levels of viral genomic
79 RNA, as well as subgenomic RNA (sgRNA), were distinctly higher in the nasal turbinate
80 samples, indicating a higher transmission risk from these animals as compared to animals
81 infected with any of the other variants (McMahan et al., 2022).

82 Very recently, two distinct zoonotic transmission events from hamsters to humans were
83 reported from Hong Kong followed by human-to-human transmission (Yen, 2022), after the
84 import of pet Golden Syrian hamsters from The Netherlands, causing a re-introduction of
85 the Delta VOC into the country. Investigation of these animals revealed SARS-CoV-2 RNA
86 in 15 out of 28 Golden Syrian hamsters, but in none of the 77 analysed Dwarf hamsters
87 (Yen, 2022). These findings gave rise to serious concern about the SARS-CoV-2
88 transmission risk from pet hamsters to humans in affected households (Haagmans &
89 Koopmans, 2022; Kok et al., 2022), and more than 2.000 pet hamsters were subsequently
90 culled in Hong Kong. This highlights the importance of an evidence-based risk assessment
91 regarding SARS-CoV-2 transmissions from pet hamsters to humans and vice versa. To the
92 best of our knowledge, no systematic quantitative study on the susceptibility of Golden
93 Syrian hamsters to a SARS-CoV-2 infection has been undertaken so far. We therefore
94 infected groups of Golden Syrian hamsters with serial SARS-CoV-2 dilutions from 10^5 to
95 10^{-4} TCID₅₀, which is equivalent to 0.7 genome copy numbers per dose, as determined by
96 reverse transcriptase quantitative real-time PCR of serial dilutions of a SARS-CoV-2 RNA
97 standard. Animals infected with a dose of 1×10^{-3} TCID₅₀ shed replication competent virus,
98 and accumulated SARS-CoV-2 infectivity in their respiratory tract until 7 days post
99 inoculation (dpi). This study demonstrates the extremely high SARS-CoV-2 susceptibility
100 of Golden Syrian hamsters. For drug or vaccine efficacy studies, a moderate infection dose,
101 sufficiently high to induce reproducible viral loads and histological findings, and at the same

102 time sufficiently low to avoid a highly artificial infection should be applied, which in our
103 experimental setup was determined as 1×10^2 TCID₅₀.

104 **Results**

105 **Pronounced weight loss and high levels of viral shedding upon orotracheal inoculation**

106 Upon orotracheal and intranasal inoculation with SARS-CoV-2 (Fig. 1A), animals started
107 losing body weight from 2 dpi, and the mean weight loss at 7 dpi was 16% after orotracheal
108 inoculation and 7% after intranasal inoculation (Fig. 1 B). We observed a statistically
109 significant 5- to 10-fold increase in viral genome shedding in the nasal wash samples at 6
110 and 9 dpi after orotracheal inoculation as compared to the intranasal inoculation (Fig. 1C).
111 Upon necropsy at 14 dpi, viral RNA in the respiratory tract samples was not detectable at
112 levels allowing a quantitative comparison, and no replication competent virus was detected.
113 Based on these data, we decided to continue with the orotracheal inoculation for subsequent
114 experiments.
115

116 **1×10^{-1} TCID₅₀ SARS-CoV-2 is sufficient to induce body weight reduction and pneumonia**

117 Animals inoculated in our first study with doses exceeding 1×10^2 TCID₅₀ SARS-CoV-2
118 showed a decrease in body weight from 2 dpi, while hamsters inoculated with 1×10^1 TCID₅₀
119 gained weight until 5 dpi, before losing weight until the end of the experiment. Animals
120 inoculated with 1×10^{-1} and 1×10^0 TCID₅₀ as well as the uninfected controls continued to
121 gain weight throughout the experiment. In the second study, all groups receiving 1×10^{-1}
122 TCID₅₀ or higher lost weight until the end of the experiment, with an onset delayed by 2-3
123 day in animals infected with 1×10^1 and 1×10^0 TCID₅₀. In a third study using dilutions to
124 1×10^{-4} TCID₅₀ and continuing until 10 dpi, doses of 10^{-1} TCID₅₀ or higher induced weight
125 loss (Fig. 2). Statistical analysis revealed significant differences between the body weights
126 determined per group at the day of the necropsy, i.e. 7 and 10 dpi (Suppl. Tab. 1).
127

128 During autopsy performed at 7 or 10 dpi, the extent of reddish discoloration of the whole
129 lung was recorded. We observed decreasing estimated levels of lung affection correlated
130 with decreasing infection doses. We still noted areas of discoloration at 7 dpi in animals that
131 had been infected with 1×10^{-3} TCID₅₀ (Fig.3 A). At 10 dpi, even hamsters inoculated with
132 the lowest dose of 1×10^{-4} TCID₅₀ showed lung changes (Fig. 3 B).
133

134 Histopathology was performed on the left lung lobe of animals sacrificed at 10 dpi.
135 Archived lungs from a high-dose infection study ($1 \times 10^{4.5}$ TCID₅₀) served as positive

136 controls. Pneumonia-associated consolidation was consistently found in all hamsters
137 infected with $1*10^{-1}$ TCID₅₀ SARS-CoV-2 or higher (Fig. 3 C, D). Lungs collected at 10
138 dpi mainly showed regenerative changes with bronchial and type-2-pneumonocyte
139 hyperplasia and hypertrophy with formation of multinucleated, atypical cells. However, still
140 a moderate to severe inflammatory reaction was found, with intra-alveolar, interstitial,
141 peribronchial and perivascular inflammatory infiltrates, as well as vasculitis and/or
142 endothelialitis. Acute necrosis of the bronchial epithelium was a rare finding (Fig. 4 A - C).
143 Although not statistically significant, hamsters infected with $1*10^{-1}$ TCID₅₀ showed a
144 tendency to be less severely affected (Fig. 4 E). In general, animals infected with doses of
145 $1*10^{-2}$ TCID₅₀ and lower did not develop pneumonia-associated consolidation in the lung
146 (Fig. 4 F, G). However, focal alveolar or perivascular infiltrates or bronchial epithelial
147 hyperplasia were found in individual animals in each group indicating prior local infection,
148 even after infection with $1*10^{-4}$ TCID₅₀ (Fig. 4 H, I).

150 **Lateral flow device Rapid Test detects infections with $1*10^{-1}$ TCID₅₀ SARS-CoV-2**

151 We analysed oral swab samples collected at 6 dpi using the Nowcheck COVID-19 Rapid
152 Test, which confirmed the infection in hamsters inoculated with doses higher than $1*10^0$
153 TCID₅₀, as well as in two hamsters inoculated with $1*10^{-1}$ TCID₅₀, which translates into
154 samples with ct-values in the real-time RT-PCR below 30. All uninfected hamsters as well
155 as one hamster inoculated with $1*10^{-1}$ TCID₅₀ were negative at 6 dpi (Fig. S1). We also
156 analysed nasal washes collected at 7 dpi. Again, the infection was detectable in animals
157 challenged with doses of $1*10^{-1}$ TCID₅₀ or higher, i.e. for samples with ct-values below 30.

159 **$1*10^{-2}$ TCID₅₀ SARS-CoV-2 is sufficient to induce seroconversion**

160 To confirm a systemic infection, all sera collected during necropsies were tested in an
161 indirect ELISA based on the SARS-CoV-2 RBD antigen. ROC analysis was performed for
162 the determination of the ELISA cut-off value. We determined a specificity of 100% and a
163 sensitivity of 99.56% using the cut-off value of 14.11 percent of the positive control value
164 (PP) (Fig. S2 A). We detected seroconversion with a dose-dependent increase of PP values
165 in hamsters inoculated with infection doses of $1*10^{-2}$ TCID₅₀ or higher (Fig. S2 B).

167 **$1*10^{-3}$ TCID₅₀ SARS-CoV-2 is sufficient to induce oral and nasal viral shedding**

168 From the first day post infection, animals infected with 10^2 TCID₅₀ SARS-CoV-2 or higher
169 shed virus between $10^{3.5}$ and $10^{4.5}$ genome copy numbers / μ l RNA. By 3 dpi, all animals

170 infected with doses of 1×10^{-1} TCID₅₀ or higher shed virus (Fig. 5 A). The oral swab samples
171 contained replicating virus even in the groups infected with a dose of 10^{-1} TCID₅₀ until 6
172 dpi, and at a level of 10^2 TCID₅₀/ ml in the swab sample collected at 6 dpi from one animal
173 infected with the 1×10^{-3} TCID₅₀ dose. This result was confirmed by the detection of SARS-
174 CoV-2 sgRNA as an additional proof of a present or past virus replication. All positive
175 results for the nasal wash samples collected at 2 and 4 dpi were confirmed by re-testing for
176 sgRNA, with ct values of about 3 points above those determined for the SARS-CoV-2 N-
177 gene (Tab. 2). The peak of oral shedding of replication competent virus was observed
178 between 3 and 5 dpi, at levels reaching up to 10^3 TCID₅₀/ ml in the oral swab samples (Fig
179 5 B).

180 SARS-CoV-2 RNA levels detected in the nasal washes were generally 10- to 100- times
181 higher than those determined from the oral swab samples. We were able to detect viral RNA
182 at 2 and 4 dpi in nasal washes, even in the animals infected with the lowest dose of 10^{-4}
183 TCID₅₀ (Fig. 6 A). Significant differences were determined for the copy numbers detected
184 in the different infection groups (Tab 1). The peak of nasal shedding of replication
185 competent virus was observed at 2 dpi at levels reaching up to 10^7 TCID₅₀ / ml, while
186 $1 \times 10^{2.75}$ TCID₅₀ (equivalent to the estimated MID for humans), as well as sgRNA were
187 detected in a sample collected from one animal infected with 10^{-3} TCID₅₀ (Fig. 6 B). The
188 dose-dependent delay in onset of oral and nasal shedding is summarized in Fig. 7.

