Susceptibility of Well-Differentiated Airway Epithelial Cell Cultures from Domestic and Wild Animals to Severe Acute Respiratory Syndrome Coronavirus 2

Mitra Gultom, Matthias Licheri, Laura Laloli, Manon Wider, Marina Strässle, Philip V’kovski, Silvio Steiner, Annika Kratzel, Tran Thi Nhu Thao, Lukas Probst, Hanspeter Stalder, Jasmine Portmann, Melle Holwerda, Nadine Ebert, Nadine Stokar-Regenscheit, Corinne Gurtner, Patrik Zanolari, Horst Posthaus, Simone Schuller, Amanda Vicente-Santos, Andres Moreira-Soto, Eugenia Corrales-Aguilar, Nicolas Ruggli, Gergely Tekes, Veronika von Messling, Bevan Sawatsky, Volker Thiel, Ronald Dijkman

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread globally, and the number of worldwide cases continues to rise. The zoonotic origins of SARS-CoV-2 and its intermediate and potential spillback host reservoirs, besides humans, remain largely unknown. Because of ethical and experimental constraints and more important, to reduce and refine animal experimentation, we used our repository of well-differentiated airway epithelial cell (AEC) cultures from various domesticated and wildlife animal species to assess their susceptibility to SARS-CoV-2. We observed that SARS-CoV-2 replicated efficiently only in monkey and cat AEC culture models. Whole-genome sequencing of progeny viruses revealed no obvious signs of nucleotide transitions required for SARS-CoV-2 to productively infect monkey and cat AEC cultures. Our findings, together with previous reports of human-to-animal spillover events, warrant close surveillance to determine the potential role of cats, monkeys, and closely related species as spillback reservoirs for SARS-CoV-2.

During the past 2 decades we have observed zoonotic outbreaks of severe acute respiratory syndrome coronavirus (SARS-CoV) in 2003 and Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 (1,2). These outbreaks have been followed by the current pandemic caused by the 2019 zoonotic emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiologic agent of coronavirus disease (COVID-19) (3,4). Humans are currently seen as the main hosts, but the zoonotic

Author affiliations: Institute for Infectious Diseases, University of Bern, Bern, Switzerland (M. Gultom, M. Licheri, L. Laloli, M. Wider, M. Strässle, L. Probst, M. Holwerda, R. Dijkman); University of Bern Department of Infectious Diseases and Pathobiology, Bern (M. Gultom, L. Laloli, M. Strässle, P. V’kovski, S. Steiner, A. Kratzel, T.T.N. Thao, H. Stalder, J. Portmann, M. Holwerda, N. Ebert, N. Stokar-Regenscheit, C. Gurtner, H. Posthaus, V. Thiel, R. Dijkman); Institute of Virology and Immunology, Bern and Mittelhäusern, Switzerland (M. Gultom, L. Laloli, M. Strässle, P. V’kovski, S. Steiner, A. Kratzel, T.T.N. Thao, H. Stalder, J. Portmann, M. Holwerda, N. Ebert, V. Thiel, R. Dijkman); University of Bern Graduate School for Biomedical Science, Bern (M. Gultom, L. Laloli, S. Steiner, A. Kratzel, T.T.N. Thao, L. Probst, M. Holwerda); Institute of Veterinary Bacteriology, University of Bern, Bern (M. Strässle); Institute of Animal Pathology, University of Bern, Bern (N. Stokar-Regenscheit, C. Gurtner, H. Posthaus); Clinic for Ruminants, Vetsuisse Faculty, University of Bern, Bern (P. Zanolari); University of Bern Department of Clinical Veterinary Medicine, Bern (S. Schuller); Virology-Research Center for tropical diseases (CIET), University of Costa Rica, Montes de Oca, Costa Rica (A. Vicente-Santos, A. Moreira-Soto, E. Corrales-Aguilar); Institute of Virology, Charité-Universitätsmedizin Berlin, Corporate Member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany (A. Moreira-Soto); Institute of Virology, Justus Liebig University Giessen, Giessen, Germany (G. Tekes); Federal Institute for Vaccines and Biomedicines, Langen, Germany (V. von Messling, B. Sawatsky)

