The Lancet Microbe

Experimental transmission studies of SARS-CoV-2 in fruit bats, ferrets, pigs and chickens

--Manuscript Draft--

Manuscript Number:	THELANCETMICROBE-D-20-00091
Article Type:	Article (Original Research)
Keywords:	Sars-Cov-2; animal model; Rousettus fruit bat; ferret; pig; chicken
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Manuscript Region of Origin:	GERMANY
Abstract:	Background A novel zoonotic SARS-related coronavirus emerged in China at the end of 2019. The novel SARS-CoV-2 became pandemic within weeks and the number of human infections and severe cases is increasing. The role of potential animal hosts is still understudied. Methods We intranasally inoculated fruit bats (Rousettus aegyptiacus ; n=9), ferrets (n=9), pigs (n=9) and chickens (n=17) with 10 5 TCID 50 of a SARS-CoV-2 isolate per animal. Animals were monitored clinically and for virus shedding. Direct contact animals (n=3) were included. Animals were humanely sacrificed for virological and immune-pathohistological analysis at different time points. Findings Under these settings, pigs and chickens were not susceptible to SARS-CoV-2. All swabs as well as organ samples and contact animals remained negative for viral RNA, and none of the animals seroconverted. Rousettus aegyptiacus fruit bats experienced a transient infection, with virus detectable by RT-qPCR, immunohistochemistry (IHC) and in situ hybridization (ISH) in the nasal cavity, associated with rhinitis. Viral RNA was also identified in the trachea, lung and lung associated lymphatic tissue. One of three contact bats became infected. More efficient virus replication but no clinical signs were observed in ferrets with transmission to all direct contact animals. Prominent viral RNA loads of up to 10 4 viral genome copies/ml were detected in the upper respiratory tract. Mild rhinitis was associated with viral antigen detection in the respiratory and olfactory epithelium. Both fruit bats and ferrets developed SARS-CoV-2

	reactive antibodies reaching neutralizing titers of up to 1:1024. Interpretation Pigs and chickens could not be infected intranasally by SARS-CoV-2, whereas fruit bats showed characteristics of a reservoir host. Virus replication in ferrets resembled a subclinical human infection with efficient spread. These animals might serve as a useful model for further studies e.g. testing vaccines or antivirals. Funding Intramural funding of the German Federal Ministry of Food and Agriculture provided to the Friedrich-Loeffler-Institut.

Experimental transmission studies of SARS-CoV-2 in fruit bats, ferrets, pigs and chickens

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Lancet Microbe

Main Text: max 3500 words: 3579

Abstract: max 300 words: 300

References: max 30: 30

Abstract

Background

A novel zoonotic SARS-related coronavirus emerged in China at the end of 2019. The novel SARS-CoV-2 became pandemic within weeks and the number of human infections and severe cases is increasing. The role of potential animal hosts is still understudied.

Methods

We intranasally inoculated fruit bats (*Rousettus aegyptiacus*; n=9), ferrets (n=9), pigs (n=9) and chickens (n=17) with 10^5 $TCID_{50}$ of a SARS-CoV-2 isolate per animal. Animals were monitored clinically and for virus shedding. Direct contact animals (n=3) were included. Animals were humanely sacrificed for virological and immuno-pathohistological analysis at different time points.

Findings

Under these settings, pigs and chickens were not susceptible to SARS-CoV-2. All swabs as well as organ samples and contact animals remained negative for viral RNA, and none of the animals seroconverted. *Rousettus aegyptiacus* fruit bats experienced a transient infection, with virus detectable by RT-qPCR, immunohistochemistry (IHC) and in situ hybridization (ISH) in the nasal cavity, associated with rhinitis. Viral RNA was also identified in the trachea, lung and lung associated lymphatic tissue. One of three contact bats became infected. More efficient virus replication but no clinical signs were observed in ferrets with transmission to all direct contact animals. Mild rhinitis was associated with viral antigen detection in the respiratory and olfactory epithelium. Prominent viral RNA loads of up to 10^4 viral genome copies/µl were detected in the upper respiratory tract of both species, and both species developed SARS-CoV-2 reactive antibodies reaching neutralizing titers of up to 1:1024.

Interpretation

Pigs and chickens could not be infected intranasally by SARS-CoV-2, whereas fruit bats showed characteristics of a reservoir host. Virus replication in ferrets resembled a subclinical human infection with efficient spread. These animals might serve as a useful model for further studies e.g. testing vaccines or antivirals.

Funding

Intramural funding of the German Federal Ministry of Food and Agriculture provided to the Friedrich-Loeffler-Institut.

Research in context

Evidence before this study

While the first SARS-CoV pandemic could be controlled at an early stage before substantial spread occurred, SARS-CoV-2 has disseminated globally within weeks, and the number of infected humans continues to increase at alarming rates. Although the pandemic is driven by human-to-human transmission, the large number of infected humans also raises the question whether anthropo-zoonotic infections occur by contact of infected humans with animals, which may lead to further spread and endemicity of SARS-CoV-2 in companion and farmed animals. However, contact with zoo and wild animals is also relevant, since bats are considered as reservoir hosts. Infection of ferrets and cats by SARS-CoV has been demonstrated experimentally and naturally. Field infections of pigs were also reported, while poultry did not appear to be affected. In addition to exploring potentially important epidemiological animal reservoirs, suitable animal models for testing vaccines and antiviral drugs are urgently required. For SARS-CoV, non-human primate and ferret models were used. First reports now indicate similar results for SARS-CoV-2. However, data on the susceptibility of bat species, as well as detailed analyzes including viral loads and histopathology of SARS-CoV-2 in ferrets and their contact animals are lacking. Furthermore, the first study on the inoculation of pigs and chickens requires confirmation and extension.

Added value of this study

In our study, four relevant animal species were intranasally inoculated: fruit bats, ferrets, pigs and chickens. Neither pigs (n = 9) nor chickens (n = 17) showed any signs of infection and none of the contact animals became infected. This is of particular importance for risk analysis in these farmed animals, which are kept in large numbers in contact with humans. Interestingly, this differs to the findings reported after infection of pigs with SARS-CoV. In contrast, the virus replicated in the upper respiratory tract of fruit bats, and was transmitted to contact animals. This indicates that fruit bats, which are kept and bred in captivity can serve as reservoir host model, but also emphasizes the risk to free-living bats e.g. in ecological bat protection programs. Finally, ferret infections resulted in a very high replication rate of SARS-CoV-2 in the nasal cavity, as confirmed by immunohistochemistry and in situ hybridization. The transmission to contacts was highly efficient and high virus titers were detected in the nasal cavity of contacts. We demonstrate by next-generation sequencing that no viral adaptions occurred during infection of ferrets with a human SARS-CoV-2 isolate. Our results suggest that

the ferret is a highly suitable model for testing vaccines and antiviral treatment for their effect on viral excretion and transmission.

Implications of all available evidence

Our results are in accordance with all so far available study results, indicating a negligible risk of anthropo-zoonotic transmission to pigs and chickens, but relevant for bats and ferrets. Fruit bats show a different pattern of infection than ferrets, but both can serve as model animals. However, ferrets next to non-human primates, most closely mimic human infection and are therefore suggested as animal model for testing vaccines and antivirals.

Introduction

Coronaviruses are enveloped viruses with a large single-stranded RNA genome of positive polarity (ICTV; (1)). While numerous coronaviruses have been identified in animals or humans (2), two recent ß-coronaviruses are remarkable: the Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) (3, 4); and the Middle East Respiratory Syndrome coronavirus (MERS) (5, 6). Both viruses presumably originate from bats (7), but adapted to further animals like palm civets (8) or dromedary camels (6) from which sporadic or sustained spill-over infections occurred resulting in abundant (SARS-CoV) (9), or limited human-to-human infection chains (MERS-CoV) (10), which finally could be controlled.