190 **Replication competent virus in lungs after infection with 1×10^{-1} TCID₅₀, and in nasal conchae** 191 **after infection with 1×10^{-3} TCID₅₀**

192 Similar levels of viral RNA were detected in nasal conchae samples of all animals sacrificed
193 at 7 dpi, independent of the inoculation dose, including the group infected with a dose of
194 1×10^{-3} TCID₅₀ (Fig. 8 A), while at 10 dpi, viral RNA was only detected in the samples
195 collected from the infection groups 1×10^2 to 1×10^{-1} TCID₅₀ (Fig. 8 B). Interestingly at 7 dpi,
196 the highest levels of viral RNA were detected in trachea and lung samples collected in
197 groups inoculated with the lowest infection dose of 1×10^{-3} TCID₅₀. At this time point, the
198 highest virus titers were determined for the nasal conchae samples collected from animals
199 infected with SARS-CoV-2 doses between 10^0 and 10^{-3} TCID₅₀ (Fig. 8 C), which was
200 confirmed by the detection of sgRNA (Tab. 2), while only nasal conchae samples from
201 animals infected with 10^{-1} TCID₅₀ contained detectable levels of replication competent virus
202 at 10 dpi (Fig. 8 D). Statistically different levels of replication competent virus were

203 detected in lung samples collected from animals inoculated with different infection doses
204 (Suppl. Tab. 1).

205 **Sequence analysis of SARS-CoV-2 retrieved from animals infected with a high or a low dose**

206 We exemplarily analysed SARS-CoV-2 whole-genome sequences retrieved from nasal
207 conchae samples of two animals. Animal H310 (L5497) from a high-dose infection study
208 ($1 \times 10^{4.5}$ TCID₅₀) autopsied at 10 dpi, whereas animal H318 (L5537) was infected with 1×10^{-1}
209 TCID₅₀. Both animals and was sacrificed at 10 dpi. The sequence of the first sample is
210 identical to the virus stock 2019_nCoV Muc-IMB-1 and shows only one SNV, a transition
211 in the M gene (C26877T) with 52% variant frequency (strand bias 51%) that was already
212 detected as minor variant with 18% variant frequency in the virus stock. The sequence of
213 the second animal is identical to the virus stock and shows only one transition in the ORF1a,
214 nsp6 (T11639C) and no further SNVs. Both described changes are silent mutations. The
215 genome sequences are available under ENA study accession number PRJEB51977.
216

217 **Discussion**

218 Today, Golden Syrian hamsters are the leading animal model in SARS-CoV-2 research, in
219 particular for vaccine and drug efficacy studies (Chiba et al., 2022; Meseda et al., 2021;
220 Taylor et al., 2021; Yadav et al.), since they mirror the moderate disease phenotype of
221 human patients with a complete recovery within 14 days (Gruber, Firsching, Trimpert, &
222 Dietert, 2021). When establishing this model, we initially used an infection dose of 1×10^5
223 TCID₅₀, and found the orotracheal infection route to induce a more prominent weight loss
224 and viral shedding, the latter exceeding those obtained after intranasal infection by at least
225 a factor of 5. Therefore, this route was used for all subsequent studies. Since the intranasal
226 route was used in almost all other published studies (Chiba et al., 2022; Meseda et al., 2021;
227 Taylor et al., 2021; Yadav et al.), and to the best of our knowledge no report of an
228 orotracheal SARS-CoV-2 inoculation of hamsters has been published so far, a direct
229 comparison between previously published experiments and our study is not feasible.
230

231 In our study, we were able to show that an infection dose of 1×10^{-1} TCID₅₀ was sufficient
232 to induce a significant weight loss, oral and nasal shedding as well as accumulation of
233 replication competent virus in the respiratory tract, and SARS-CoV-2 related pneumonia.
234 In this group, the onset of weight loss and lethargy were delayed by 3 days, presumably due
235 to an initial virus replication at the inoculation site, before spreading throughout the
236

237 respiratory tract. Infection doses of 1×10^{-2} TCID₅₀ or higher were sufficient to induce a
238 dose-dependent seroconversion within 7 days, therefore the virus must have reached the
239 lymphatic system within the first 2-3 days post infection. As no blood samples were
240 available for time points before 7 dpi, a possible viremia within the first 3 days post infection
241 could not be monitored. The estimated proportion of affected lung tissue also showed a clear
242 correlation to the infection dose. Even after challenge with 1×10^{-3} TCID₅₀, we observed
243 macroscopically visible lung affections, oral and nasal shedding as well as accumulation of
244 replication competent virus in the nasal conchae and the trachea. We therefore define the
245 MID for Golden Syrian hamsters upon orotracheal infection as 1×10^{-3} TCID₅₀. This result
246 not only proves the exceptionally high susceptibility of Golden Syrian hamsters to a SARS-
247 CoV-2 infection, but also indicates the low sensitivity of the Vero E6 virus titration assay
248 with a detection limit of $1 \times 10^{1.5}$ TCID₅₀, as compared to 1×10^{-3} TCID₅₀ in the Golden Syrian
249 hamster model. In another Golden Syrian hamster susceptibility study published by Rosenke
250 and co-workers (Rosenke et al., 2020) using intranasal infection doses between 1×10^3 and
251 1×10^0 TCID₅₀, a dose of 5 TCID₅₀ was postulated as MID. The data obtained for the lower
252 dose group resembles what we observed for the group inoculated with 1×10^{-2} TCID₅₀, which
253 may be due to the increased efficiency of the orotracheal route. Moreover, since data were
254 only collected until 5 dpi, a delayed onset of virus shedding and virus propagation in the
255 respiratory tract after challenge with very low doses would have remained unnoticed.

256 We propose an optimal SARS-CoV-2 infection dose for drug or vaccine efficacy studies in
257 Golden Syrian hamsters of 10^2 TCID₅₀, as this is sufficient to induce a reproducible (as
258 confirmed by three independent experiments) weight loss, macroscopically visible lung
259 affection, pneumonia, shedding of replication competent virus from 1 dpi, and the presence
260 of high levels of replication competent virus in the respiratory tract on day 7 post infection,
261 which will therefore allow a quantification of therapeutic effects. Infection doses of 10^5
262 TCID₅₀ that are generally used in Golden Syrian hamsters (Chan et al., 2020; Dowall et al.,
263 2021; Francis et al., 2021; Johnson et al., 2022) may be too high to allow a disease
264 progression at least partly resembling the distinctly slower processes in humans after a
265 natural infection (Karimzadeh et al., 2021).

266 Replication competent virus as well as SARS-CoV-2 sgRNA were detectable at dose
267 dependent levels in nasal washes of all groups until 4 dpi except the group infected with
268 1×10^{-4} TCID₅₀. Thus, by this time, the virus must have already disseminated from the
269 trachea and lung (inoculation site) to the upper respiratory tract, which may have occurred
270 either via mucus transport from the lower respiratory tract, or via the bloodstream. Since we

271 did not determine seroconversion in the animals receiving an infection dose of 1×10^{-2}
272 TCID₅₀ or lower, virus dissemination via active mucus transport seems most plausible. The
273 positive sgRNA results also indicate a past active viral replication in the nasal epithelium,
274 as sgRNAs are transcribed only in infected cells (Wolfel et al., 2020). However, positive
275 sgRNA results cannot be interpreted as an equivalent to the detection of replication
276 competent virus by TCID₅₀ (Alexandersen, Chamings, & Bhatta, 2020).

277 To allow a first insight whether the exposition to immune reactions in an animal inoculated
278 with a low dose may stimulate the formation of gene mutations, we analysed the SARS-
279 CoV-2 sequences retrieved from nasal conchae samples from one animal each infected with
280 a high dose and a low dose, and found no indication for such an effect.

281 The confirmation of SARS-CoV-2 infections in animals infected with 1×10^{-1} TCID₅₀ or
282 higher by LFD confirms the suitability of these assays for the screening of hamster swab
283 samples, which may be relevant in households with COVID-19 patients where Golden
284 Syrian hamsters are kept as pets. Upon natural infection with a low dose, these animals will
285 most probably not develop clinical signs, but will shed replication competent virus for at
286 least six days at levels possibly exceeding the estimated human MID of 1×10^2 TCID₅₀, thus
287 allowing a zoonotic infection cycle between humans and Syrian Gold hamsters, as it has
288 been observed very recently in Hong Kong (Yen, 2022).

289 Our data demonstrate the exceptionally high susceptibility of Golden Syrian hamsters to an
290 infection with a German SARS-CoV-2 isolate obtained during an outbreak in Munich in
291 January 2020 (Wolfel et al., 2020). Other groups reported an almost identical disease
292 progression and virus shedding pattern for VOCs Alpha, Beta, and Delta as for the ancestral
293 virus in hamsters (S. Mohandas et al., 2021; O'Donnell et al., 2021), rendering the titration
294 experiments with these VOCs dispensable. Meanwhile, a low pathogenicity combined with
295 high up to 100-fold increased viral loads in the upper respiratory tract were reported for
296 hamsters infected with the Omicron variant (Abdelnabi et al., 2022; McMahan et al., 2022).
297 Therefore, an Omicron titration study in hamsters similar to what we describe here for the
298 ancestral virus may be rewarding.