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*Current affiliation: Institute for Infectious Diseases, University of Bern, Bern, Switzerland.
origins and intermediate and potential spillback host reservoirs of SARS-CoV-2 are not yet well defined. Several reports indicate that SARS-CoV-2 spillover events from human to other animal species can occur (5–7). These zoonotic events are likely driven by close human–animal interactions and the conservation of crucial receptor binding motif (RBM) residues in the angiotensin-converting enzyme 2 (ACE2) orthologs, potentially facilitating SARS-CoV-2 entry (8,9). This knowledge gap highlights the need to assess the potential host spectrum for SARS-CoV-2 to support current pandemic mitigation strategies.

Besides their use in determining the host spectrum, animal models will be needed for viral pathogenesis studies, as well as for testing novel antiviral drugs, immunotherapies, and vaccines against SARS-CoV-2. Typically, in such studies a large variety of animal species are tested for susceptibility (10–12). However, such experiments have several drawbacks, including the availability of diverse animal models and the need for dedicated personnel, housing facilities, and most important, ethics approval. Some of these factors are especially limiting when applied to wildlife and livestock animals, such as pigs, cattle, and other ruminants; when working with companion animals and nonhuman primates, there are additional socioemotional and ethical considerations.

In this study, we evaluated the susceptibility of several mammal species to SARS-CoV-2 by recapitulating the initial stages of infection in a controlled in vitro model, in compliance with the reduction, refinement, and replacement principles in animal experimentation, while at the same time circumventing traditional in vivo experimental constraints. We used a unique well-differentiated airway epithelial cell (AEC) culture repository from the primary tracheobronchial airway tissue of 12 mammal species comprising companion animals, animal model candidates, livestock, and wild animals to assess their susceptibility to SARS-CoV-2 infection. To control for the quality of the AEC, we used influenza viruses that have known broad host tropism (13–15).

Materials and Methods

Conventional Cell Culture
We cultured Vero E6 cells in Dulbecco Modified Eagle medium supplemented with 10% volume/volume percent (vol/vol) heat-inactivated fetal bovine serum, 1 mmol/L sodium pyruvate, 1xGlutaMAX, 100 µg/mL streptomycin, 100 IU/mL penicillin, 1% vol/vol nonessential amino acids, and 15 mMol/L HEPES buffering agent (GIBCO; https://www.thermofisher.com). We maintained cells at 37°C in a humidified incubator with 5% CO2.

Establishment of Animal AEC Culture Repository
We isolated tracheobronchial epithelial cells from 12 different animal species from postmortem tracheobronchial tissue that was obtained from slaughterhouses, veterinary hospitals, or domestic or international research institutes that euthanize their animals for diagnostic purposes or as part of their licensed experimental work in accordance with local regulations and ethics guidelines. We isolated and cultured the cells as described elsewhere (16). To establish well-differentiated AEC cultures from diverse mammal species, we introduced several modifications to the composition of the air-liquid interface (ALI) medium (Table 1). We maintained all animal ALI cultures at 37°C in a humidified incubator with 5% CO2. While the differentiated ALI cultures were developing over 3–4 wk, we changed media every 2–3 d.

