

1 **SARS-CoV-2 is transmitted via contact and via the air between ferrets.**

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12 **SARS-CoV-2, a coronavirus that newly emerged in China in late 2019^{1,2} and spread rapidly**
13 **worldwide, caused the first witnessed pandemic sparked by a coronavirus. As the pandemic**
14 **progresses, information about the modes of transmission of SARS-CoV-2 among humans is critical**
15 **to apply appropriate infection control measures and to slow its spread. Here we show that SARS-**
16 **CoV-2 is transmitted efficiently via direct contact and via the air (via respiratory droplets and/or**
17 **aerosols) between ferrets. Intranasal inoculation of donor ferrets resulted in a productive upper**
18 **respiratory tract infection and long-term shedding, up to 11 to 19 days post-inoculation. SARS-**
19 **CoV-2 transmitted to four out of four direct contact ferrets between 1 and 3 days after exposure**
20 **and via the air to three out of four independent indirect recipient ferrets between 3 and 7 days**
21 **after exposure. The pattern of virus shedding in the direct contact and indirect recipient ferrets**
22 **was similar to that of the inoculated ferrets and infectious virus was isolated from all positive**
23 **animals, showing that ferrets were productively infected via either route. This study provides**
24 **experimental evidence of robust transmission of SARS-CoV-2 via the air, supporting the**
25 **implementation of community-level social distancing measures currently applied in many**
26 **countries in the world and informing decisions on infection control measures in healthcare**
27 **settings³.**