Since the end of 2019, another SARS-CoV-related zoonotic ß-coronavirus - Severe Acute Respiratory Syndrome coronavirus 2 (SARS-CoV-2) – has been spreading pandemically from Wuhan, China. As for SARS-CoV and MERS-CoV, ß-coronaviruses very closely related to SARS-CoV-2 were found in bats (11, 12) and Pangolins (13). Whether the pandemic started by a direct spill-over transmission of the SARS-CoV-2 ancestor from bats to humans or via another intermediate mammalian host providing further adaptation to the human host, is still under debate.

Due to the zoonotic origin of SARS-CoV-2 from the likely bat reservoir, several questions concerning the susceptibility of animals arise: (i) susceptibility of putative reservoir hosts like bats, (ii) risk of possible anthropo-zoonotic spill-over infections to farmed animals, and (iii) suitable animal models of human infection to study antivirals and vaccine prototypes. Viral receptor structure may be used as an important predictive factor of susceptibility: Recently it was shown, that SARS-CoV and SARS-CoV-2 employ the same receptor molecule, ACE2 (14), for contact with the receptor-binding-domain (RBD) of the spike (S) protein. Based on molecular studies the ACE2 proteins of human primates, pigs, cats and ferrets closely resembled the human ACE2 receptor. Therefore, these species may be susceptible to SARS-CoV-2 infection as has been shown for SARS-CoV and MERS-CoV (15, 16). During the last influenza A virus H1N1 pandemic in 2009, the virus was transmitted from humans to pigs, and is now endemic in pig holdings worldwide (17), posing a continuous risk of zoonotic spill-back infections. The potential impact of a SARS-CoV-2 infection of pigs therefore is very high. In this context, it is also very important to prove that chickens are not susceptible to SARS-CoV-2. Finally, bats as a major reservoir host of ß-coronaviruses and especially SARS-CoV-related viruses (18) need to be further studied to better understand the viral replication, shedding, transmission or persistence in a putative reservoir host species.

Here, we intranasally inoculated fruit bats, ferrets, pigs and chickens with SARS-CoV-2 and investigated virus replication and shedding, the clinical course, pathohistological changes as well as transmission.

Materials and methods

Ethics

The animal experiments were evaluated and approved by the ethics committee of the State Office of Agriculture, Food safety, and Fishery in Mecklenburg – Western Pomerania (LALLF M-V: LVL MV/TSD/7221.3-2-010/18-12). All procedures were carried out in approved biosafety level 3 (BSL3) facilities.

Animals & study design

Twelve Egyptian fruit bats (*Rousettus aegyptiacus*, mixed sexes and ages, originating from the FLI breeding colony), twelve ferrets (*Mustela putorius*, female, nine-twelve month old, originating from the FLI breeding colony), twelve male pigs (*Sus scrofa domesticus*, nine weeks old; raised by BHZP GmbH (Dahlenburg, Germany)) and twenty chickens (*Gallus gallus domesticus* (white leghorn, five weeks old, mixed sexes, hatched from SPF-eggs (VALO BioMedia GmbH, Osterholz-Scharmbeck, Germany)) were used. Fruit bats as well as pigs were kept in groups of four and six in different cages and stables, respectively. Ferrets were kept altogether in one cage and chickens were kept in free run conditions with nests and perches. All animals were offered water ad-libitum, and were fed and checked for clinical scores daily and by video supervision during the 21-day study period. All animals tested negative for SARS-CoV-2 genome and antibodies prior to the experiment.

Nine fruit bats, ferrets and pigs were infected intranasally while the 17 chickens received oculo-oronasally 10^5 $TCID_{50}$ SARS-CoV-2 2019_nCoV Muc-IMB-1 per animal (kindly provided by R. Woelfel, German Armed Forces Institute of Microbiology, Munich, Germany). The inoculum was administered to both nostrils using a pipette (fruit bats, ferrets and chickens) or an intranasal spraying device (pigs) (Teleflex Medical GmbH, Germany). To test viral transmission by direct contact, three naïve sentinel animals were added 24 hours post inoculation. Animals were monitored for body temperature (pigs, fruit bats, ferrets) and body weight (fruit bats, ferrets) throughout the experiment. Viral shedding was tested on nasal washes and rectal swabs (ferrets), oral swabs and pooled feces samples (fruit bats), nasal and rectal swabs (pigs) or oropharyngeal and cloacal swabs (chicken) on 2, 4, 8, 12, 16, and 21 days post infection (dpi). On day 4 (animals #1,#2), day 8 (animals #3,#4) and 12 dpi (animals #5,#6), two or three (chickens) inoculated animals of each species were sacrificed. All remaining animals, including the sentinels, were euthanized on day 21 pi (Fig. 1). All animals were subjected to autopsy. For virus detection and histopathology: nasal conchae, trachea, lung,

tracheobronchial lymph node (not for chicken), heart, liver, spleen, duodenum, colon/cecum, pancreas, kidney, adrenal gland, skeletal muscle, skin, brain were collected.

Further materials and methods

For details on virus, cells, virus titration, RNA extraction, RT-qPCR, next-generation sequencing, antibody detection, histopathology, immunohistochemistry and in-situ hybridization, please refer to the materials&methods section in the supplement.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. MB had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Egyptian fruit bats

No clinical signs (such as anorexia or respiratory signs), elevated temperatures, body weight loss or mortality were observed in any of the bats.

Oral virus shedding was observed in infected bats from 2 to 12 dpi, with one out of the three remaining infected bats still virus positive at 12 dpi (fruit bat #8), the other ones were sacrificed as scheduled on 4 and 8 dpi. Oral shedding was also detected in two out of three contact animals until 8 dpi (fruit bats #10 & #11, Fig 2A). Virus was isolated from one oral swab on day 2 pi ($10^{1.75}$ $TCID_{50}$/ml, fruit bat #8) (Fig 3A). Fecal shedding was observed in all three cages at 2 and 4 dpi with Cq values ranging from 29.54 to 36.43 (data not shown). SARS-CoV-2 genome (Cq values between 23.16 and 38.97; $1.96x10^4$ to $1.32x10^1$ genome copies/µl RNA) was detected in the nasal epithelium in seven of nine infected bats sacrificed at 4, 8 and 21 dpi, with one animal each giving negative results at 8 and 12 dpi respectively. Interestingly, the nasal epithelium of one contact animal contained viral RNA on day 21 pi (Cq value 32.89; 3.12 genome copies/µl RNA). At 4 dpi, genome was also detected in respiratory tissues (trachea (2/2), lung (1/2) and lung associated lymphatic tissue (2/2)) and at lower levels in heart, skin, duodenum and adrenal gland (one animal at 4 dpi) and in duodenum, skin and adrenal gland (one animal) on 8 dpi (Fig 2C). Virus could be cultivated from the trachea ($10^{2.25}$ $TCID_{50}$/ml) and the nasal epithelium ($10^{1.75}$ $TCID_{50}$/ml) of fruit bat #2 at 4 dpi. For all other RT-qPCR positive samples, cultivation of replicating virus was impossible.

SARS-CoV-2 reactive antibodies were observed in all inoculated bats by iIFA starting from 8 dpi as well as in one contact bat (#10) on day 21 with titers ≥16. Only a slight increase in antibody levels could be observed between day 8 and day 21 (with varying titers between 16 and 64). Neutralizing antibodies could be detected in the same fruit bats with titers up to 64 (Table 1A).