Virus Propagation
We propagated SARS-CoV-2 (SARS-CoV-2/München-1.1/2020/929) virus stock in Vero E6 cells for 48 h then cleared virus-containing supernatant from cell debris by centrifuging for 5 min at 500 × g before aliquoting and storing it at −80°C. We determined viral titer by plaque forming unit (PFU) assay on Vero E6 cells as described elsewhere (17). We prepared working stocks of influenza A virus (IAV) A/Hamburg/4/2009 strain in the pHW2000 reverse genetic backbone by propagating the rescued virus in MDCK-II cells for 72 h in the infection medium, which was composed of Eagle Minimum Essential Medium, supplemented with 0.5% of bovine serum albumin, 100 µg/mL streptomycin and 100 IU/mL penicillin solution, 1 µg/mL trypsin acetylated from bovine pancreas (Sigma-Aldrich, https://www.sigmaaldrich.com), and 15 mMol/L HEPES buffer. We determined viral titer by plaque assay on MDCK-II cells as described elsewhere (18,19). We propagated influenza D virus (IDV, D/bovine/Oklahoma/660/2013 strain) stocks in the human rectal tumor cell line HRT-18G (ATCC [American Type Culture Collection] CRL11663, https://www.atcc.org) for 96 h in the infection medium, with the adjustment of using 0.25 µg/mL of trypsin. We determined viral titer by TCID50 assay on HRT-18G cells as described elsewhere (20).

Infection of Animal AEC Cultures
We infected well-differentiated AEC cultures from 12 different species with 30,000 PFU of SARS-CoV-2, or 10,000 TCID50 of either IAV or IDV, as described...
elsewhere (16). We monitored progeny virus release at 24-h intervals for 96 h, through the application of 100 µL of HBSS onto the apical surface and incubated 10 min before collection. We diluted collected apical washes 1:1 with virus transport medium and stored them at –80°C for later analysis. After the collection of the apical washes, we exchanged the basolateral medium with fresh ALI medium. We repeated each experiment as 2 independent biologic replicates using AEC cultures established from either 1 or 2 biologic donors of each species depending on the availability of procured animal tissue (Table 1).

**Immunofluorescence Analysis**

We fixed virus-infected animal AEC cultures with 4% vol/vol neutral-buffered formalin at 96 hours postinfection (hpi) for SARS-CoV-2 or 48 hpi for IAV- or IDV-infected AEC cultures and processed them as described elsewhere (16). To detect SARS-CoV-2, we incubated fixed animal AEC cultures with a Rockland (https://rockland-inc.com) 200-401-A50 rabbit polyclonal antibody against SARS-CoV nucleocapsid protein, which has previously been shown to cross-react with SARS-CoV-2 (17). We used an Abcam (https://www.abcam.com) ab128193 mouse antibody against IAV clone C43 nucleoprotein to detect IAV-infected cells and a custom-made rabbit polyclonal antibody against the nucleoprotein of influenza D/bovine/Oklahoma/660/2013 strain (GenScript, https://www.genscript.com) to detect IDV-infected cells. To visualize the distribution of ACE2 in the AEC cultures, we used Abcam ab15348 and Biorbyt (https://www.biorbyt.com) orb582208 rabbit polyclonal antibody to visualize the distribution of ACE2 in the AEC cultures (Table 1).

We extracted viral RNA from 100 µL of 1:1 diluted apical wash using the NucleoMag VET (Macherey-Nagel AG, https://www.mn-net.com), according to the manufacturer’s guidelines, on a Kingfisher Flex
purification system (Thermo Fisher Scientific). We amplified 2 µL of extracted RNA using TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific) according to the manufacturer’s protocol. We used a forward primer, adapted from primers described elsewhere (23): 5′-ACAGTGATAGTTAATAGTGAATACGTGATCTTT-3′, reverse 5′- ACAATTGCAAGCGTACGCACA-3′, and probe 5′-FAM-ATCCITACT-GCGCTTCGA-MGB-Q530-3′ (Microsynth, https://www.microsynth.ch), targeting the envelope gene of SARS-CoV-2 (GenBank accession no. MN908947.3). As a positive control, we included a serial dilution of in vitro–transcribed RNA containing regions of the RNA-dependent RNA polymerase, envelope, and N genes derived from a SARS-CoV-2 synthetic construct (GenBank accession no. MT108784) to determine the genome copy number. We performed measurements and analysis using an Applied Biosystems ABI7500 instrument and associated software package (Thermo Fisher Scientific).