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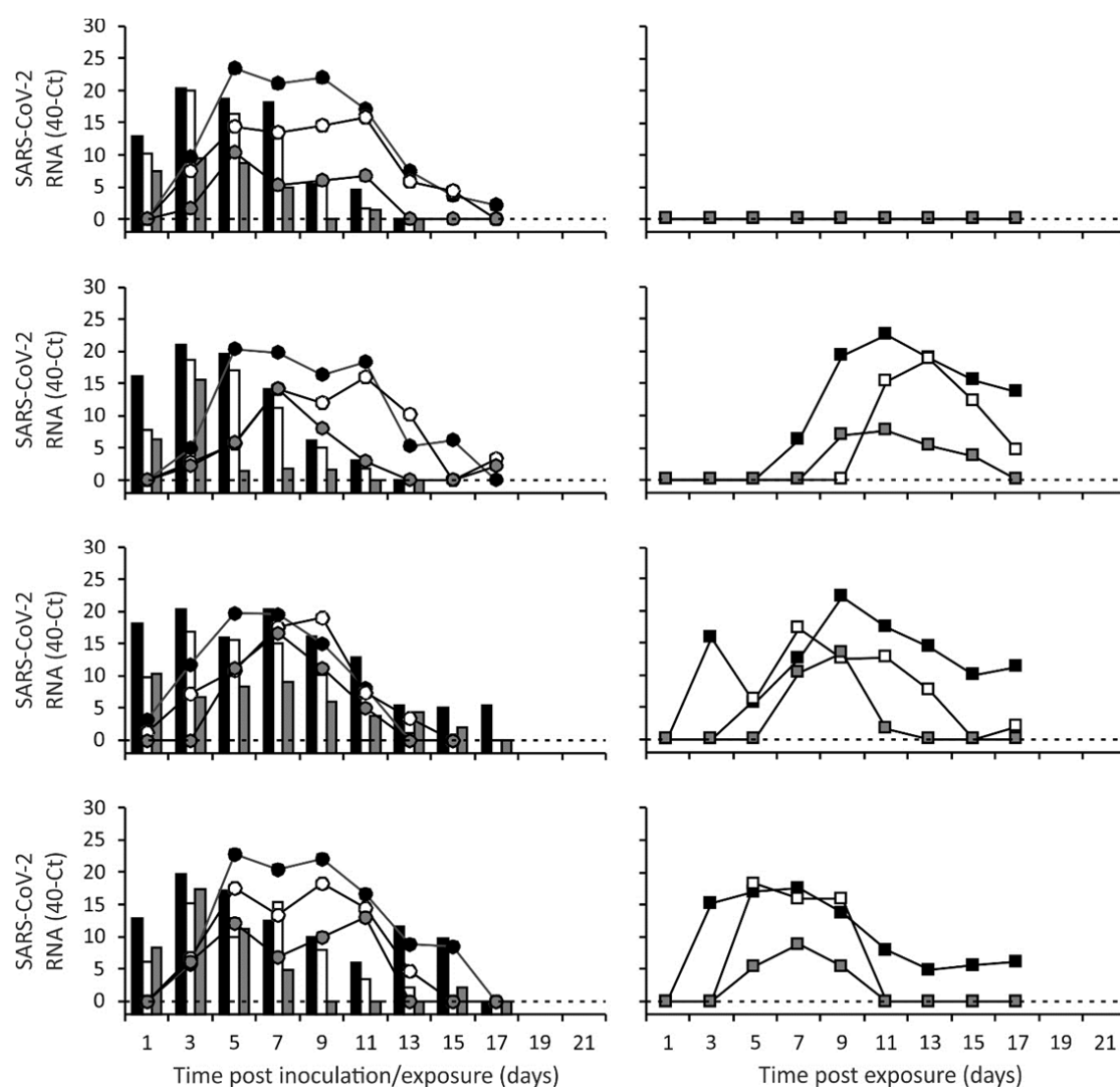
29 In late December 2019, clusters of patients in China presenting with pneumonia of unknown etiology
30 were reported to the World Health Organization (WHO) ¹. The causative agent was rapidly identified
31 as being a virus from the *Coronaviridae* family, closely related to the severe acute respiratory
32 syndrome coronavirus (SARS-CoV) ^{2,4,5}. The SARS-CoV epidemic affected 26 countries and resulted in
33 more than 8000 cases in 2003. The newly emerging coronavirus, named SARS-CoV-2 ⁶, rapidly spread
34 worldwide and was declared pandemic by the WHO on March 11, 2020 ⁷. The first evidence
35 suggesting human-to-human transmission came from the descriptions of clusters among the early
36 cases ^{8,9}. Based on epidemiological data from China before measures were taken to control the
37 spread of the virus, the reproductive number R_0 (the number of secondary cases directly generated
38 from each case) was estimated to be between 2 and 3 ¹⁰⁻¹². In order to apply appropriate infection
39 control measures to reduce the R_0 , the modes of transmission of SARS-CoV-2 need to be elucidated.
40 Respiratory viruses can be transmitted via direct and indirect contact (via fomites), and through the
41 air via respiratory droplets and/or aerosols. Transmission via respiratory droplets ($> 5 \mu\text{m}$) is
42 mediated by expelled particles that have a propensity to settle quickly and is therefore reliant on
43 close proximity between infected and susceptible individuals, usually within 1 m of the site of
44 expulsion. Transmission via aerosols ($< 5 \mu\text{m}$) is mediated by expelled particles that are smaller in
45 size than respiratory droplets and can remain suspended in the air for prolonged periods of time,
46 allowing infection of susceptible individuals at a greater distance from the site of expulsion ¹³.
47 Current epidemiological data suggest that SARS-CoV-2 is transmitted primarily via respiratory
48 droplets and contact ^{8-10,14,15}, which is used as the basis for mitigation of spread through physical and
49 social distancing measures. However, scientific evidence that SARS-CoV-2 can be efficiently
50 transmitted via the air is weak.
51 Previous studies have shown that ferrets were susceptible to infection with SARS-CoV ¹⁶⁻²⁰, and that
52 SARS-CoV was efficiently transmitted to co-housed ferrets via direct contact ¹⁶. Here, we used a
53 ferret transmission model to assess whether SARS-CoV-2 spreads through direct contact and/or
54 through the air (via respiratory droplets and/or aerosols). For this purpose, individually housed