Necropsy revealed no pathological alterations in any of the inoculated or contact bats. At 4 dpi, minimal to mild rhinitis was found, with epithelial necrosis, edema, infiltrating lymphocytes and neutrophils, and intraluminal cellular debris (Fig 4A). Immunohistochemistry (IHC) revealed viral antigen, restricted to foci in the nasal respiratory epithelium and single cells of the non-respiratory, stratified epithelium (fruit bats #1,2; Fig. 4B,C), confirmed by in situ hybridization (ISH). Although viral antigen was absent at later time points, moderate rhinitis was detected at 8 dpi (fruit bats #3, #4), 12 dpi (fruit bat #6), and to a milder extent at 21 dpi (fruit bats #7, #11), indicating previous replication sites. Despite the detection of viral RNA by

RT-qPCR, no viral antigen was detectable in the lung. However, single infected animals at 4, 8 and 12 dpi as well as one contact animal presented with interstitial, mixed cellular infiltrates and in one case also with perivascular lymphocytic cuffs (Table S.1, Fig. S.1A-C). Minimally increased numbers of alveolar macrophages were found at all time points. None of the other organs were found positive for viral antigen and no further relevant morphological changes were detected.

Ferrets

None of the ferrets showed clinical signs or loss of body weight during the study period. Body temperatures remained normal.

Viral shedding was detected in nasal washes in eight out of nine infected ferrets between day 2 and day 8 pi with Cq values ranging from 21.77 to 36.35 ($8.44x10^3$ to 0.34 genome copies/µl RNA). Virus isolation was successful from nasal washes collected on days 2 pi (ferret #2,3,4: $10^{2.5} – 10^{2.875}$ $TCID_{50}$/ml) and 4 pi (ferret #4; $10^{2.75}$ $TCID_{50}$/ml) (Fig. 3B). All three naïve ferrets were infected by direct contact to the other inoculated ferrets. The first RT-qPCR positive nasal wash sample in a contact ferret was observed on 8 dpi. Ferret #12 showed viral shedding on 8 and 12 dpi (Cq values 37.03 and 28.59, respectively). Ferret #11 had positive results in nasal washes between day 12 and 21 pi (Cq values 37.39, 26.15 and 36.93) and ferret #10 on day 16 and 21 pi (Cq values 28.04 and 30.00) (Fig. 2B). Analysis of the rectal swabs showed minor amounts of viral RNA in individual ferrets at singular time points with Cq values between 33.97 and 38.45 (data not shown).

The two ferrets (ferret #1,#2) sacrificed at 4 dpi were RT-qPCR positive in different tissues (lung, muscle, skin, trachea, lung lymph node and colon) with the highest viral genome load in the nasal conchae (Cq values 24.31 and 26.21; $1.93x10^3 – 5.26x10^2$ genome copies/µl RNA). The two ferrets euthanatized at 8 dpi (ferret #3,#4) were positive in the nasal conchae (Cq values 34.77 and 21.57; $1.61 – 1.21x10^4$ genome copies/µl RNA). On 12 dpi, one of two ferrets was also positive in the nasal conchae (ferret #6, Cq value 29.26). The last three inoculated ferrets were sacrificed at 21 dpi. These animals showed only very weak RT-qPCR positivity in the cerebrum (ferret #7, Cq value 37.78) and in the caecum (ferret #9, Cq value 37.47). The three contact ferrets euthanized on the same day (21 dpi) were all positive in the nasal conchae (Cq values between 26.29 and 36.51). In addition, RT-qPCR positive samples were collected from muscle, lung, cerebrum, cerebellum and trachea tissue, which were all positive in ferret #10 and #11 whereas lung lymph node, skin and adrenal gland were only positive in one animal (Fig. 2D).

Antibodies against SARS-CoV-2 were detected by iIFA from day 8 pi in all inoculated ferrets with varying titers (64 to 8192). One of three contact animals also showed high antibody titers (ferret #12, highest reactive serum dilution 8192), whereas the others remained negative. Neutralizing antibodies were observed in three inoculated ferrets (ferret #7 128; ferret #8 1024 and ferret #9 1024 as the highest effective serum dilution) sacrificed on day 21 pi and one contact animal (ferret #12, 256) by VNT (Table 1B).

Post mortem examination did not identify relevant pathological alterations. At 4 dpi, viral antigen was associated with rhinitis, showing epithelial degeneration and necrosis, intraluminal cellular debris and mild inflammation (Fig. 4D-F). A more pronounced rhinitis developed at day 8 and 12 pi. At 21 dpi, rhinitis was only slightly detectable (ferret #7) or absent (ferret #8,#9). We also observed an antigen associated rhinitis in the contact ferrets (#10,#11). Viral antigen was detected in the nasal cavity at days 4 pi (ferret #1,#2), 8 pi (ferret #3), and 21 pi (contact ferret #10#11) in the nasal respiratory and olfactory epithelium. Remarkably, the olfactory epithelium of the vomero-nasal organ was affected (ferret #11; Fig. S2A-C). IHC results were confirmed by ISH (Fig. S3A-B). No viral antigen was identified in the lung. Single infected animals at days 4 and 8 pi and all contact animals showed interstitial, mixed cellular infiltrates and in some cases also perivascular lymphocytic cuffs (Table S1, Fig. S1D-F). Minimally increased numbers of alveolar macrophages were found at all time-points. None of the other organs was found positive for viral antigen, and no further relevant morphological alterations were detected.

Pigs and chickens

No clinical signs, including elevated body temperatures, were observed in any of the 12 pigs or 20 chickens. All collected samples were negative for SARS-CoV-2 genome. SARS-CoV-2 reactive antibodies were not detected. Histopathology was inconspicuous (animals sacrificed at 4, 8, and 12 dpi) or not performed on tissues obtained from animals sacrificed at 21 dpi. Three porcine cell lines (PK-15, SK-6 and ST) as well as embryonated chicken eggs inoculated with SARS-CoV-2 proved to be non-permissive (data not shown).

Discussion

Our study focused on four animal species, which are potentially relevant as models (fruit bats, ferrets) or could pose a risk as a viral reservoir following anthropo-zoonotic spill-over infections into food-producing animals (pigs, chickens).

Neither pigs (n = 9) nor chickens (n = 17) were susceptible to SARS-CoV-2 by intranasal or oculo-oronasal infection. All swabs as well as organ samples and contact animals (three animals in direct contact) remained negative for SARS-CoV-2 RNA and did not seroconvert. Non-permissiveness of chickens to SARS-CoV-2 infection parallel previous reports on the lack of susceptibility of chicken to SARS-CoV (20) and confirm recently reported results (21). We showed that this extends to embryonated chicken eggs, which are a classical substrate for isolation and propagation of a plethora of zoonotic viruses. The chicken data are also in agreement with studies on the chicken ACE2 receptor (22) that contains alterations in three of five critical residues (K31E; E35R, M82R). In contrast, similar predictions suggested that pigs as well as ferrets would likely be susceptible to SARS-CoV-2 due to their matching ACE2 receptor-binding site (22). In contrast to such *in silico* predictions, our study as well as the report by Shi et al (21) ruled out any susceptibility of pigs by the intranasal inoculation route. We extend these findings further by showing non-permissiveness of three universal porcine cell lines (PK-15, SK-6 and ST cells).