Titration of SARS-CoV-2 in the Apical Washes
To quantify SARS-CoV-2, we titrated apical washes by plaque assay on Vero E6 cells. In brief, we seeded 1 × 10^5 cells/well in 24-well plates 1 d before titration and inoculated them with 10-fold serial dilutions of virus solutions. We removed inoculums 1 hpi and replaced them with overlay medium consisting of Dulbecco Modified Eagle Medium supplemented with 1.2% Avicel (DuPont, https://www.pharma.dupont.com), 10% heat-inactivated fetal bovine serum, 100 µg/mL streptomycin, and 100 IU/mL penicillin. We incubated cells at 37°C with 5% CO2 and inoculated them with 10-fold serial dilutions of SARS-CoV-2 stock and the 96 hpi apical washes from SARS-CoV-2–infected monkey and cat AEC cultures. In contrast, for the remaining animal AEC cultures we detected either a continuous or declining level of viral RNA load throughout the entire time course (Figure 1, panels A, B; Appendix 2, https://wwwnc.cdc.gov/EID/article/27/7/20-4660-App2.pdf, Figure 1, panels).
Susceptibility of Animals to SARS-CoV-2

Because productive progeny virus production was only observed in the well-differentiated tracheobronchial epithelial cell cultures from rhesus macaques and cats, we wondered whether this was because of incompatibility with the cellular receptor used by SARS-CoV-2 for cellular entry (27,35). To assess whether this corresponds to the amino acid sequence conservation of RBM in ACE2, we performed in silico analysis on the ACE2 protein sequences of the species included in this study (27,28). This process revealed that the amino acid identity of the ACE2 RBM regions interacting with the receptor-binding domain of SARS-CoV-2 in humans were more similar
to those in rhesus macaques and cats than in other species (Appendix 2 Figure 2, panel A).

Apart from receptor compatibility as a limiting factor for virus infection, it has been demonstrated previously that partially differentiated AEC cultures are poorly permissive to respiratory virus infection (36). To investigate whether the lack of replication in ferret cells, for example, was not caused by poor differentiation of our cell cultures, we validated the AEC cultures by infecting culture samples with the 2009 pandemic IAV A/Hamburg/4/2009 and ruminant-associated IAV D/bovine/Oklahoma/660/2013 strains. Both viruses are members of Orthomyxoviridae and are known to have a broad host spectrum, including ferrets (13–15,37). We inoculated the AEC cultures from 12 different species (rhesus macaque, cat, ferret, dog, rabbit, pig, cattle, goat, llama, camel, and 2 neotropical bats) with 10,000 TCID₅₀ of either IAV or IDV and incubated them at either 33°C or 37°C. At 48 hpi, we fixed the AEC cultures and processed them by

![Figure 2. Tropisms of SARS-CoV-2, IAV, and IDV in infected airway epithelial cell cultures from diverse mammal species. We inoculated well-differentiated animal airway epithelial cell cultures with either 30,000 PFU of SARS-CoV-2 (SARS-CoV-2/München-1.1/2020/929), 10,000 50% tissue culture infective dose of IAV/Hamburg/4/2009, or IDV (D/bovine/Oklahoma/660/2013). We incubated virus-infected airway epithelial cell cultures at 33°C or 37°C and formalin-fixed them at 96 hours postinfection (for SARS-CoV-2) or 48 hours postinfection (for influenza viruses). After fixation, we stained virus-infected cultures using antibodies against either SARS-CoV-2, IAV, or IDV NP (green), and β-tubulin (cilia, red). We acquired images using an EVOS FL Auto 2 Imaging System equipped with a 40x air objective. Scale bar indicates 50 µm. *Sturnira lilium bat; †Carollia perspicillata bat. IAV, influenza A virus; IDV, influenza D virus; NP, nucleocapsid protein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.]
Susceptibility of Animals to SARS-CoV-2