299 In summary, we determined the extremely high susceptibility of Golden Syrian hamsters to
300 a SARS-CoV-2 infection, and defined the MID as 1×10^{-3} TCID₅₀. A very close monitoring
301 of pet Golden Syrian hamsters that are kept in households with COVID-19 patients is
302 therefore strongly recommended, for instance by using rapid tests. COVID-19 patients
303 should strictly avoid any direct or indirect contact to their pet hamsters. The optimal
304 infection dose for drug efficacy studies was determined as 1×10^2 TCID₅₀. These conclusions

not only apply to Golden Syrian hamsters, but likely also to Chinese hamsters (*Cricetulus griseus*) and Roborovski Dwarf hamsters (*Phodopus roborovskii*), since these two species have been shown to also be highly susceptible (Bertzbach et al., 2021; Trimpert et al., 2020).

Materials and Methods

Experimental Design

This study was performed to quantify the susceptibility of Golden Syrian hamsters to an infection with SARS-CoV-2. All SARS-CoV-2 experimental studies were performed in the biosafety level 3 facilities at the Friedrich-Loeffler-Institut, Insel Riems, Germany.

Cell line and virus

Vero E6 cells (Cell Culture Collection in Veterinary Medicine, FLI) were cultured in minimal essential medium (MEM) containing 10% fetal calf serum (FCS) at 37°C and 5% CO₂. SARS-CoV-2 isolate 2019_nCoV Muc-IMB-1 (accession number LR824570; (Wolfel et al., 2020)) was kindly provided by Bundeswehr Institute of Microbiology, Munich, Germany. Virus propagation was maintained in Vero E6 cells in DMEM supplemented with 2% FCS. Prior to inoculation into hamsters, the virus stock was sequenced by using a generic metagenomics sequencing workflow as described previously (Wylezich, Papa, Beer, & Hoper, 2018) with some modifications. For reverse-transcribing RNA into cDNA, SuperScriptIV First-Strand cDNA Synthesis System (Invitrogen, Germany) and the NEBNext Ultra II Non-Directional RNA Second Strand Synthesis Module (New England Biolabs, Germany) were used, and library quantification was done with the QIAseq Library Quant Assay Kit (Qiagen, Germany). Libraries were sequenced without applying further SARS-CoV-2 enrichment using an Ion 530 chip and chemistry for 400 base pair reads on an Ion Torrent S5XL instrument (Thermo Fisher Scientific, Germany).

Virus titration - tissue culture infectious doses₅₀

Samples were serially diluted in MEM containing 2% FCS and 100 Units Penicillin / 0.1 mg Streptomycin (P/S) (Millipore Sigma, Germany). Vero E6 cells were incubated with 100 µl of ten-fold dilutions of sample dilutions added in quadruplicates for 1 h at 37°C before 100 µl MEM containing 2% FCS and P/S were added per well and plates were incubated for 5 days at 37°C and 5% CO₂. Supernatant was removed and cells were fixed with 4% formalin. Next, plates were stained with 1% crystal violet and titers were determined following the Spearman Kaerber method (Atkinson, 1961).

339 **Animal studies**

340 Male Golden Syrian hamsters (*Mesocricetus auratus*), 5-7 weeks old with a body weight of
341 80 – 100 g, were obtained from Janvier Labs, France. Three hamsters were housed in
342 individually ventilated cages (IVC). Animals had *ad libitum* access to food and water. The
343 animals' well-being and body weight were checked daily. Handling and sampling were
344 performed starting with the uninfected group and continuing from the low dose groups to
345 the high dose groups to minimize the contamination risk.

346 *Inoculation routes*

347 To determine the optimal inoculation route for SARS-CoV-2, we inoculated groups of eight
348 hamsters under isoflurane anaesthesia in parallel by the intranasal and orotracheal route with
349 100 µl containing 1×10^5 TCID₅₀. For the orotracheal challenge, the inoculum was
350 administered on the root of the tongue (Fig. 1a), ensuring aspiration of the inoculum with
351 the following inhalation. Oral swab samples were collected in DMEM containing P/S daily,
352 starting one day before inoculation. Nasal washes were collected under isoflurane
353 anaesthesia at days 2, 4, 6, 9, 11 and 13 by flushing 200 µl PBS along the animal's nose. At
354 14 dpi, hamsters were euthanized by deep isoflurane anaesthesia, cardiac exsanguination
355 and cervical dislocation, and nasal conchae, trachea and lung samples were collected and
356 stored at -80°C for virological analysis. Tissue samples were also stored in 4% neutral-
357 buffered formalin for histopathological analysis. Serum was separated from the collected
358 blood.

359 *Titration in hamsters*

360 To define the MID, three sets of experiments were performed, as the endpoint in the first
361 two studies was not reached. For animal welfare reasons, we continued the dilutions in the
362 second and third experiment, including an overlap ensuring the comparability of result.

363 First, hamsters were inoculated with a dose of 1×10^5 TCID₅₀ SARS-CoV-2 and with serial
364 dilutions from 1×10^3 to 1×10^{-1} TCID₅₀. Oral swab samples were collected at 1, 3, 5, and 6
365 dpi, nasal washes were collected at 2 and 4 dpi as described above. At 7 dpi, hamsters were
366 euthanized and sampled as described above.

367 In the second study, hamsters were inoculated with a serial dilution from 1×10^4 to 1×10^{-3}
368 TCID₅₀ SARS-CoV-2. Sampling and autopsies were performed as described above.

369 In the third study, hamsters were inoculated with 1×10^2 TCID₅₀, and with serial dilutions
370 from 1×10^0 to 1×10^{-4} TCID₅₀ SARS-CoV-2. This experiment was conducted for 10 days to
371 allow the follow-up of clinical and virological data after a 2 - 3 day delayed disease onset
372 in the groups inoculated with low doses. Oral swab samples were collected at 1, 3, 5, 6, 8

373 and 9 dpi, while nasal washes were collected at 2, 4 and 7 dpi. At 10 dpi, animals were
374 sacrificed and autopsied as described above.

375 *Ethical statement*

376 Ethical approval for this study was obtained from the competent authority of the Federal
377 State of Mecklenburg-Western Pomerania, Germany upon consultation with the Ethic
378 Committee of Mecklenburg-Western Pomerania (file number: 7221.3-1.1-049/20), on the
379 basis of national and European legislation, namely the EU council directive 2010/63/EU.
380 Animal studies are continuously monitored by the Animal Welfare Officer and were
381 approved by the Institutional Animal Care and Use Committee (IACUC).

382 **Total RNA extraction and SARS-CoV-2 detection**

383 Total RNA was extracted from swab, nose fluid and tissue samples as described earlier
384 (Schlottau et al., 2020). SARS-CoV-2 RNA was detected using “Envelope (E)-gene Sarbeco
385 6-carboxyfluorescein quantitative RT-PCR” (Corman et al., 2020) as described previously
386 (Schlottau et al., 2020). Viral genome copy numbers were calculated from standard curves
387 determined for 10^{-2} to 10^{-5} dilutions containing known copy numbers of SARS-CoV-2.

388 Selected samples were analysed for the presence of subgenomic RNA (sgRNA) as an
389 indication of virus replication, using a published protocol (Alexandersen et al., 2020;
390 Hoffmann et al., 2021). Quantitative Realtime PCR was performed with the qScript XLT
391 One-Step RT-qPCR ToughMix (QuantaBio/VWR). Primer sequences for the ORF 7a
392 detection are available upon request.

393 **Indirect SARS-CoV-2 RBD ELISA**

394 SARS-CoV-2 specific antibodies were detected using a published protocol (Wernike et al.,
395 2021) with the modification of using a 1:30.000 dilution of Protein A/G (Thermo Fisher) in
396 exchange of the multi-species conjugate.

397 To determine a cut-off-value and the diagnostic sensitivity of this modified assay, we tested
398 53 negative hamster sera and 227 sera of SARS-CoV-2 infected hamsters. The area under
399 the receiver operating characteristic (ROC) curve was used to determine the ELISA cut-off-
400 value. Statistical analyses were performed using MedCalc for Windows, version 19.4
401 (MedCalc Software, Ostend, Belgium). p-value < 0.01 was regarded as statistically
402 significant.

403 **Lateral flow device (LFD) Rapid Test**

404 Oral swab samples of groups inoculated with 1×10^3 to 1×10^2 TCID₅₀ SARS-CoV-2 were
405 collected at 6 dpi in lysis buffer supplied with the Nowcheck COVID-19 LFD (concile
406 GmbH, Freiburg, Germany) testkit. 120 μ l of the suspension was applied on the LFD and

407 incubated at RT for 15 min. We also analysed nasal wash samples collected at 7 dpi by
408 diluting 25 µl of the fluid into 300 µl of the supplied lysis buffer and applying 120 µl of this
409 mixture to the LFD. Evaluation of the control (C) and test (T) bands was performed
410 according to the instructions. LFDs were imaged and densitometry was performed on the C
411 and T bands using ImageJ (ImageJ 1.52a, Wayne Rasband, NIH, USA). These band
412 quantifications were used to calculate a T/C ratio. Standard deviation was calculated from
413 the T/C ratios within each respective group.

414 **Pathology of lung samples collected at 10 dpi**

415 During autopsy, the percentage of dark red discoloration per total lung tissue was estimated.
416 The left lung lobe was carefully removed, immersion-fixed in 10% neutral-buffered
417 formalin, paraffin-embedded, and 2-3-µm sections were stained with hematoxylin and eosin
418 (HE). Slides were scanned using a Hamamatsu S60 scanner, and evaluated using the
419 NDPview.2 plus software (Version 2.8.24, Hamamatsu Photonics, K.K. Japan). The lung
420 tissue was evaluated using a 500x500µm grid, and the extent of pneumonia-associated
421 consolidation was recorded as percentage of affected lung fields. Further, the lung was
422 examined for the presence of SARS-CoV2-characteristic lesions described for hamsters, i.e.
423 intra-alveolar, interstitial, peribronchial and perivascular inflammatory infiltrates, alveolar
424 edema, necrosis of the bronchial and alveolar epithelium, diffuse alveolar damage, vasculitis
425 or endothelialitis, pneumocyte type 2 hyperplasia/hypertrophy with bronchialisation and
426 atypical cells, and hypertrophy/hyperplasia of the bronchial epithelium. Archived lung
427 tissues from hamsters infected with $1 \times 10^{4.5}$ TCID₅₀ were included as positive controls.
428 Evaluation and interpretation were performed by a board-certified pathologist (DipLECVP)
429 following a post examination masking approach (Meyerholz & Beck, 2018).