On the other hand, we present here to our knowledge first data on the intranasal inoculation of nine *Rousettus aegyptiacus* fruit bats, which resulted in a transient infection in the respiratory tract and virus shedding. SARS-CoV-2 genome could be detected by RT-qPCR in nasal conchae, trachea, lung and lung lymph node in fruit bat #1 and fruit bat #2 as well as in skin and duodenum of fruit bat #2, dissected on day 4 pi (Fig. 2C). Infectious virus was isolated from nasal conchae and trachea tissues from the same animal. Virus shedding was detectable by RT-qPCR in oral swabs up to day 12 pi, but infectious virus could only be isolated from fruit bat #2 at 2 dpi (Fig 2A and 3A). In total, seven out of nine inoculated fruit bats had viral genome in their nasal cavity, as confirmed by IHC and ISH at 4 dpi. Rhinitis was the detectable lesion associated with presence of viral antigen, mainly in the respiratory epithelium. Despite the absence of viral antigen at later time points, rhinitis was still identifiable, indicating earlier replication sites. Some infected animals as well as contact fruit bat #10 presented with mild inflammation in the lung. Its occurrence and significance should be addressed in future studies, because no lesion-associated antigen was detectable. Starting from 8 dpi, a weak immune-response developed as demonstrated in iIFA and VNT. The virus was transmitted to one out of

the three naïve contact fruit bats (fruit bat #10). The other two naïve contact animals remained seronegative. Interestingly, in fruit bat #10 an early pregnancy was determined during necropsy. Several studies show a higher virus detection rate in bats during the reproductive phase, probably due to the associated immunosuppression (23). ß-coronaviruses were shown to infect a variety of bat species with limited clinical signs even during active virus shedding (24). Moreover, low antibody titers are typical for bats (25). Although Egyptian fruit bats express ACE2 in the intestine and respiratory tract, an earlier study revealed very limited evidence of virus replication and seroconversion after infection with SARS-like coronaviruses, however, serum samples of some of these bats, collected prior to the infection, turned out to be already reactive with SARS S or N proteins (26). In the present study, SARS-CoV-2 transiently replicated in particular in the respiratory epithelium as shown by RT-qPCR, IHC and ISH. Our data suggest that intranasal infection of *Rousettus aegyptiacus* could reflect reservoir host status. Furthermore, we demonstrate that bat-to-bat transmission is possible. Consequently, bats are at risk of being infected anthropo-zoonotically by SARS-CoV-2. It is therefore highly recommended, that during the pandemic, all contacts to bats, e.g. during research programs or ecological analyses should be avoided.

SARS-CoV-2 replicated most efficiently in ferrets. Eight of nine intranasally infected ferrets shed virus between day 2 and 8 pi. Viral genome was detected by RT-qPCR in nasal washes and infectious virus isolated from two animals at 2 and 4 dpi (Fig. 2B and 3B). Only ferret #5 remained RT-qPCR negative during the observation period and developed only a weak iIFA titer. All other inoculated ferrets showed increasing SARS-CoV-2 reactive antibodies starting from day 8 pi. In general, the measured antibody levels were much higher in ferrets than in bats (Table 1), indicating a more prominent virus dissemination in the infected animals. For iIFA, this might also be explained by the use of different secondary antibodies. Neutralizing antibodies were only detected at later time points (21 dpi), but also with high titers of up to 1024 in ferrets, while we detected neutralizing antibodies in bats from day 8 dpi at comparably low titers of 16 – 64 (Table 1B). This might represent a reservoir host infection, which deserves more detailed analysis in future studies.

SARS-CoV-2 was efficiently transmitted to three naïve ferrets by direct contact. In those animals, viral RNA was present in nasal washes starting from day 12 pi and detected by RT-qPCR mostly in the nasal conchae, but also lung, trachea, lung lymph node or cerebrum and cerebellum (Fig. 2D). Viral antigen within the upper respiratory tract was confirmed by strong positive IHC and ISH in the nasal cavity. In the case of SARS-CoV, the virus was found to replicate in the upper and lower respiratory tract, and the animals developed no or mild clinical

disease characterized by nasal discharge, sneezing and fever (27). We also used high-throughput sequencing to analyze the complete genome of the used virus inoculum as well of samples from the inoculated ferrets. Complete sequence identity demonstrates that the virus did not adapt during ferret inoculation and that no additional mutations were required for an efficient infection of these animals with a human SARS-CoV-2 isolate.

Our results are in line with two recent reports that were also able to show productive SARS-CoV-2 infection of ferrets with no, or only mild clinical signs (21, 28). However, histopathology and tissue tropism data were very limited in both studies. Our report adds important detailed histopathology substantiating the restriction of the main SARS-CoV-2 replication site to the nasal cavity. Presence of viral antigen in the nasal respiratory and olfactory epithelium, including the vomero-nasal organ, was associated with rhinitis. Interestingly, the lesions were still present at later time points despite absence of viral antigen. Nevertheless, no viral antigen was identified in the lung, although several animals showed pulmonary inflammation.

In general, RT-qPCR detected viral genome in a significantly broader spectrum of tissues as IHC. The differences could be explained by (i) a higher sensitivity of RT-qPCR, (ii) the restriction of labelling to cell associated antigen whereas RT-qPCR detects viral RNA in blood, secretions and excretions (i.e. tracheal and bronchial mucus, saliva on the fur), and not least (iii) viral antigen was found in restricted foci of the nasal cavity only, that might be missed in tissue sections although several areas have been analyzed. Although less sensitive, IHC is an excellent tool to localize and identify infected target cells. To avoid cross contamination at necropsy, instruments were washed in sodium hypochlorite-based reagents after each tissue sample. Numerous extraction controls were executed and questionable results were confirmed by a second RT-qPCR assay. Therefore, we assume that our RT-qPCR results are highly reliable. Testing a broader tissue spectrum, including salivary glands, the lower urinary tract, full gastrointestinal tract and the cerebrospinal fluid will help to increased understanding of the source of viral RNA in secretions, excretions as well as in the brain.

In summary, farmed animals like chickens and pigs were resistant against intranasal SARS-CoV-2 inoculation under our experimental conditions. This is relevant for risk assessment and epidemiology of the infection. Furthermore, our study demonstrated that ferrets and *Rousettus* fruits bats could be productively infected. Especially SARS-CoV-2 infection in ferrets, which resembles a mild infection of humans, might serve as a useful animal model for testing prototypic COVID-19 vaccines and antivirals.

Acknowledgment

The authors are very grateful to Roman Wölfel (German Armed Forces Institute of Microbiology) for providing the SARS-CoV-2 isolate used in this study. We thank Bernd Köllner for generating the anti-bat monoclonal antibody. We also acknowledge Mareen Lange, Christian Korthase, Silvia Schuparis, Gabriele Czerwinski and Patrick Zitzow for their excellent technical assistance and Frank Klipp, Doreen Fiedler, Harald Manthei, René Siewert, Christian Lipinski, Ralf Henkel and Domenique Lux for their excellent support in the animal experiments.

Author's contribution

KS, MR, AG, JS, DHo, ABB, TH and ChG performed the animal experiments. KS; MR; AG and JS did molecular, serological and classical virological analyses. AB and JuS did animal necropsies, AB did histopathology, immunohistochemistry and in situ hybridization analysis. DHö, CW and BH added sequencing and quantification data. KS; AG, DHo, TH, TM, ABB and MB designed the study. KS; MR; AG; AB and MB wrote the manuscript. All authors critically evaluated and approved the manuscript.

Declaration of Interests

All authors declare no competing interest.