In the immunofluorescence analysis, we also incorporated an antibody against β-tubulin marker to discern ciliated and nonciliated cell populations. For both rhesus macaques and cats, SARS-CoV-2 antigen-positive cells predominantly overlapped with the nonciliated cell populations, at either incubation temperature. Using polyclonal antibodies against ACE2, we found that the cellular receptor expression in rhesus macaques and cats predominantly overlapped with SARS-CoV-2 cell tropism, similar to ACE2 distribution in human AEC cultures (Appendix 2 Figure 2, panel B) (17). Unfortunately, because of limited availability of well-differentiated AEC cultures, we could not assess the ACE2 expression in goat, cattle, and rabbit AEC cultures. Nevertheless, for most species, including ferrets, that did not support efficient replication of SARS-CoV-2, we observed that ACE2 was expressed on the cell surface (Appendix 2 Figure 2, panel B). This finding suggests that ACE2 expression alone does not per se confer permissiveness to SARS-CoV-2.

It has previously been shown that SARS-CoV-2 can undergo rapid genetic changes in vitro (39). Because we observed efficient replication in rhesus macaque and cat AEC cultures, we assessed whether any mutations suggestive of viral adaptation had occurred. We performed whole-genome sequencing (Oxford Nanopore Technologies) on the viral inoculum used, as well as on the progeny viruses collected from the rhesus macaque and cat AEC cultures incubated at 33°C or 37°C. SARS-CoV-2/Wuhan-Hu-1 (GenBank accession no. MN908947.3) was used as the reference sequence. P, passage; UTR, untranslated region.

Figure 3. Whole-genome sequencing analysis using Nanopore sequencing technology (Oxford Nanopore Technologies, https://nanoporetech.com). A graphical representation of variants found in the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) stock P1 and P2, as well as the apical washes from SARS-CoV-2–infected monkey and cat airway epithelial cell cultures with either P1 or P2 stock 96 hpi at 33°C or 37°C. SARS-CoV-2/Wuhan-Hu-1 (GenBank accession no. MN908947.3) was used as the reference sequence. P, passage; UTR, untranslated region.
**Discussion**

Our study used an in vitro AEC culture repository composed of various domestic and wildlife animal species to assess the spectrum of potential intermediate and spillback host reservoirs for SARS-CoV-2. Inoculation of AEC cultures from rhesus macaque, cat, ferret, dog, rabbit, pig, cattle, goat, llama, camel, and 2 neotropical bat species with SARS-CoV-2 revealed that tracheobronchial cells only from rhesus macaque monkeys and cats supported efficient replication of SARS-CoV-2. Whole-genome sequencing indicated that the currently circulating SARS-CoV-2 D614G variant can efficiently infect rhesus macaque and cat AEC. Our data highlight that these 2 animals are potential models for evaluating therapeutic mitigation strategies for circulating viral variants. Our findings, in conjunction with information from previously documented spill-over events, indicate that close surveillance of these animals and closely related species, whether in the wild, captivity, or households, is warranted.

To date, there have been several reports published evaluating the suitability of animal models, including cats, rhesus macaques, dogs, pigs, rabbit and ferrets, for testing SARS-CoV-2 infection (32,33,40–43). We observed that SARS-CoV-2 did not efficiently replicate in tracheobronchial AEC derived from rabbits and ferrets, although ferrets are used as an animal model for SARS-CoV-2. This finding may be because viral infections in rabbits and ferrets are mainly restricted to the nasal conchae, are dose-dependent and, in addition, the origin of the cells used as input for the AEC cultures may not recapitulate the cells of the nasal mucosa (34,40,42,43). Differences exist in cellular composition and host determinent expression levels along proximal and distal regions of the respiratory tract (44). In addition, SARS-CoV-2 might use a different cellular receptor in ferrets, although ACE2 could be detected on the cell surface (Appendix 2 Figure 2, panel B) (45). Therefore, it would be of interest to complement our current repository with AEC cultures from different anatomic regions of animals such as rabbits and ferrets and to evaluate whether ACE2 is the cellular receptor employed by SARS-CoV-2 in these various animal species.