430 **Sequence analysis**

431 Full genome sequences were generated via reference mapping with the Genome Sequencer
432 software suite (version 2.6; Roche; default software settings for quality filtering and
433 mapping), using SARS-CoV-2 strain 2019_nCoV_Muc_IMB1 (accession number
434 LR824570) as reference. Consensus sequences and underlying sequence reads were
435 visualized using Geneious Prime (10.2.3; Biomatters, Auckland, New Zealand). The
436 presence of single nucleotide variants (SNVs) was checked using the variant analysis tool
437 implemented in Geneious Prime (default settings, minimum variant frequency 0.02).

438 **Statistical analysis**

439 Mean values determined for the experimental groups were compared using analysis of
440 variance (ANOVA) with Tukey's post-hoc tests for multiple comparisons and non-

441 parametric Kruskal-Wallis test followed by the Dunn's method for multiple comparisons.
442 Data were analyzed using GraphPad Prism (version 9; GraphPad Software, Inc., CA, USA)
443 and SPSS software (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version
444 20.0, IBM Corporation, Armonk, NY, USA). *P*-value < 0.05 was considered statistically
445 significant.

446 For histopathology, data were tested for Gaussian distribution using the Shapiro-Wilk test,
447 followed by one-way ANOVA with Tukey's post-hoc tests for multiple comparison.

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466 All authors have read and agreed to the published version of the manuscript.

467 **Competing interests:**

468 The authors declare they have no competing interests.

469 **Data and materials availability:**

470 All data needed to evaluate the conclusions in the paper are present in the paper and/or the
471 Supplementary Materials.

Figures and Tables

Figure 1: Orotacheal SARS-CoV-2 inoculation results in increased weight loss and viral shedding. (A)

Inoculation technique. Anaesthetized animals are fixed with a stretched neck and extended tongue, inoculum is instilled on the root of the tongue. (B) Body weight curves of uninfected control group (grey), groups infected by the orotracheal (red) and nasal routes (green); $N = 19$. (C) Oral and nasal shedding between 1 and 13 dpi after orotracheal (red) and intranasal (green) inoculation; $N = 16$.

Figure 2: Mean and standard deviation of body weight per infection dose group. Changes in body weight (%) in relation to 0 dpi. Statistically significant differences in daily body weight changes are marked by an asterisk. $N = 69$, $P = 0.0001$ for all days. Further details on statistical analysis are shown in Suppl. Tab. 1.

Figure 3: Levels of lung affection after challenge with different infection doses. (A) macroscopically assessed level of affection of whole lung (%) during autopsy at 7 dpi and (B) at 10 dpi displayed as the mean value and standard deviation for each group. (C-D) Histopathology of hamster lungs, 10 days after orotracheal SARS-CoV-2 infection. Pneumonia-associated consolidation (C) and representative overviews (D) of the entire left lung lobe of (i) uninfected control; (ii) high dose control; (iii) infected with 1×10^2 TCID₅₀; (iv) infected with 1×10^0 TCID₅₀; (v) infected with 1×10^{-1} TCID₅₀; (vi) infected with 1×10^{-2} TCID₅₀; (vii) infected with 1×10^{-3} TCID₅₀; (viii) infected with 1×10^{-4} TCID₅₀. (B-D) $n = 3$.

Figure 4: Detailed histopathology of hamster lungs, 10 days after orotracheal SARS-CoV-2 infection.

Hematoxylin and eosin staining, bar 50 μm . (i) Uninfected control, no lung lesion, (ii) after $1 \times 10^{4.5}$ TCID₅₀ infection, showing vasculitis (arrow), type 2 pneumocyte hyperplasia and bronchialisation of alveoli (arrowhead), intra-alveolar erythrocytes (asterisk), (iii) after 1×10^2 TCID₅₀ infection, with vasculitis (arrow), type 2 pneumocyte hyperplasia and bronchialisation of alveoli (arrowhead) and perivascular infiltrates (asterisk), (iv) after 1×10^0 TCID₅₀ infection exhibiting perivascular (arrow) and alveolar (asterisk) infiltrates, type 2 pneumocyte hyperplasia and bronchialisation of alveoli (arrowhead), (v) after 1×10^{-1} TCID₅₀ infection, showing alveolar infiltrates (asterisk), type 2 pneumocyte hyperplasia and bronchialisation of alveoli (arrowhead), (vi) after 1×10^{-2} TCID₅₀ infection, with focal alveolar infiltrates (arrow) and edema (asterisk), (vii) after 1×10^{-3} TCID₅₀ infection, exhibiting focal hypertrophy/hyperplasia of bronchial epithelium (arrow), (viii) after 1×10^{-4} TCID₅₀, with focal alveolar infiltrates (arrow), (ix) after 1×10^{-4} TCID₅₀ showing focal perivascular infiltrates (arrow).

Figure 5: Viral shedding in oral swab samples. (A) Genome copy number (Log_{10}) of viral RNA and (B) replication competent virus (Log_{10} TCID₅₀/ml) detected in the oral swab samples of all hamsters. All groups were analysed, negative results are not shown. p-values are indicated for statistically significant differences in RNA levels. Details on statistical analysis are shown in Suppl. Tab. 1. Colors match and represent results from the same hamsters as shown before and following.

Figure 6: Viral shedding in nasal washes. (A) Genome copy number (Log_{10}) of SARS-CoV-2 RNA and (B) replication competent virus (Log_{10} TCID₅₀/ml) detected in the nasal wash samples of all hamsters. All groups were analysed, negative results are not shown. Details on statistical analysis are shown in Suppl. Tab. 1. Colors match and represent results from the same hamsters as shown before and following.

Figure 7: Time-dependent shedding of replication competent virus and change in body weight after infection with decreasing SARS-CoV-2 doses. Body weight development is shown by arrows; \uparrow increasing from 1-7 or 10 dpi; \downarrow decreasing from 1 – 7 or 10 dpi; $\downarrow\uparrow$ decrease until 7 dpi followed by increase; $\uparrow\downarrow$ increase until 4 dpi, followed by decrease until 10 dpi. Colors match and represent results from the same hamsters as shown before and following.

515 **Figure 8: Genome copy number (Log₁₀) of SARS-CoV-2 RNA and replication competent virus (Log₁₀**
516 **TCID₅₀/ml) detected in the respiratory tract.** (A) Genome copy number (Log₁₀) of viral RNA and (B) replication
517 competent virus (Log₁₀ TCID₅₀/ml) detected in the respiratory tract samples at 7 dpi. (C) Genome copy number (Log₁₀)
518 of viral RNA and (D) replication competent virus (Log₁₀ TCID₅₀/ml) detected in the respiratory tract samples at 10 dpi.
519 Statistically significant differences in RNA levels were determined at 7 dpi. Further details on statistical analysis are
520 shown in Suppl. Tab. 1 and Suppl. Tab. 3. Colors match and represent results from the same hamsters as shown before.

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Figure 1

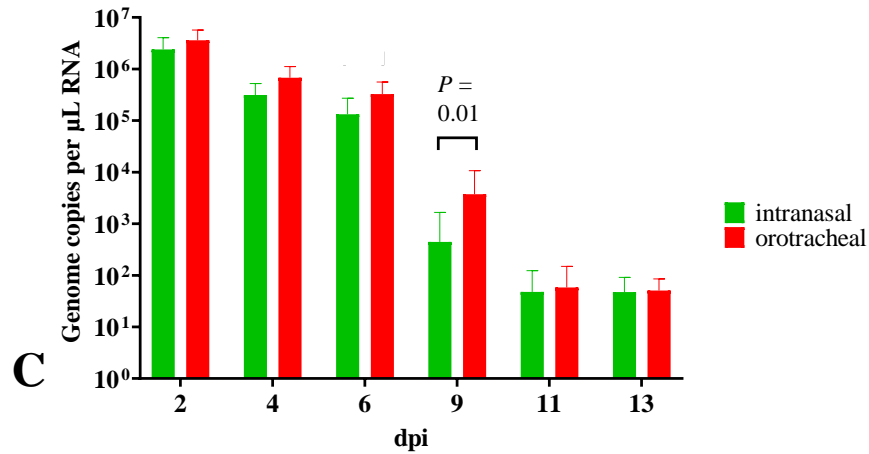
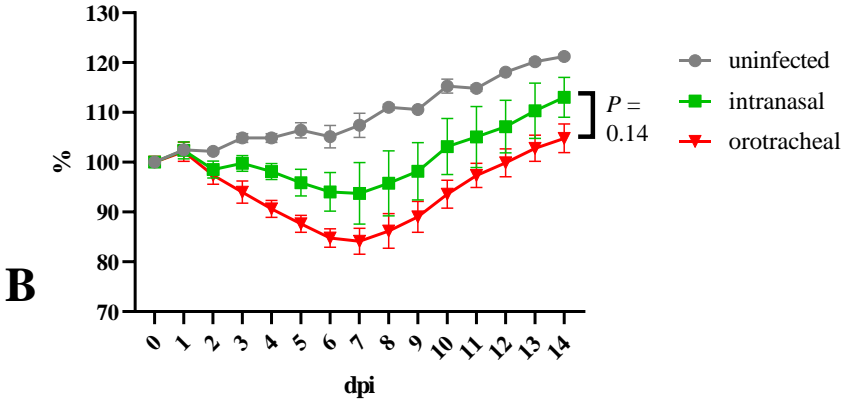


