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Distinct phenotype of SARS-CoV-2 Omicron BA.1 in human primary cells but no increased host range in cell lines of putative mammalian reservoir species

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ABSTRACT

SARS-CoV-2's genetic plasticity has led to several variants of concern (VOCs). Here we studied replicative capacity for seven SARS-CoV-2 isolates (B.1, Alpha, Beta, Gamma, Delta, Zeta, and Omicron BA.1) in primary reconstituted airway epithelia (HAE) and lung-derived cell lines. Furthermore, to investigate the host range of Delta and Omicron compared to ancestral SARS-CoV-2, we assessed replication in 17 cell lines from 11 non-primate mammalian species, including bats, rodents, insectivores and carnivores. Only Omicron's phenotype differed *in vitro*, with rapid but short replication and efficient production of infectious virus in nasal HAEs, in contrast to other VOCs, but not in lung cell lines. No increased infection efficiency for other species was observed, but Delta and Omicron infection efficiency was increased in A549 cells. Notably replication in A549 and Calu3 cells was lower than in nasal HAE. Our results suggest better adaptation of VOCs towards humans, without an extended host range, and may be relevant to the search for the putative intermediate host and reservoirs prior to the pandemic.

Introduction

Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is the etiological agent of coronavirus disease 19 (COVID19) that has caused one of the biggest public health challenges of modern times. Few mutational changes were observed in SARS-CoV-2 during the first year of the pandemic, most notably the Spike D614G mutation. This mutation enhanced angiotensin converting enzyme-2 (ACE-2) binding by stabilizing the spike trimers (Juraszek et al., 2021;Zhang et al., 2021), providing a fitness advantage, and was responsible for the first pandemic wave (Zhou et al., 2021). By late 2020, SARS-CoV-2 variants had emerged, characterised by numerous mutations, mainly in Spike. These were classified as variants of concern (VOC), variants of interest (VOI) or variants under monitoring (VUMs), based on their genetic, clinical and epidemiological characteristics. To date, five VOCs have been designated: Alpha, Beta, Gamma, Delta and Omicron. VOCs are characterized by a rapid increase in case numbers, quickly outcompeting earlier strains in their region of emergence. Omicron has the most observed mutations,

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with the majority located in Spike, causing the strongest escape from prior immunity so far. It also shows signs of higher transmissibility and secondary attack rates. In addition, VUMs and VOIs were co-circulating along with VOCs, including the (former) VOI Zeta used in our study here, that arose alongside the Gamma VOC in South America during a surge in local cases, but nearly all have disappeared.

Variant evolution does not yet follow a pattern, and most variants have not evolved from each other, but arose independently from basal circulating viruses (Markov et al., 2022). Indeed, the closest known lineage to Omicron was last detected in mid-2020 (Viana et al., 2022). There are three main hypotheses on VOC origins (Burki, 2022). The first holds that they developed in populations that were not covered by genomic surveillance. The second involves reverse zoonotic events, with transmission from humans into intermediate hosts, and then back to humans. While a large range of animal species are known to be susceptible to SARS-CoV-2, no plausible VOC progenitors have been found in animals (Meekins et al., 2021). Additionally, aside from white-tailed deer in the USA, detection of sustained transmission in similar wildlife populations in Europe has only been sporadic (Moreira-Soto et al., 2022). Other species that have tested positive often have frequent contact with humans (such as pets and common rodent pests), and any spillback would be expected frequently, and thus with less change between spillbacks. The third hypothesis describes intra-host evolution during chronic infections in immunocompromised patients. Such cases have already been reported, with prolonged shedding of mutated infectious virus (Cele et al., 2022; Nussenblatt et al., 2021; Weigang et al., 2021).

VOCs have shown distinct epidemiological and partial clinical differences. While genomic surveillance can inform about mutations and the phylogenetic relationships, it cannot directly conclude on biological properties resulting from these mutations, thus the *in vitro* assessment of phenotypic differences is of immediate relevance whenever new variants arise. Understanding the mechanisms of enhanced fitness, the origins of VOCs and their risk for reverse zoonotic events are crucial for the further control of this pandemic. Here, we have assessed replicative capacity and potential *in vitro* phenotypes of ancestral SARS-CoV-2 (B.1), VOCs Alpha, Beta, Gamma, Delta, Omicron (BA.1) and former VOI Zeta on human primary cells and lung-derived cell lines and on mammalian cell lines derived from bats, rodents, insectivores and carnivores to investigate potential host range.