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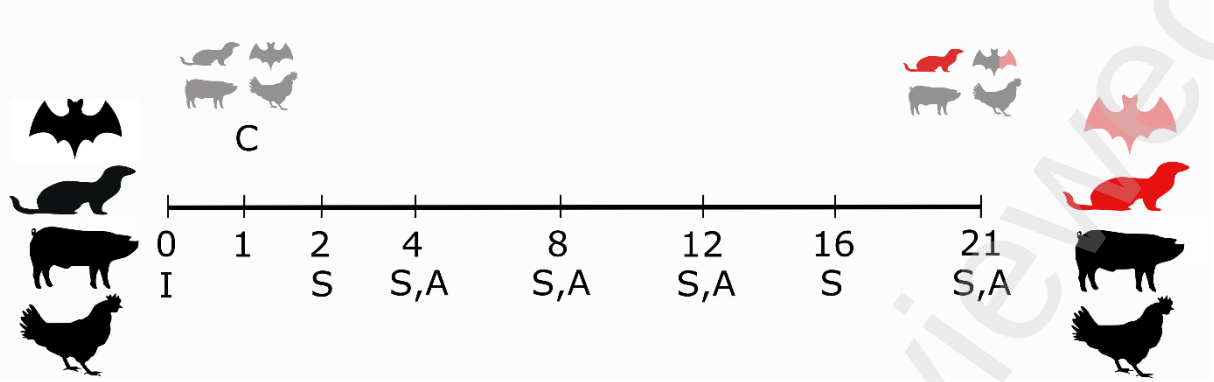
Tables

Table 1: Serological evidence of SARS-CoV-2 infection in A) fruit bats and B) ferrets.

A)	iIFA	VNT
fruit bat #1, day 4	< 1:16	< 1:16
fruit bat #2, day 4	< 1:16	< 1:16
fruit bat #3, day 8	**1:16**	**1:32**
fruit bat #4, day 8	**1:16**	**1:32**
fruit bat #5, day 12	**1:16**	**1:32**
fruit bat #6, day 12	**1:32**	**1:16**
fruit bat #7, day 21	**1:64**	**1:64**
fruit bat #8, day 21	**1:32**	**1:32**
fruit bat #9, day 21	**1:64**	**1:32**
fruit bat #10, day 21	**1:16**	**1:16**
fruit bat #11, day 21	< 1:16	< 1:16
fruit bat #12, day 21	< 1:16	< 1:16

B)	iIFA	VNT
ferret #1, day 4	< 1:16	< 1:16
ferret #2, day 4	< 1:16	< 1:16
ferret #3, day 8	**1:128**	< 1:16
ferret #4, day 8	**1:512**	< 1:16
ferret #5, day 12	**1:64**	< 1:16
ferret #6, day 12	**1:4096**	< 1:16
ferret #7, day 21	**1:4096**	**1:128**
ferret #8, day 21	**1:8192**	**1:1024**
ferret #9, day 21	**1:4096**	**1: 1024**
ferret #10, day 21	< 1:16	< 1:16
ferret #11, day 21	< 1:16	< 1:16
ferret #12, day 21	**1:8192**	**1:256**

Figures



I - infection; C - contact animals; S - swabbing; A - Autopsy

Figure 1: Outline of the *in vivo* experiments with an observation period of 21 days. Procedure of the trials with fruit bats, ferrets, domestic pigs and chickens are shown. Black-colored animals (n=9 for each species, except chickens n=17) were inoculated intranasally (or oculo-oronasally for chicken) with 10^5 $TCID_{50}$; grey animals (n=3 for each species) depict direct contact animals associated after day 1 post inoculation; black- and grey-colored animals on the right were found not susceptible; red animals became infected and showed strong viral shedding; rose/pink animals were infected but displayed only minute shedding of virus.

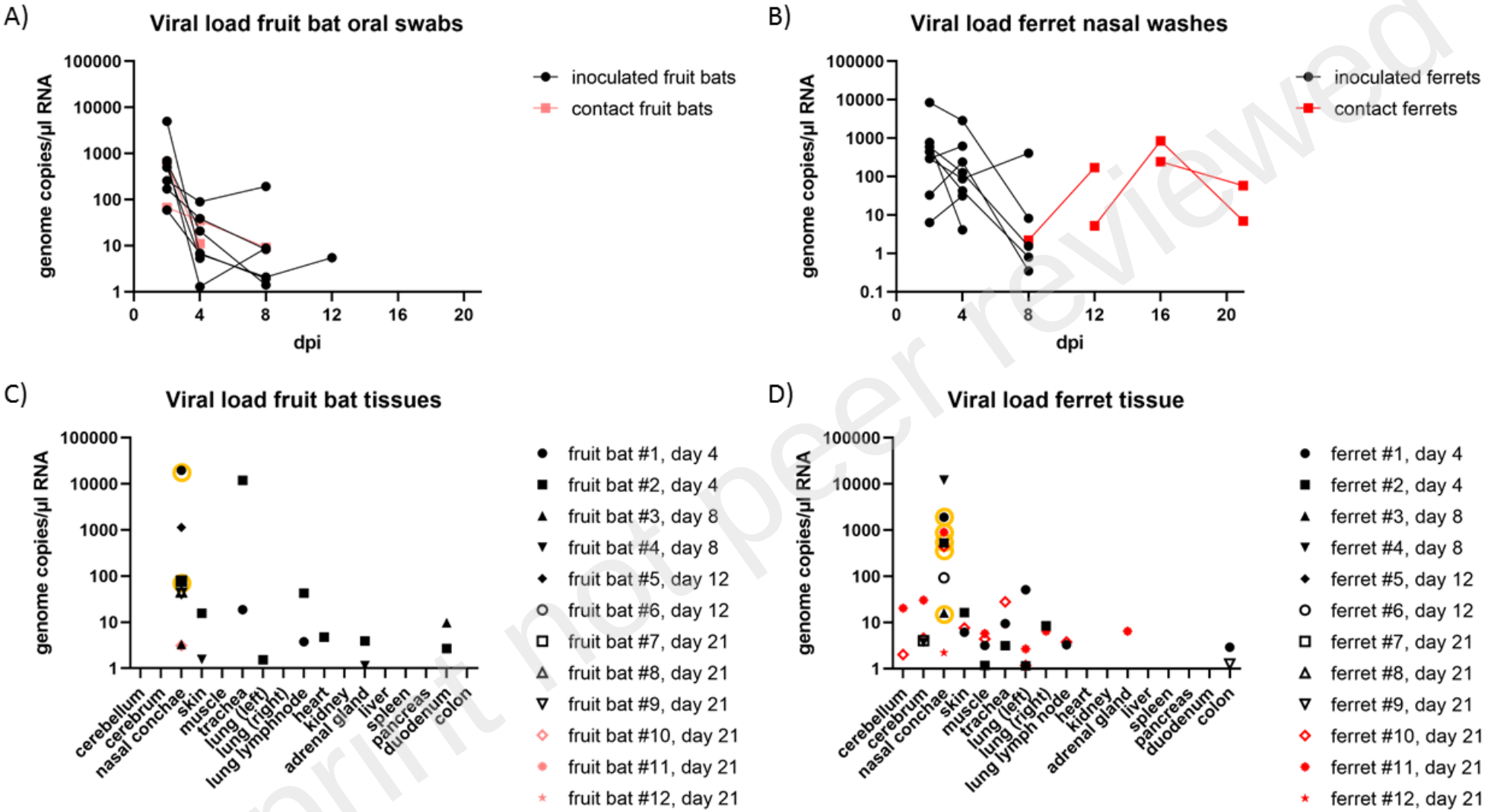


Figure 2: SARS-CoV-2 viral genome loads in A) oral swabs of fruits bats, B) nasal washes of ferrets, tissues collected from C) fruit bats and D) ferrets experimentally infected with SARS-CoV-2 and the contact animals, respectively. Genome copies per µl RNA template were calculated based on a quantified standard RNA. Red-colored animals became infected and showed strong viral shedding; rose/pink animals were infected but displayed only minute shedding of virus. Organs with positive IHC results were marked with an orange ring.