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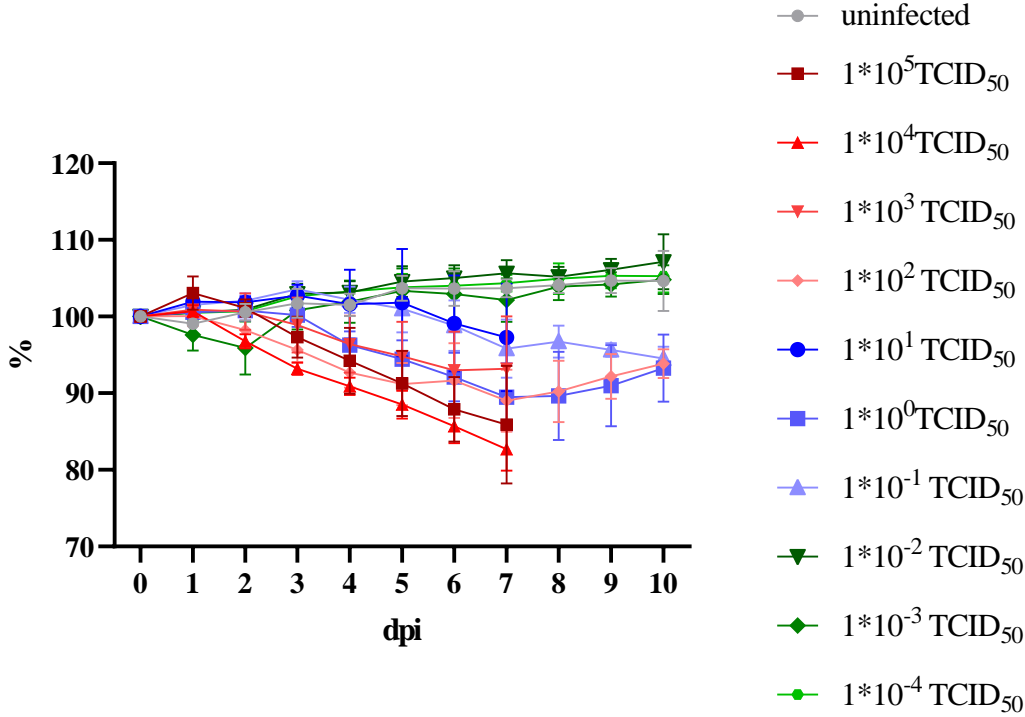


Figure 3 A + B

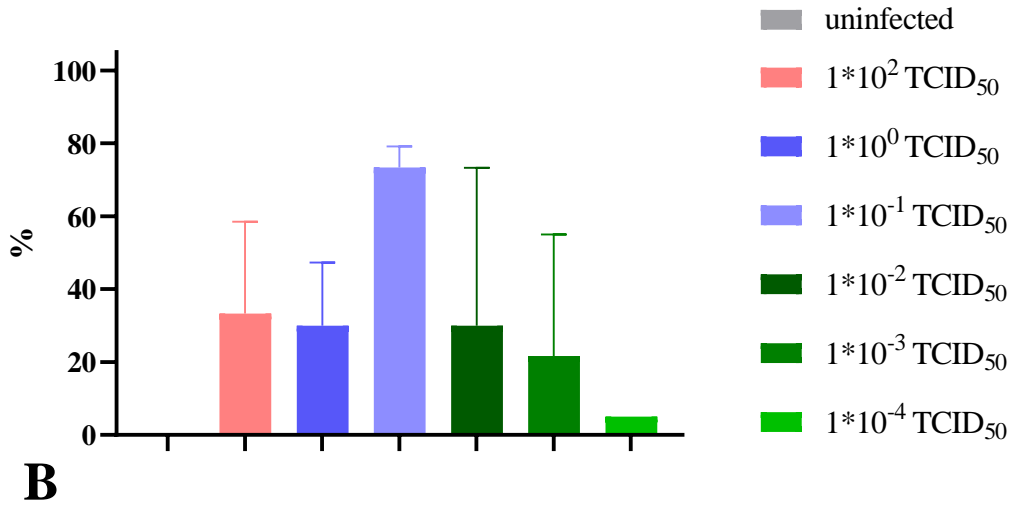
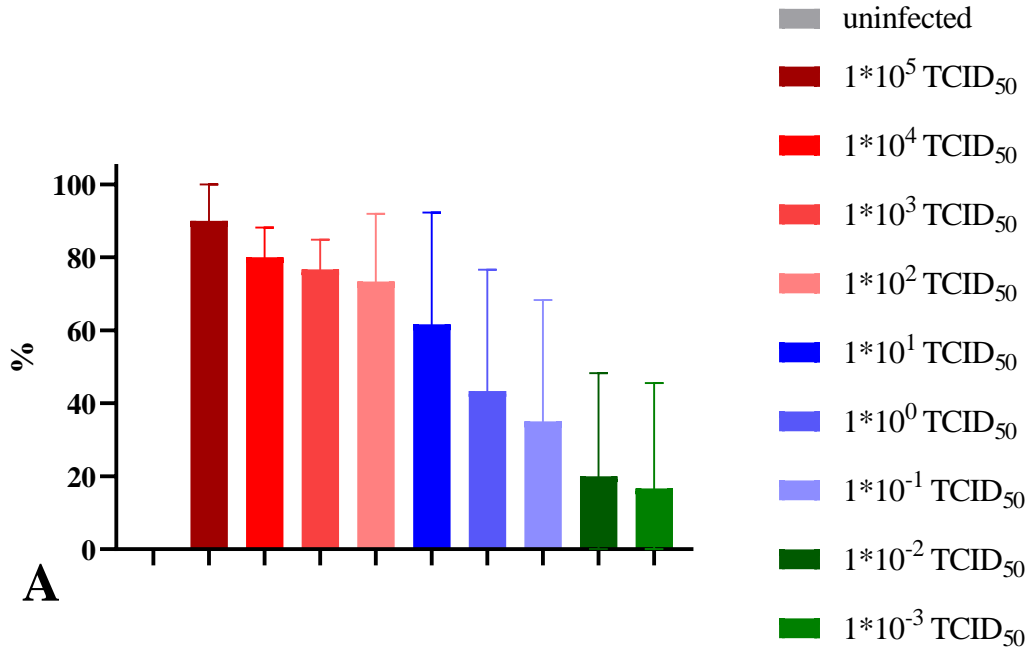


Figure 3 C + D

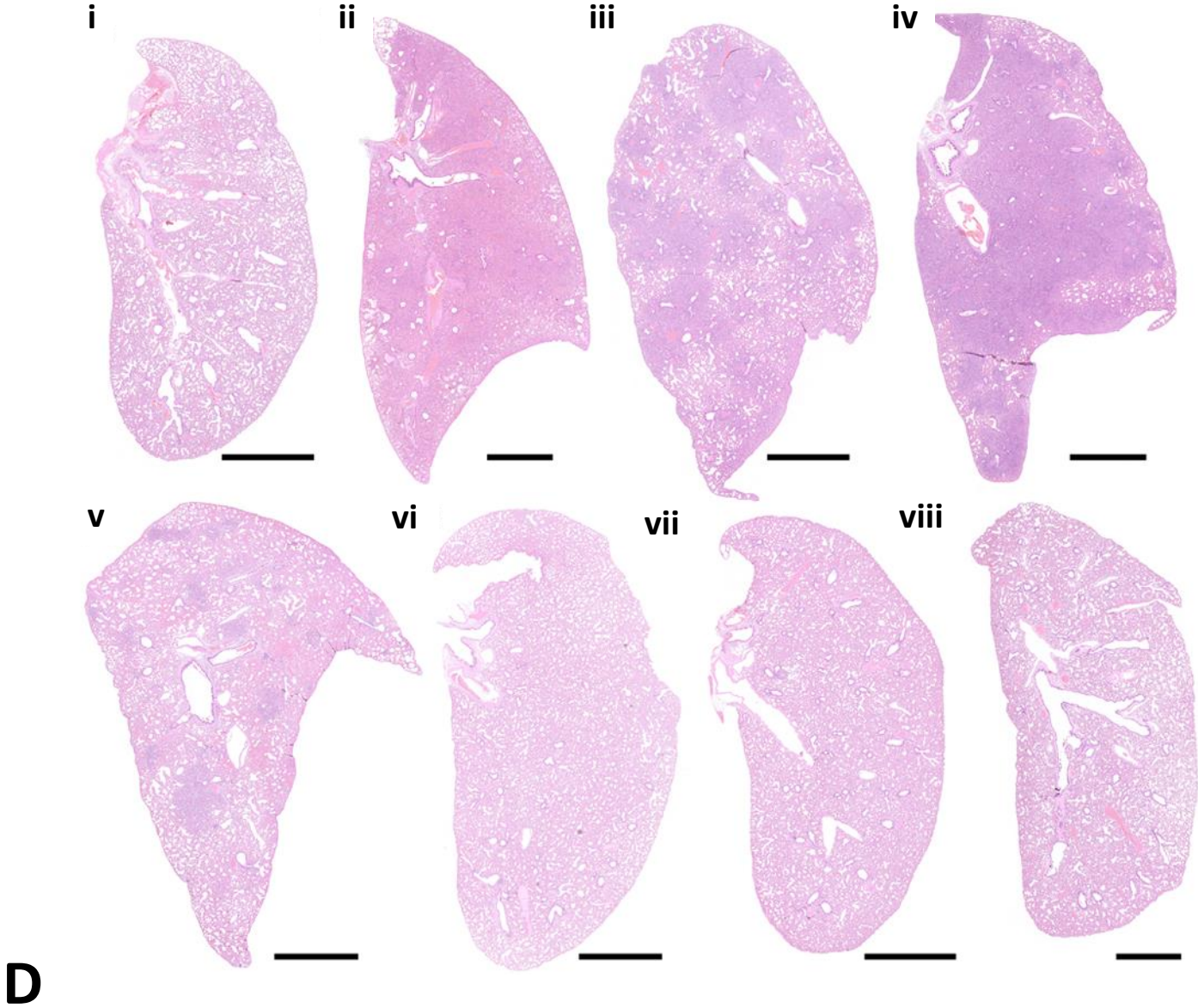
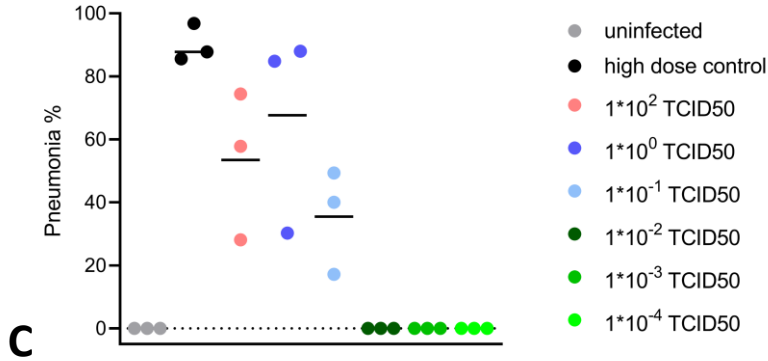