55 donor ferrets were inoculated intranasally with a strain of SARS-CoV-2 isolated from a German
56 traveller returning from China. Six hours post-inoculation (hpi), a direct contact ferret was added to
57 each of the cages. The next day, indirect recipient ferrets were placed in adjacent cages, separated
58 from the donor cages by two steel grids, 10 cm apart, allowing viruses to be transmitted only via the
59 air (Supplementary Figure 1). On alternating days to prevent cross-contamination, throat, nasal and
60 rectal swabs were collected from each ferret in the inoculated and direct contact groups and from
61 the indirect recipient group, followed by SARS-CoV-2 detection by RT-qPCR and virus titration.
62 Ferrets were productively infected by SARS-CoV-2 upon intranasal inoculation, as demonstrated by
63 the robust and long-term virus shedding from the donor ferrets (Figure 1, Supplementary Figure 2).
64 SARS-CoV-2 RNA levels peaked at 3 days post-inoculation (dpi) and were detected up to 11 dpi in
65 two animals and up to 15 and 19 dpi in the other two animals (Figure 1, Supplementary Figure 2).
66 SARS-CoV-2 was transmitted to direct contact ferrets in four out of four independent experiments
67 between 1 and 3 days post-exposure (dpe) and viral RNA was detected up to 13 to 15 days (i.e. 13 to
68 17 dpe) (Figure 1, Supplementary Figure 2). Interestingly, SARS-CoV-2 was also transmitted via the
69 air to three out of four indirect recipient ferrets. SARS-CoV-2 RNA was detected from 3 to 7 dpe
70 onwards these indirect recipient ferrets and for 13 to 19 days (Figure 1, Supplementary Figure 2).

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75 **Figure 1. SARS-CoV-2 shedding in ferrets in the transmission experiment.** SARS-CoV-2 viral RNA was

76 detected by RT-qPCR in throat (black), nasal (white) and rectal (grey) swabs collected from

77 inoculated donor ferrets (bars; left panels), direct contact ferrets (circles; left panels) and indirect

78 recipient ferrets housed in separate cages (squares; right panels). Swabs were collected from each

79 ferret every other day until no viral RNA was detected in any of the three swabs. The dotted line

80 indicates the detection limit.

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82 Whereas donor ferrets were inoculated with a high virus dose, direct contact and indirect recipient
83 ferrets are likely to have received a low infectious dose via direct contact or via the air. In spite of
84 this, the pattern of virus shedding from the direct contact and indirect recipient ferrets was similar
85 to that of the inoculated donor ferrets, both in terms of duration and SARS-CoV-2 RNA levels,
86 corroborating robust replication of SARS-CoV-2 upon transmission via direct contact and via the air,
87 independent of the infectious dose. In general, higher SARS-CoV-2 RNA levels were detected in the
88 throat swabs as compared to the nasal swabs. SARS-CoV-2 RNA levels in the rectal swabs were
89 overall the lowest. From each SARS-CoV-2 RNA positive animal, infectious virus was isolated in
90 VeroE6 cells from throat and nasal swabs for at least two consecutive days (Supplementary Table 1).
91 In contrast, no infectious virus was isolated from the rectal swabs. Infectious virus titers ranged from
92 $10^{0.75}$ to $10^{2.75}$ TCID₅₀/ml (median tissue culture infectious dose per ml) in the donor ferrets, from
93 $10^{0.75}$ to $10^{3.5}$ TCID₅₀/ml in the direct contact ferrets and from $10^{0.75}$ to $10^{4.25}$ TCID₅₀/ml in the indirect
94 recipient ferrets. All SARS-CoV-2 positive ferrets seroconverted 21 dpi/dpe, and the antibody levels
95 were similar in donor, direct contact and indirect recipient ferrets (Figure 2). The indirect recipient
96 ferret, in which no SARS-CoV-2 was detected, did not seroconvert as expected.
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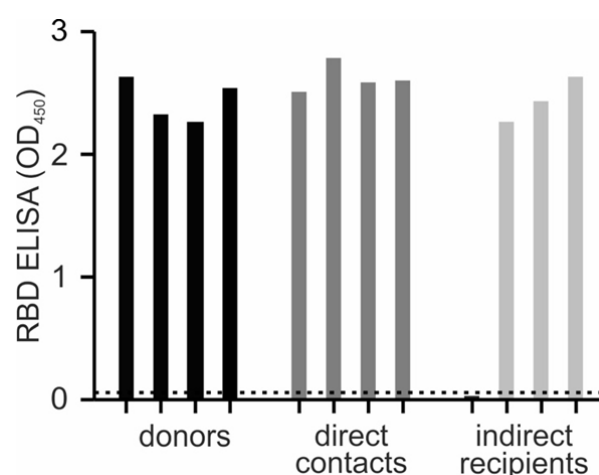