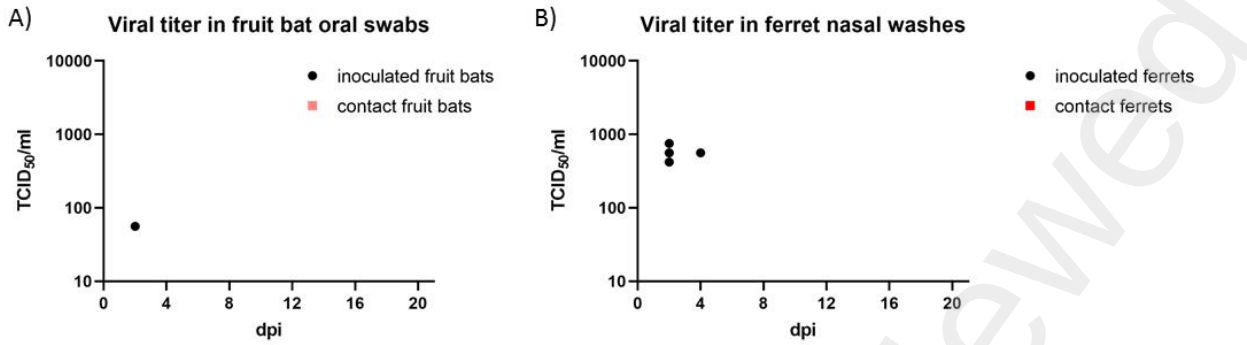


Figure 3: Shedding of infectious SARS-CoV-2 in A) fruit bat oral swabs and B) ferret nasal washes. Given are $TCID_{50}$/ml values for every day with a RT-qPCR positive result. All other samples were $<10^{1}TCID_{50}$/ml for fruit bats or $<10^{2.5}TCID_{50}$/ml for ferrets.

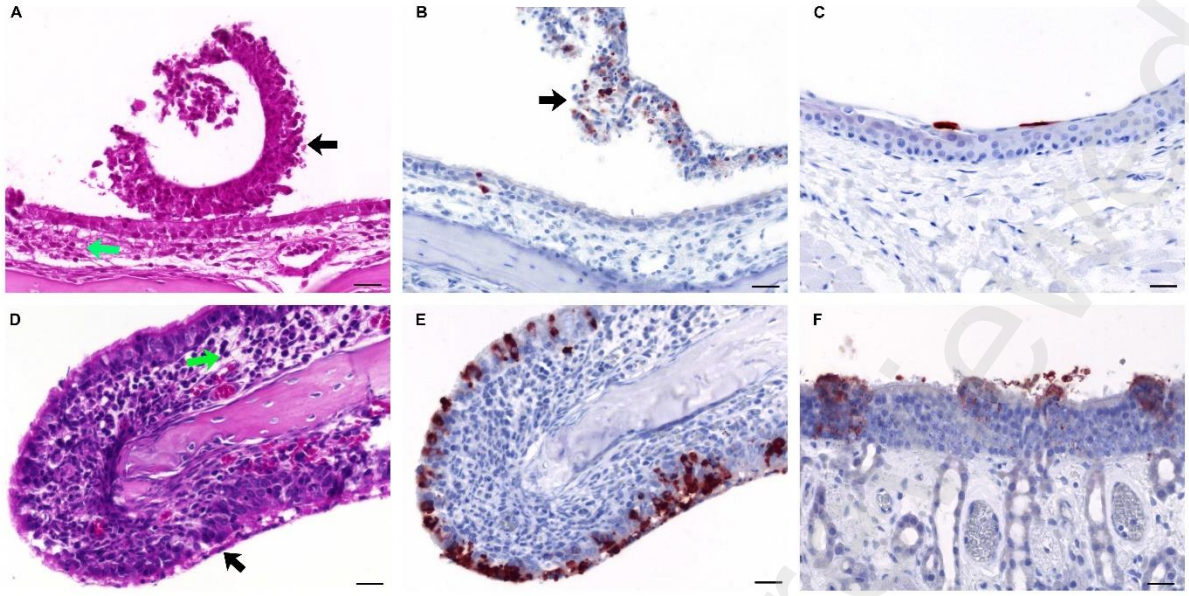


Figure 4. SARS-CoV-2 associated rhinitis and antigen detection at day 4 pi in a bat (A-C) and a ferret (D-F). (A) Bat, rhinitis, with intraluminal debris (black arrow), slight mucosal edema and minimal inflammation (green arrow), (B) Bat, nasal respiratory epithelium, intralesional viral antigen mainly within intraluminal debris, (C) Bat, non-respiratory epithelium, with single antigen positive cells, no inflammation. (D) Ferret, rhinitis, with degeneration and necrosis of the respiratory epithelium (black arrow), slight mucosal edema and numerous infiltrates (green arrow), (E) Ferret, nasal respiratory epithelium, intralesional, abundant viral antigen, (F) Ferret, olfactory epithelium, multifocal, intralesional viral antigen (A, D) Histopathology, H&E stain, bar 20 µm (B, C, E, F) Immunohistochemistry, ABC method, AEC chromogen (red-brown), Mayer's hematoxylin counter stain (blue), bar 20 µm.

Supplementary

Supplementary Material & Methods

Virus and cells

SARS-CoV-2 isolate 2019_nCoV Muc-IMB-1 was kindly provided by German Armed Forces Institute of Microbiology (Munich, Germany). The complete sequence of this isolate is available through GISAID under the accession ID_EPI_ISL_406862 and name "hCoV-19/Germany/BavPat1/2020". The virus was propagated once in Vero E6 in a mixture of equal volumes of Eagle MEM (Hanks' balanced salts solution) and Eagle MEM (Earle's balanced salts solution) supplemented with 2mM L-Glutamine, nonessential amino acids, adjusted to 850 mg/L, NaHCO3, 120 mg/L sodium pyruvate, 10% fetal bovine serum (FBS), pH 7.2. No contaminants were detected within the virus stock preparation and the sequence identity of the passaged virus (study accession number: PRJEB37671) was confirmed by metagenomics analysis employing previously published high throughput sequencing procedures using Illumina MiSeq sequencing (29). The virus was harvested after 72h, titrated on Vero E6 cells and stored at -80°C until further use.

RNA extraction and detection of SARS-CoV-2

Total RNA was extracted from oral, nasal and rectal samples, nasal washes, fecal samples and tissue samples collected at different time points using the NucleoMagVet kit (Macherey&Nagel, Düren, Germany) according to the manufacturer's instructions. Tissue samples were homogenized in 1 ml cell culture medium and a 5 mm steel bead in a TissueLyser (Qiagen, Hilden, Germany). Fecal samples were vortexed in sterile NaCl and the supernatant was sterile filtered (22 µm) after centrifugation. Swab samples were transferred into 0.5-1 ml of serum-free tissue culture media and further processed after 30 min shaking.