Figure 4

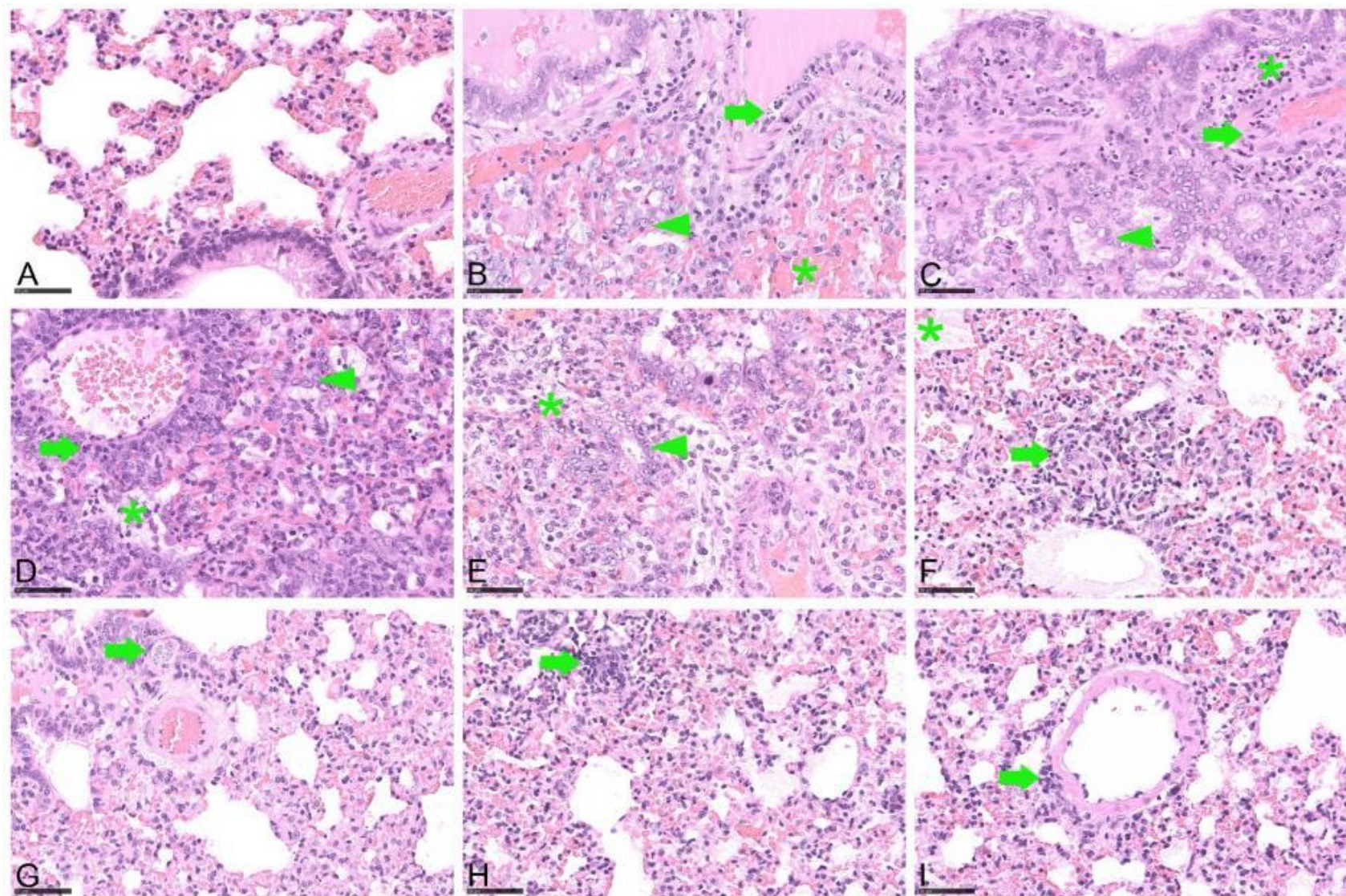


Figure 5

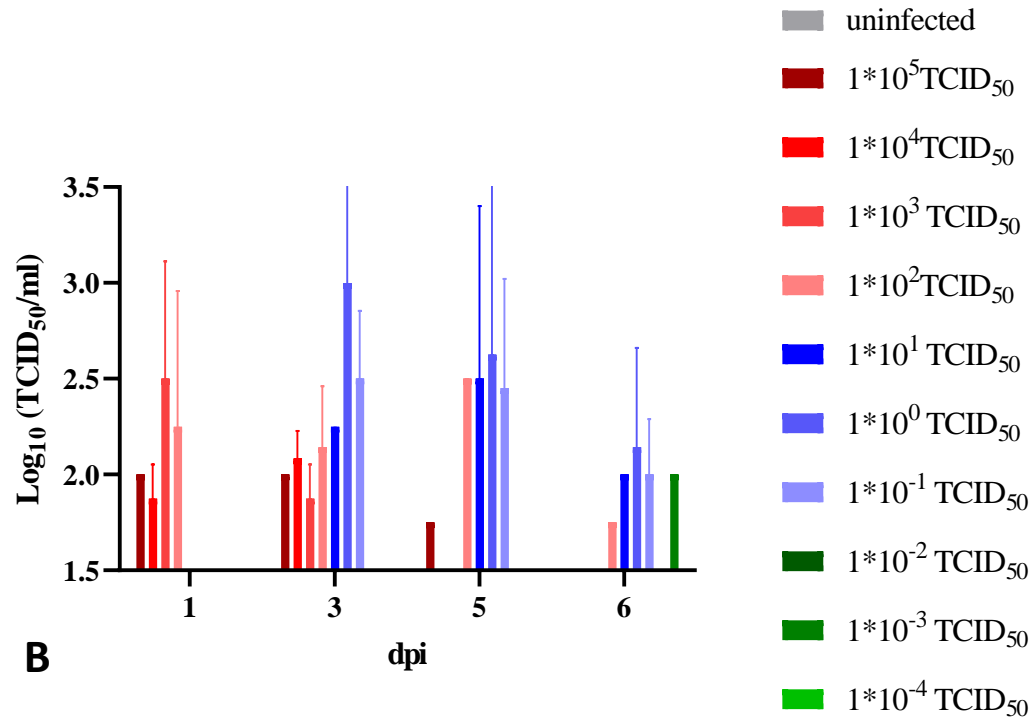
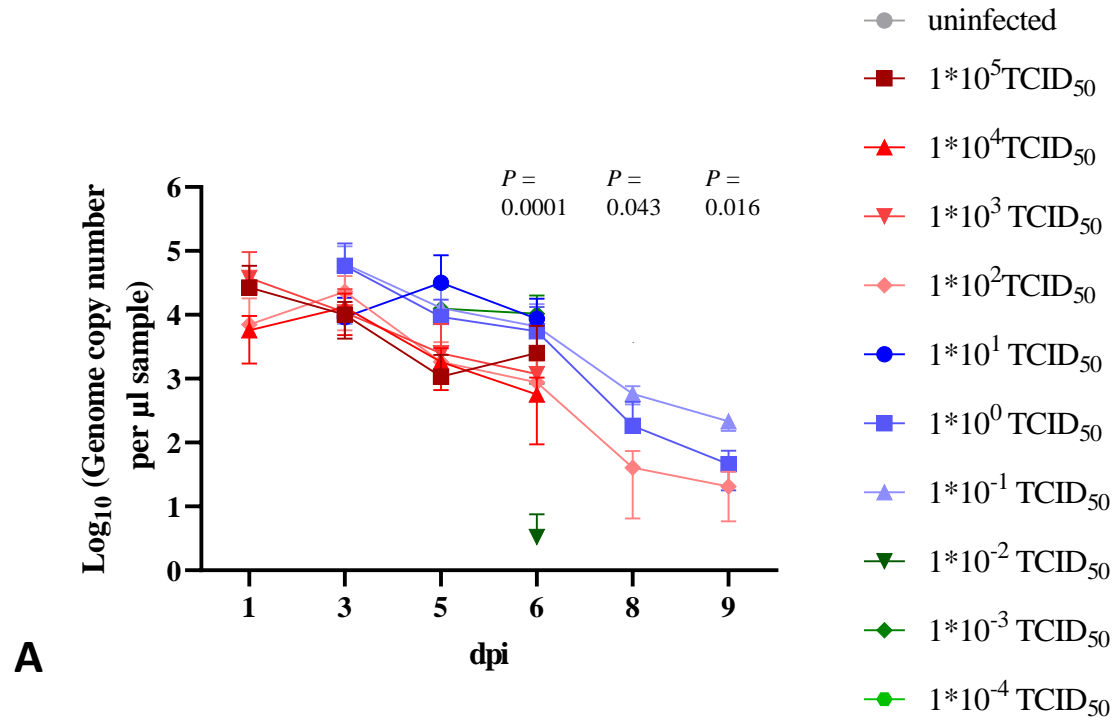