Figure 2. Antibody responses in donor, direct contact and indirect recipient ferrets at 21 dpi/dpe.

Sera were collected from the donor, direct contact and indirect recipient ferrets at 21 dpi/dpe and IgG responses were assessed using a SARS-CoV-2 receptor binding domain (RBD) ELISA. The dotted line indicates the background of the assay.

SARS-CoV-2 transmission in experimental animal models has recently also been described by others. SARS-CoV-2 direct contact transmission between ferrets²¹ and hamsters²² was reported, with similar efficiency as observed in our study. In addition, SARS-CoV-2 was also found to be transmitted via the air in two out of six ferrets²¹, and in two out of six cats²³. However, only low levels of SARS-CoV-2 RNA were detected in nasal washes and feces of the indirect recipient ferrets, and no infectious virus was isolated²¹. Furthermore, virus shedding was shorter as compared to the donor animals and only one out of the two SARS-CoV-2 RNA positive indirect recipient ferrets seroconverted. Similarly, the transmission via the air between cats was not efficient. SARS-CoV-2 RNA was detected in the feces and tissues of one cat at 3 and 11 dpi respectively and in nasal washes of another cat, but no infectious virus was isolated. Both SARS-CoV-2 RNA positive indirect recipient cats seroconverted. In contrast, the present study showed that SARS-CoV-2 was efficiently transmitted via the air between ferrets, as demonstrated by long-term virus shedding and the presence of infectious virus in the indirect recipient animals, which is comparable to the transmissibility of pandemic influenza viruses in the ferret model²⁴.

To date, there is no evidence of fecal-oral transmission of SARS-CoV-2 in humans. However, the prolonged detection of RNA in consecutive stool samples²⁵ and the environmental contamination of sanitary equipment²⁶ may suggest that the fecal-oral route could be a potential route of transmission of SARS-CoV-2. Here, no infectious virus was retrieved from any of the rectal swabs. Despite this, it cannot be fully excluded that SARS-CoV-2 was also transmitted from donors to direct contact ferrets partly via the fecal-oral route. In the study by Kim *et al.*, ferret fecal material was used to inoculate ferrets, resulting in a productive infection, indicating that infectious SARS-CoV-2 was shed in fecal specimens²¹.

Our experimental system does not allow to assess whether SARS-CoV-2 was transmitted via the air through respiratory droplets, aerosols or both, as donor and indirect recipient ferret cages are placed only 10 cm apart from each other. In a recent study, SARS-CoV-2 remained infectious in aerosols for at least 3h after aerosolization at high titers in a rotating drum, comparable to SARS-CoV²⁷. Although it is informative to compare the stability of different respiratory viruses in the air, our study provides the additional information that infectious SARS-CoV-2 particles can actually be expelled in the air and subsequently infect recipients. In two other studies, the presence of SARS-CoV-2 in air samples collected in hospital settings was investigated. However, no SARS-CoV-2 RNA was detected in the air sampled in three isolation rooms²⁶, or 10 cm from a symptomatic patient who was breathing, coughing or speaking²⁸. Nevertheless, RNA was detected on the air exhaust outlet of one of the isolation rooms in the first study, suggesting that virus-laden droplets may be displaced by airflows²⁶.