It has been proposed that SARS-CoV-2 spillover into humans, as with SARS-CoV, originally occurred from bats, either directly or through an intermediate reservoir (3,46). With >1,400 bat species comprising >20% of all mammal species, we restricted our experiments with SARS-CoV-2 to our established AEC cultures from the 2 neotropical bat species *C. perspicillata* and *S. lilium* (M. Gultom et al., unpub. data). We showed that these 2 neotropical bats express ACE2 but are not susceptible to SARS-CoV-2, suggesting that they are not likely reservoir hosts for SARS-CoV-2 despite the detection of other coronaviruses and presumptive ACE2 receptor usage by SARS-CoV-2 in closely related bat species (25,47). In fact, a 2020 study described susceptibility to SARS-CoV-2 infection in fruit bats (*Rousettus aegyptiacus*) (33). Future research should therefore include AEC cultures from this bat species, as well as from horseshoe bat species (genus *Rhinolophus*), which have previously been characterized as reservoir hosts for viruses with a close genetic relationship to the coronavirus associated with the 2003 SARS outbreak (33,46). In summary, our results highlight that in vitro well-differentiated animal AEC culture models can be used as an alternative to traditional animal experimentation models to evaluate and provide insight into the host spectrum of SARS-CoV-2.

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**About the Author**

Ms. Gultom is a PhD student at the University of Bern, Bern, Switzerland. Her research interest lies with the establishment of primary airway epithelial cell cultures to study emerging respiratory viruses.

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Address for correspondence: Ronald Dijkman, Institute for Infectious Diseases, University of Bern, Friedbühlstrasse 51, 3001 Bern, Switzerland; email: ronald.dijkman@ifik.unibe.ch
Susceptibility of Well-Differentiated Airway Epithelial Cell Cultures from Domestic and Wild Animals to Severe Acute Respiratory Syndrome Coronavirus 2

Appendix 2

Appendix 2 Figure 1. Mock-treated cells from animal airway epithelial cell (AEC) cultures infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), influenza A virus (IAV), and influenza D virus (IDV). Mock-treated AEC cultures were incubated at 33°C or 37°C in parallel with
virus-infected cells for 96 hpi (for SARS-CoV-2) or 48 hpi (for IAV and IDV). Afterwards, cells were fixed and stained using antibodies against either SARS-CoV, IAV, or IDV nucleocapsid proteins (green), β-tubulin in cilia (red), and tight-junctions (white) (panel A). Images were acquired using an EVOS FL Auto 2 imaging system (Thermo Fisher Scientific, https://www.thermofisher.com) equipped with a 40x air objective. Scale bar = 50 µm. In parallel with the SARS-CoV-2–infected cells, apical washes from the mock-treated cells were collected every 24 hours and analyzed by qualitative reverse transcription PCR (panels B,C) and plaque titration assays on Vero E6 cells (panels D,E). Error bars represent the average of 2 independent biological replicates using AEC cultures established from 1 or 2 biological donors. The dotted lines on panels D and E indicate the detection limit of the assay. IAD, influenza A virus; IDV, influenza D virus, LOA, limit of assay; NP, nucleoprotein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; *, Sturnira lilium; †, Carollia perspicillata
Appendix 2 Figure 2. Angiotensin-converting enzyme 2 (ACE2) analysis among different animal species. A) Protein sequence alignment of ACE2 from diverse animals in the residues interacting with severe acute respiratory syndrome coronavirus 2. The alignment was constructed using ClustalW program (https://www.genome.jp/tools-bin/clustalw). B) To visualize the ACE2 distribution in the animal airway epithelial cell cultures, formalin-fixed cells were stained with antibodies against ACE2 (green), β-tubulin (cilia, red), and ZO-1 (tight junctions, white). Image acquisition was performed using an EVOS FL Auto 2 Imaging System equipped with a 40x air objective. Scale bar is 20 µm.