Figure 6

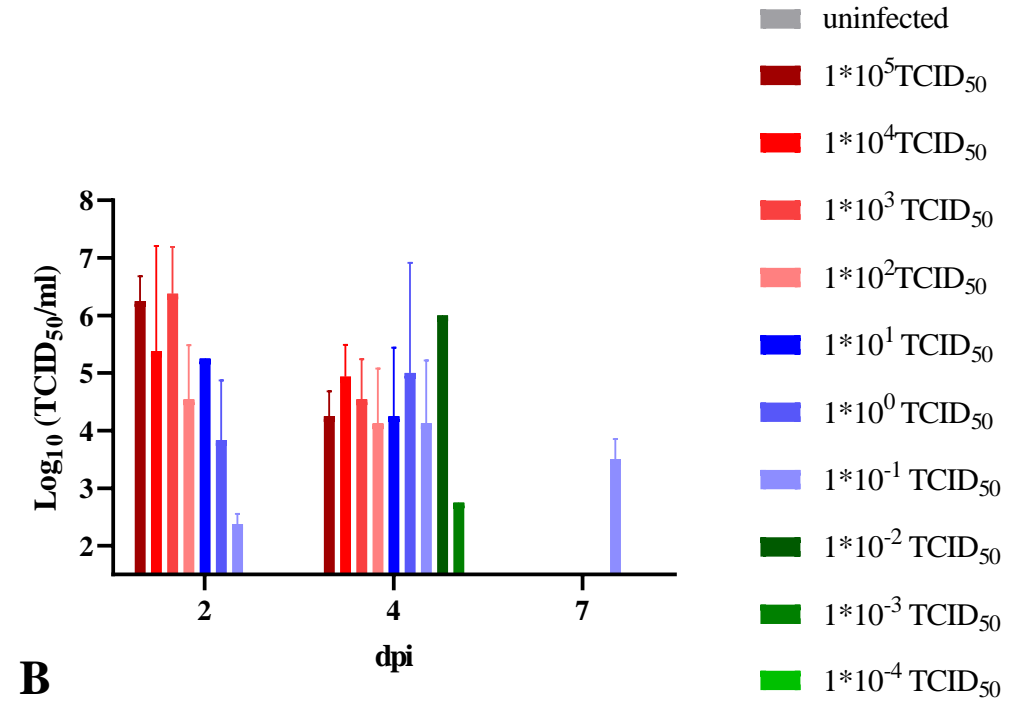
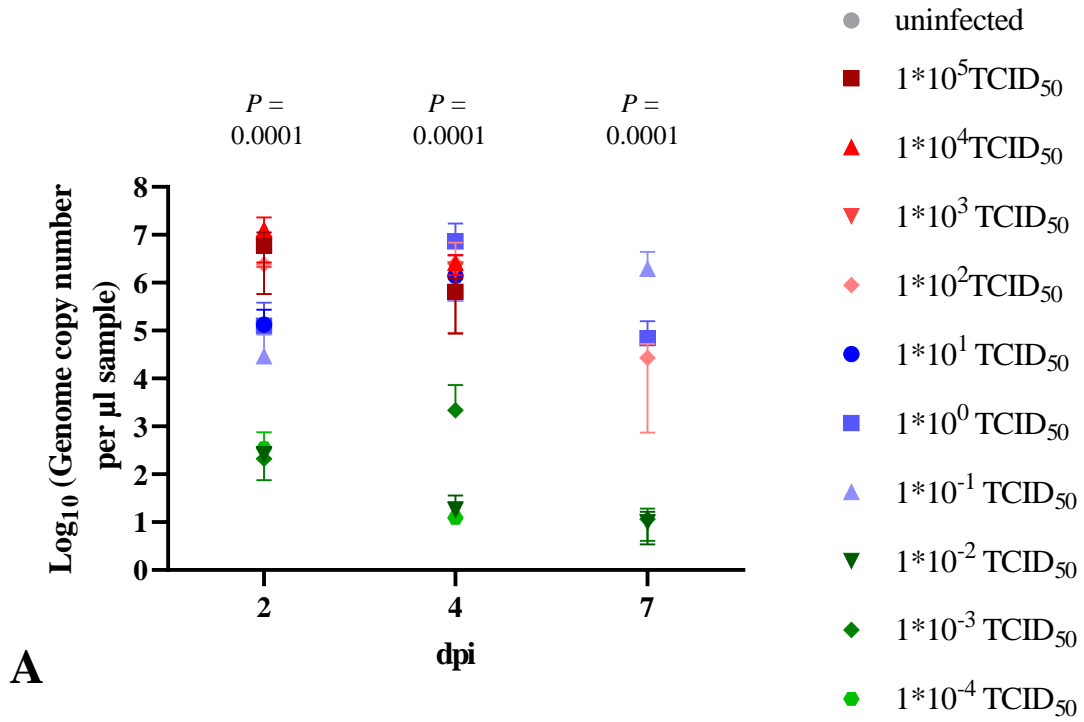