Here we provide the first experimental evidence that SARS-CoV-2 can be transmitted efficiently via the air between ferrets, resulting in a productive infection and the detection of infectious virus in indirect recipients, as a model for human-to-human transmission. Although additional experiments on the relative contribution of respiratory droplets and aerosols to the transmission of SARS-CoV-2 are warranted, the results of this study corroborate the WHO recommendations about transmission precautions in health care settings and the social distancing measures implemented in many

144 countries around the globe to mitigate the spread³. The ferret transmission model will also be
145 useful to understand transmission dynamics and the molecular basis of the transmissibility of SARS-
146 Cov-2 and other betacoronaviruses, which, in the context of the current SARS-CoV-2 pandemic and
147 future pandemic threats, is clearly of utmost importance.
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Methods

Virus and cells

SARS-CoV-2 (isolate BetaCoV/Munich/BavPat1/2020; kindly provided by Prof. Dr. C. Drosten) was propagated to passage 3 on VeroE6 cells (ATCC) in Opti-MEM I (1X) + GlutaMAX (Gibco), supplemented with penicillin (10,000 IU mL⁻¹, Lonza) and streptomycin (10,000 IU mL⁻¹, Lonza) at 37°C in a humidified CO₂ incubator. VeroE6 cells were inoculated at an moi of 0.01. Supernatant was harvested 72 hpi, cleared by centrifugation and stored at -80°C. VeroE6 cells were maintained in DMEM (Gibco) supplemented with 10% foetal calf serum (Greiner), 2 mM of L-glutamine (Gibco), 10 mM Hepes (Lonza), 1.5 mg ml⁻¹ sodium bicarbonate (NaHCO₃, Lonza), penicillin (10,000 IU/mL) and streptomycin (10,000 IU/mL) at 37°C in a humidified CO₂ incubator. All work was performed in a Class II Biosafety Cabinet under BSL-3 conditions at the Erasmus Medical Center.

Ferret transmission experiment

All relevant ethical regulations for animal testing have been complied with. Animals were housed and experiments were performed in strict compliance with the Dutch legislation for the protection of animals used for scientific purposes (2014, implementing EU Directive 2010/63). Influenza virus, SARS-CoV-2 and Aleutian Disease Virus seronegative 6 month-old female ferrets (*Mustela putorius furo*), weighing 700–1000 g, were obtained from a commercial breeder (TripleF (USA)). Research was conducted under a project license from the Dutch competent authority (license number AVD1010020174312) and the study protocol was approved by the institutional Animal Welfare Body (Erasmus MC permit number 17-4312-02). Animal welfare was monitored on a daily basis. Virus inoculation of ferrets was performed under anesthesia with a mixture of ketamine/medetomidine (10 and 0.05 mg kg⁻¹ respectively) antagonized by atipamezole (0.25 mg kg⁻¹). Swabs were taken under light anesthesia using ketamine to minimize animal discomfort.

Four donor ferrets were inoculated intranasally with 6.10^5 TCID₅₀ of SARS-CoV-2 virus diluted in 500 µl of phosphate-buffered saline (PBS) (250 µl instilled dropwise in each nostril) and were housed individually in a cage. Six hpi, direct contact ferrets were placed in the same cage as the donor ferrets. One day later, indirect recipient ferrets were placed in an opposite cage separated by two steel grids, 10 cm apart, to avoid contact transmission (Figure S1). Throat, nasal and rectal swabs were collected from the animals every other day, to prevent cross-contamination, until they were negative for SARS-CoV-2 RNA or maximum for 21 dpi/dpe by determined by real-time RT-qPCR as described below. Swabs were stored at -80 °C in transport medium (Minimum Essential Medium Eagle with Hank's BSS (Lonza), 5 g L⁻¹ lactalbumine enzymatic hydrolysate, 10% glycerol (Sigma-Aldrich), 200 U ml⁻¹ of penicillin, 200 mg ml⁻¹ of streptomycin, 100 U ml⁻¹ of polymyxin B sulfate (Sigma-Aldrich), and 250 mg ml⁻¹ of gentamicin (Life Technologies)) for end-point titration in VeroE6 cells as described below. Ferrets were euthanized at 21 dpi/dpe by heart puncture under anaesthesia. Blood was collected in serum-separating tubes (Greiner) and processed according to the manufacturer's instructions. Sera were heated for 1h at 60 °C and used for the detection of specific antibodies against SARS-CoV-2 as described below. [All animal experiments were performed in class III isolators in a negatively pressurized ABSL3+ facility.](#)