SARS-CoV-2 RNA was detected by the "E-gene Sarbeco FAM" published by Corman et al. (30). The RT-qPCR reaction was prepared using the AgPath-ID-One-Step RT-PCR kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) in a volume of 12.5 µl including 1 µl of E-gene Sarbeco FAM mix, 1 µl of ß-Actin-mix2-HEX as internal control) and 2.5 µl of extracted RNA. The reaction was performed for 10 min at 45°C for reverse transcription, 5 min at 95°C for activation, and 42 cycles of 15 sec at 95°C for denaturation, 20 sec at 57°C for annealing and 30 sec at 72°C for elongation. Fluorescence was measured during the annealing phase. All RT-qPCRs were performed on a BioRad real-time CFX96 detection system (Bio-Rad, Hercules, USA). Absolute quantification was done using a standard quantified by the QX200

Droplet Digital PCR System in combination with the 1-Step RT-ddPCR Advanced Kit for Probes (BioRad, Hercules, USA).

Nasal conchae samples from ferret #3 and #4 were subjected to high-throughput sequencing and viral genomes compared to the inoculum (study accession number: PRJEB37671) by employing previously published high throughput sequencing procedures using Ion Torrent S5XL instrument (29).

Detection of SARS-CoV-2 reactive antibodies

Serum samples collected before the start of the experiments as well as on necropsy were tested for the presence of SARS-CoV-2 reactive antibodies by indirect immunofluorescence assay (iIFA) and virus neutralization test (VNT).

Confluent Vero E6 cells in a 96 well plate were infected with 0.1 MOI of SARS-CoV-2 or cell culture medium for negative control cells. After 24h, cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton-X-100. Serum samples were heat inactivated at 56°C for 30 min. For antibody detection, 50 µl of a 2-fold dilution series of the serum samples (starting from 1:20) were added in parallel to the SARS-CoV-2 positive and negative cells. After 1h incubation, cells were washed and incubated for 1h with a goat-anti-ferret-IgG-FITC antibody (1:250, Bethyl, Texas, USA), mouse-anti-bat-IgG #6 (1:100, FLI produced) combined with a goat-anti-mouse-Cy3 (1:400, Jackson Immunoresearch, Pennsylvania, USA), goat-anti-pig-FITC IgG (1:2000, antibodies-online, Aachen, Germany), goat-anti-chicken-IgG-FITC (1:400, OriGene Technologies GmbH, Maryland, USA), respectively. After final washing, cells were analyzed by fluorescence microscopy.

For virus neutralization assay, 50 µl of medium containing $10^{3.3}$ $TCID_{50}$ SARS-CoV-2 were mixed with 50 µl of diluted serum. Each sample was tested in triplicates. After 1h incubation at 37°C the mixture was transferred to confluent Vero E6 cells in a 96 well plate. Viral replication was assessed after 5 days at 37°C, 5% CO_2 by the detection of CPE.

Virus titration

Virus titer used for infection experiments was confirmed by titration on Vero E6 cells and evaluation of CPE after 5 days. RT-qPCR positive nasal washes and tissue samples were titrated on Vero E6 cells as well.

Pathology: Necropsy, histopathology, immunohistochemistry, in situ hybridization

Full necropsies were performed on all animals according to a standard protocol under BSL3 conditions. The following tissues were collected and fixed in 10% neutral-buffered formalin: Nasal conchae (non-respiratory, respiratory and olfactory region), trachea, lung (inflated with formalin, left and right cranial as well as caudal lobe), tracheobronchial lymph node, heart (left ventricle), liver, spleen, duodenum, colon, pancreas, kidney, adrenal gland, skeletal muscle, skin, brain. Tissues of ferrets and fruit bats were embedded in paraffin, and 3 µm sections were stained with hematoxylin and eosin for light microscopical examination.

For SARS-CoV-2 antigen detection, tissue sections of all bats and ferrets were deparaffinized and rehydrated according to standardized procedures. Antigen heat retrieval was performed (citrate buffer, pH 6, 12 min, microwave 600 Watt). Nonspecific antibody binding was blocked with goat normal serum for 30 min at room temperature. Polyclonal rabbit anti SARS-CoV-2 antibody (dilution 1:200, Novus Biologicals # NB100-56576, Centennial, CO, USA) was incubated over night at room temperature, followed by washing steps and incubation with a secondary biotinylated goat anti-rabbit antibody (dilution 1:200; Vector Laboratories, Burlingame, CA, USA) for 30 min at room temperature. Freshly prepared avidin-biotin-peroxidase complex (ABC) solution (Vectastain Elite ABC Kit; Vector Laboratories) was applied, and a bright red antigen labelling was produced with the 3-amino-9-ethylcarbazole substrate (AEC, Dako, Agilent, Santa Clara, CA, USA). The sections were counterstained with hematoxylin, rehydrated, and mounted on coverslips. In each run, we included consecutive sections incubated with negative rabbit control serum, historical tissue sections from SARS-CoV-2 negative ferrets and bats (negative control), and sections of cell pellets infected with SARS-CoV-2 and fixed after 24 h (positive control).

To confirm IHC, RNA in situ hybridization (ISH) was performed on tissues of selected animals with RNAScope 2-5 HD Reagent Kit-Red (ACD, Advanced Cell Diagnostics, Newark, CA) according to the manufacturer's instructions. For hybridization, RNAScope® probes were custom designed by ACD for SARS-CoV-2 NSP. The specificity of the probes was verified using a positive control probe peptidylprolyl isomerase B (cyclophilin B, ppib) and a negative control probe dihydrodipicolinate reductase (DapB). Evaluation and interpretation of pathology data were performed by a board-certified pathologist (DiplECVP).

Susceptibility of different porcine cell lines to SARS-CoV-2

Porcine cell lines, porcine kidney-15 (PK-15), swine kidney-6 (SK-6) and swine testicle (ST) cells that are routinely used for porcine virus isolation attempts at FLI, were investigated for their permissivity to SARS-CoV-2. Cells were maintained in modified Eagle medium (MEM)

supplemented with 10% FBS. Nearly confluent cells were infected with SARS-CoV-2 at a titer of $10^{5.5}$ $TCID_{50}$ in a microtitration format in 96well plates or were mock-infected with medium only. Vero E6 cells were used as a highly permissive control. Cells were observed for cytopathic effects (CPE) daily until six days post infection.

Susceptibility of embryonated chicken eggs

Six 9-day-old SPF chicken eggs were inoculated by allantoic sac route, using 0.1 ml with $5.5x10^4$ $TCID_{50}$. Amnotic-Allantoic fluid (AAF) was harvested after incubation for 7 days and tested by RT-qPCR and virus isolation on Vero E6 cells.

Supplementary Tables

Table S1: Histopathologic findings in the lungs of inoculated and contact Egyptian fruit bats and ferrets. For all animals, the left and right, cranial as well as caudal lung lobes (= 4 in total) were examined.