Figure 7

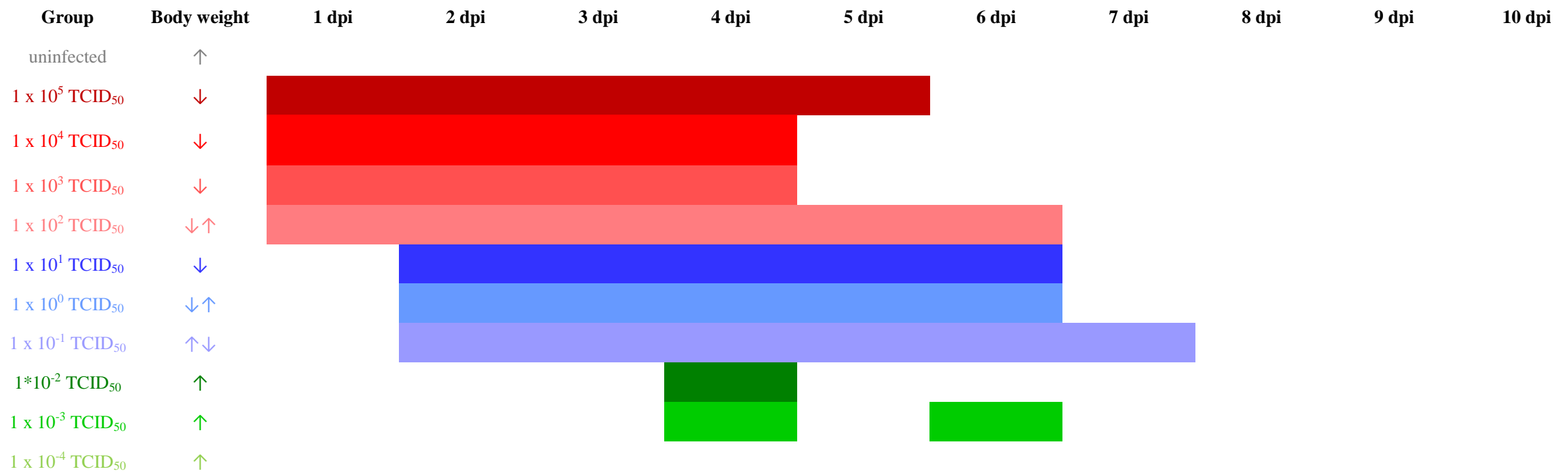
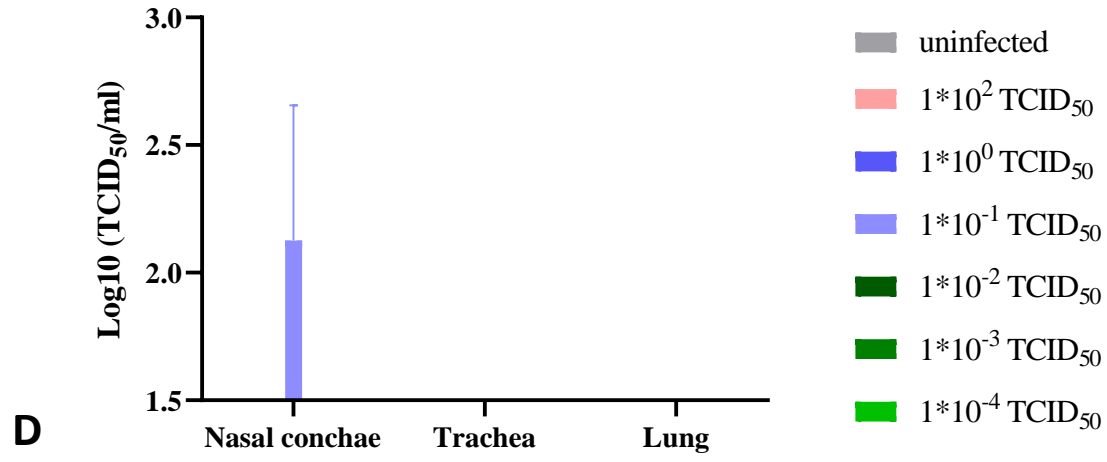
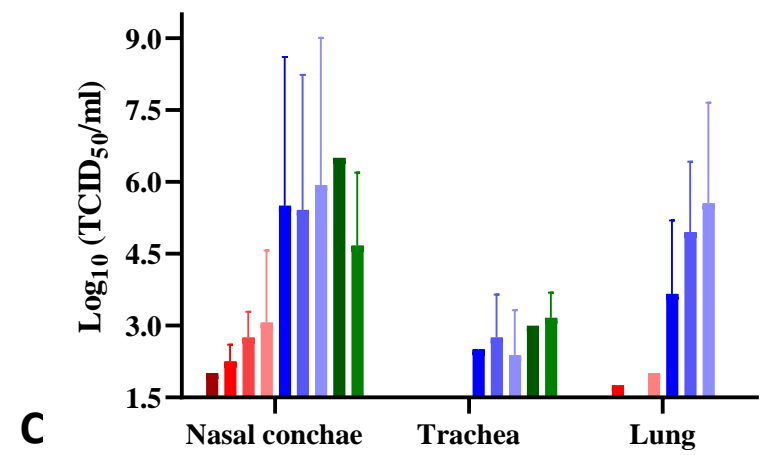
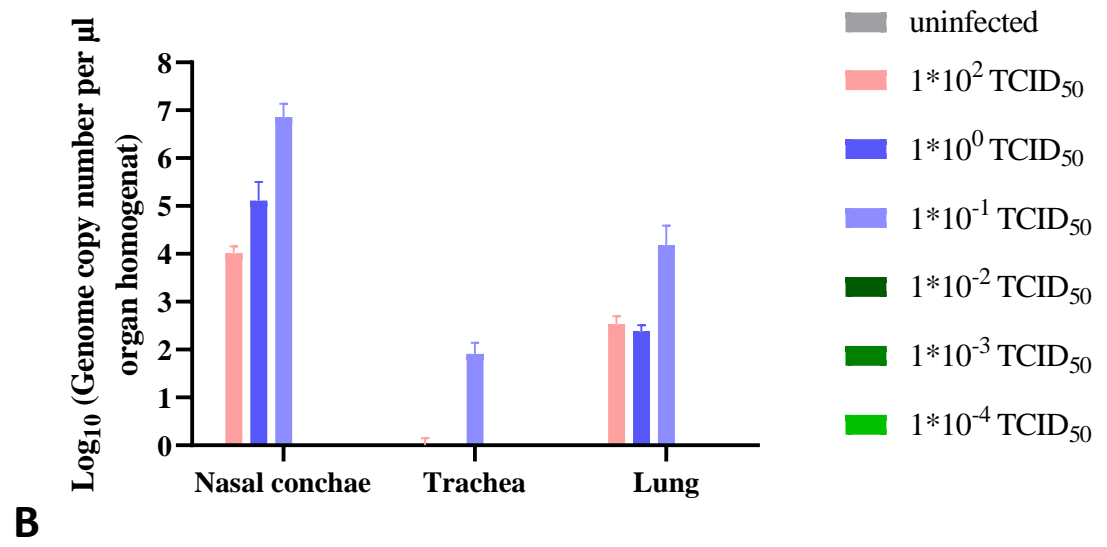
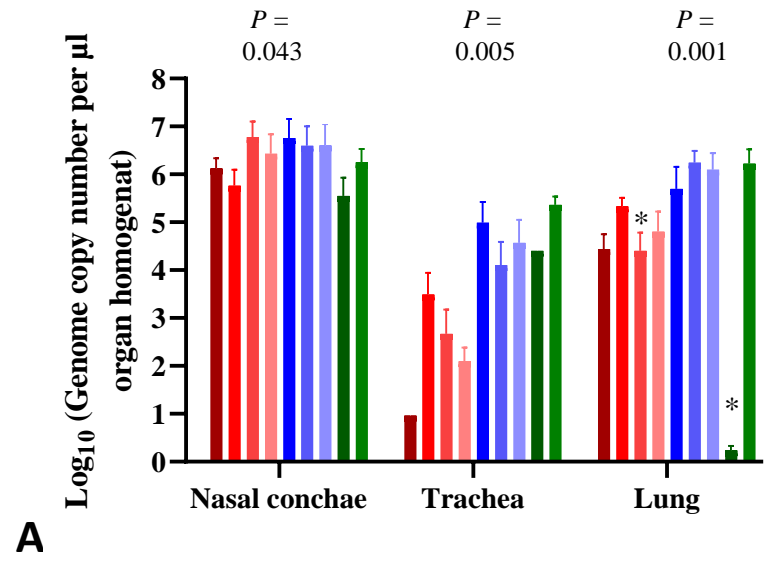
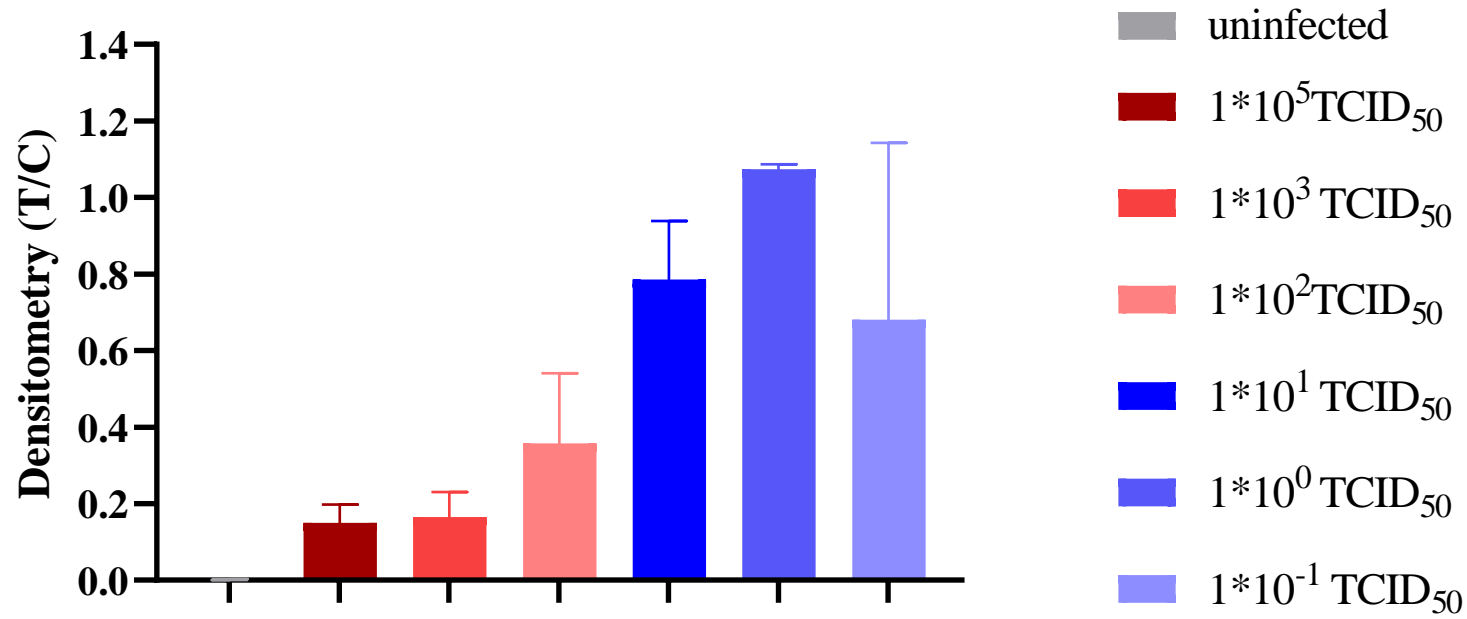


Figure 8

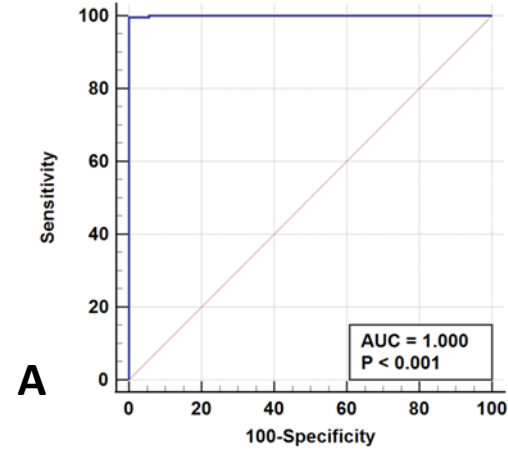


Supplementary Figure 1



Group	uninfected	$1 \cdot 10^5$ TCID ₅₀	$1 \cdot 10^3$ TCID ₅₀	$1 \cdot 10^2$ TCID ₅₀	$1 \cdot 10^1$ TCID ₅₀	$1 \cdot 10^0$ TCID ₅₀	$1 \cdot 10^{-1}$ TCID ₅₀
<i>N</i>	3	3	3	3	3	3	3
Ct (SD)	-	27,88 (2,68)	33,04 (0)	28,95 (1,65)	27,13 (4,38)	24,37 (1,53)	27,52 (1,13)
TCID50		$< 10^{1,5}$	$< 10^{1,5}$	$< 10^{1,5}$	$10^{1,74}$	$10^{2,56}$	$< 10^{1,5}$
TCID50 SD		0	0	0	$10^{1,51}$	$10^{1,65}$	0

Supplementary Figure 2



ROC analysis

Area under the ROC curve (AUC)

Area under the ROC curve (AUC)	1.000
Significance level P (Area=0.5)	<0.0001

Criterion values and coordinates of the ROC curve

Criterion	Sensitivity	Specificity
≤14.11	99.56	100