RNA isolation and RT-qPCR

RNA was isolated using an in-housed developed high-throughput method in a 96-well format. Sixty µl of sample were added to 90 µl of MagNA Pure 96 External Lysis Buffer (Roche). A known concentration of phocine distemper virus (PDV) was added to the sample as internal control for the RNA extraction²⁹. The 150 µl of sample/lysis buffer was added to a well of a 96-well plate containing 50 µl of magnetic beads (AMPure XP, Beckman Coulter). After thorough mixing by pipetting up and down at least 10 times, the plate was incubated for 15 minutes (min) at room temperature. The plate was then placed on a magnetic block (DynaMag™-96 Side Skirted Magnet (ThermoFisher Scientific)) and incubated for 3 min to allow the displacement of the beads towards the side of the magnet. Supernatants were carefully removed without touching the beads and beads

were washed three times for 30 seconds (sec) at room temperature with 200 µl/well of 70% ethanol. After the last wash, a 10 µl multi-channel pipet was used to remove residual ethanol. Plates were air-dried for 6 min at room temperature. Plates were removed from the magnetic block and 30 µl of PCR grade water was added to each well and mixed by pipetting up and down 10 times. Plates were incubated for 5 min at room temperature and then placed back on the magnetic block for 2 min to allow separation of the beads. Supernatants were pipetted in a new plate and RNA was kept at 4°C. The RNA was directly used for RT-qPCR using primers and probes targeting the E gene of SARS-CoV-2 as previously described³⁰. The primers and probe for PDV detection were described previously²⁹.

Virus titrations

Throat, nasal and rectal swabs were titrated in quadruplicates in VeroE6 cells. Briefly, confluent VeroE6 cells were inoculated with 10-fold serial dilutions of sample in Opti-MEM I (1X) + GlutaMAX, supplemented with penicillin (10,000 IU mL⁻¹), streptomycin (10,000 IU mL⁻¹). At one hpi, the first three dilutions were washed twice with media and fresh media was subsequently added to the whole plate. At six dpi, virus positivity was assessed by reading out cytopathic effects. Infectious virus titers (TCID₅₀/ml) were calculated from four replicates of each throat, nasal and rectal swabs and from 24 replicates of the virus stock using the Spearman-Kärber method.

Serology

Sera were tested for SARS-CoV-2 antibodies using a receptor binding domain (RBD) enzyme-linked immunosorbent assay (ELISA) as described previously, with some modifications³¹. Briefly, ELISA plates were coated overnight with either SARS-CoV-2 RBD. After blocking, sera were added and incubated for 1h at 37°C. Bound antibodies were detected using horseradish peroxidase (HRP)-labelled goat anti-ferret IgG (Abcam) and 3,3',5,5'-Tetramethylbenzidine (TMB, Life Technologies) as a substrate. The absorbance of each sample was measured at 450 nm.

Data availability

All data are available from the corresponding author (S.H.) on reasonable request.

No custom software was used in this study.

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Author Contributions

M.R. and S.H. conceived, designed, analysed and performed the work. M.R. and S.H. wrote the manuscript. A.K., D.M., T.B., M.L., N.O. helped with performing the work. M.F.V., B.R., B.H., M.K., R.A.M.F. helped with the design of the work, interpretation of the data and manuscript revision. All authors read and approved the final manuscript.

The authors declare no competing interests.

Additional information

Supplementary Information is available for this paper.

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