	Infiltrates, interstitial, mixed, mild	Infiltrates, perivascular, lymphocytic, mild	Infiltrates, intra alveolar, mixed, minimal	Alveolar macrophages, number increased, minimal
Day 4	Bat#1; 4/4 lobes			Bat#2; 1/4 lobes
	Ferret#2; 1/4 lobes	Ferret#2; 4/4 lobes	Ferret#1, #2; 1/4 lobes	Ferret#1, 2/4 lobes, Ferret#2; 3/4 lobes
Day 8	Bat#4; 1/4 lobes			
	Ferret#3; 3/4 lobes	Ferret#3; 1/4 lobes		Ferret#3, 4; 1/4 lobes
Day 12	Bat#5; 2/4 lobes			
				Ferret#6; 2/4 lobes
Day 21				Bat#7, 8; 1/4 lobes
				Ferret#8; 1/4 lobes
Day 21 Contact	Bat#10; 1/4 lobes	Bat#10; 1/4 lobes		Bat#10; 2/4 lobes, Bat #11; 1/4 lobes
	Ferret#10, 11; 4/4 lobes, Ferret#12, 11; 1/4 lobes	Ferret#10; 3/4 lobes		Ferret#10, 12 3/4 lobes; Ferret#11, 1/4 lobes;

Supplementary Figures

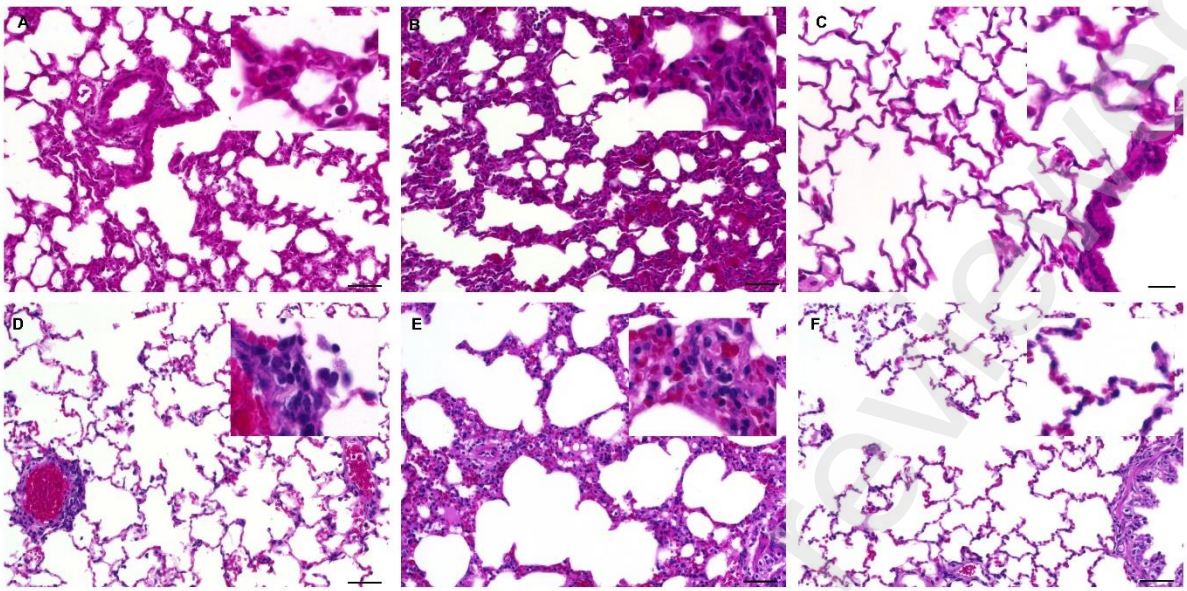


Figure S1: SARS-CoV-2 associated pulmonary lesions in bats (A-C) and ferrets (D-F). (A) Thickening of the alveolar wall by congestion and slight, neutrophilic infiltrates, bat, day 4 pi, bar 50 µm (B) Pronounced thickening by congestion and mixed cellular infiltration of the alveolar wall, contact bat, day 21, bar 50 µm, (C) No relevant findings in inoculated bats at day 21 pi, bar 50 µm (D) Perivascular, mononuclear infiltrates, ferret, day 4, bar 50 µm, (E) Thickening by congestion and infiltration of the alveolar wall, contact ferret, day 21, bar 50 µm, (F) No relevant findings in inoculated ferrets at day 21 pi, bar 50 µm.

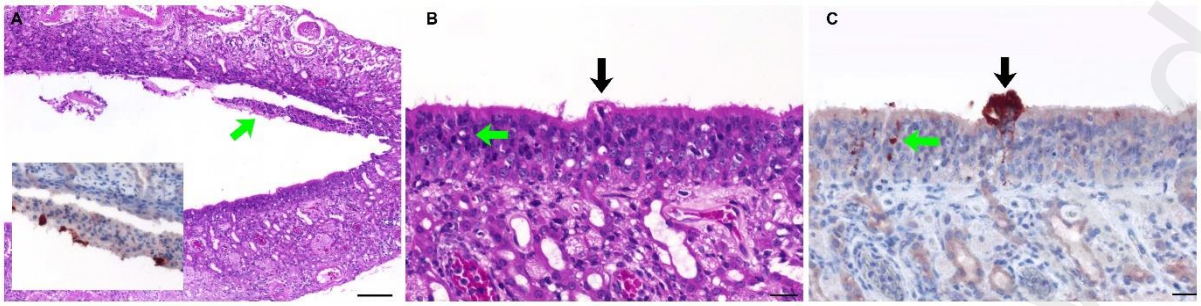


Figure S2: SARS-CoV-2 in the vomero-nasal organ of a contact ferret on day 21. (A) Intraluminal debris (green arrow), extensive degeneration, necrosis and focal loss of the olfactory epithelium, abundant mixed cellular infiltrates, intralesional viral antigen (inlay), bar 100 µm, (B) Degeneration with swelling of the olfactory epithelium (black arrow) and apoptosis (green arrow), bar 20 µm, consecutive slide (C) Viral antigen within olfactory epithelium (black and green arrow), bar 20 µm. (A, B) H&E stain, (inlay and C) Immunohistochemistry, ABC Method, AEC chromogen (red-brown), Mayer's hematoxylin counter stain (blue), bar 20 µm.

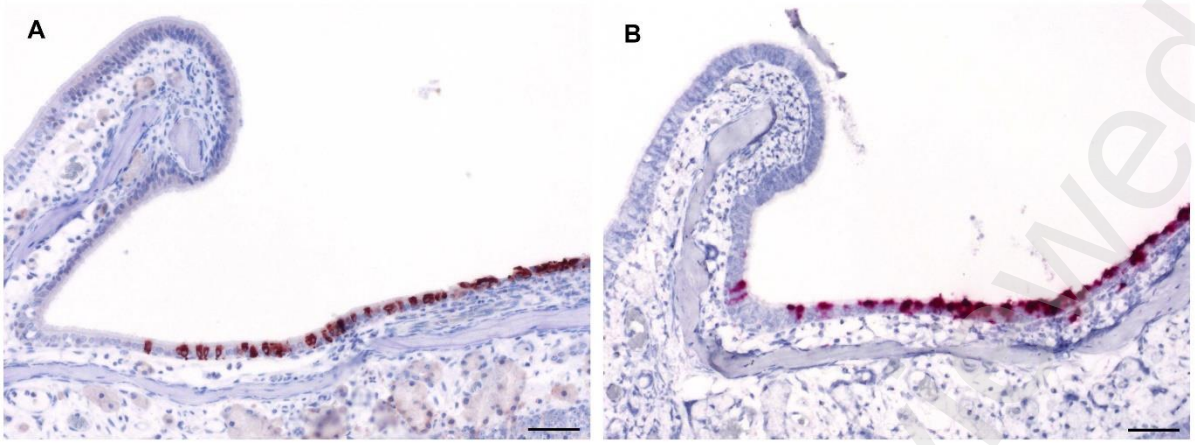


Figure S3: Comparative SARS-CoV-2 antigen and RNA detection. Immunohistochemistry and in situ hybridization yielded comparative results with respect to cell types affected and semi-quantitative antigen amount. Exemplarily shown in the respiratory epithelium, ferret, 4 dpi. (A) Immunohistochemistry, ABC Method, AEC chromogen (red-brown), Mayer's hematoxylin counter stain (blue), (B) In situ hybridization, RNAScope®, chromogenic labelling (fast red) with probes to SARS-Cov-2 NSP, Mayer's hematoxylin counter stain (blue).