2. Material and methods

2.1. Human and animal cell lines

Commercially available nasal HAE, called "MucilAir™", were purchased from Epithelix SARL [www.epithelix.com]. They are maintained in air-liquid interface culture where the medium (MucilAir™ medium, Epithelix, Plan-Les-Ouates, Switzerland) is supplemented by the basal chamber and the apical surface of the tissue is in contact with air (see Essaidi-Laziosi et al., 2021 for details).

All used animal cell lines are summarized in Table1.The corresponding species, some of them belonging to animal groups discussed as potential spill-over or spill-back reservoirs for SARS-CoV-2 (Sharun et al., 2021) and other coronaviruses (Eckerle et al., 2014a), were confirmed by sequencing of the cytochrome C subunit oxidase I gene (Alcaide et al., 2009; Ivanova et al., 2012). Five additional standard cell lines were used in this study as controls or for viral stock production, including Calu3 (human lung cancer cell), Vero-E6 (African Green Monkey kidney), A549 (human lung carcinoma), A549 cells overexpressing ACE-2 receptor and Vero-E6 overexpressing TMPRSS2. All immortalized cells were cultured in monolayer using medium (MEM Glutamax, Gibco, Waltham, MA USA) supplemented with 10 % (for cell maintenance) or 2 % (for infections) Fetal Bovin Serum (FBS, Gibco, Waltham, MA USA), 1X penicillin-streptomycin (Gibco, Waltham, MA USA) and 1 % non-essential amino-acids (MEM NEAA, Gibco). All cell cultures were performed at 37 °C under 5 % CO_2.

2.2. Viruses

Isolates were obtained from clinical samples collected in the outpatient testing center of the University Hospitals of Geneva as described previously (Bekliz et al., 2022). All SARS-CoV-2 isolates used are summarized in Table S1. B.1 and the variants Alpha, Gamma, Zeta and Delta were isolated after one passage in Vero-E6 cells. One additional passage allowed viral stock production in the same line. Vero-E6 was less permissive to Beta and Omicron variants. Hence, Beta was isolated in A549-ACE-2, after a second passage in mixed Vero-E6:A549-ACE-2 (1:1) cells, the viral stock was produced in Vero-E6 cells. Omicron was isolated after 2 passages in Vero-TMPRSS2. All viral stocks were titrated in Vero-E6 and sequenced (Table S1).

2.3. Infections of animal and human cells

Infection assays were performed at 37 °C or 33 °C (temperatures characterizing the upper and lower respiratory tract in humans, respectively) at 5 % CO_2. HAE reconstituted *in vitro* (3D culture) or immortalised cell lines in 2D culture, including Calu3 and animal cell lines (Table 1), were tested for infections as previously described (Ulrich et al., 2021) at 37 °C and an MOI of 0.1 using seven SARS-CoV-2 isolates (Table S1). Cells were washed after 1 h for Calu3 and animal cells or 3 h for HAE (to optimize virus adsorption, the inoculation time is longer in HAE, where pseudostratified cells might be less accessible compared to Calu3 and animal cells that are cultured in monolayer). Supernatant (Calu3) or apical washes (HAE) were collected at different times after infection. Infected animal cells were lysed using NucliSens easyMAG lysis buffer (BioMérieux, Petit-Lancy, Switzerland) at 4 days post infection (dpi).

2.4. Quantification of viral RNA

To determine the viral load from collected samples, RNA was extracted with NucliSens easyMAG (BioMérieux) and quantified by quantitative real time PCR (RT-qPCR) using SuperScript™ III Platinum™ One-Step qRT-PCR Kit (Invitrogen; Waltham, MA USA) in a CFX96 Thermal Cycler (BIORAD; California, USA). Viral replication was assessed by comparing residual vRNA (from the supernatant collected at 1hpi for Calu3 and from apical tissue washes 3hpi for HAE) to the amount of vRNA at 1, 2, 3 and 4dpi for HAE or at 4dpi for Calu3. Real time RT-qPCR was performed using specific sets of primers and probes targeting either genomic or sub-genomic viral RNA as previously described (Corman et al., 2020; Wolfel et al., 2020).

2.5. Assessment of infectious titer

Infectious titer of collected samples was assessed by plaque assays performed at 37 °C and 5 % CO_2 as previously described (Ulrich et al., 2021). Briefly, Vero-E6 seeded in monolayer of 2×10^5 cells/mL in 24-well plates were inoculated 2 h after using serially diluted samples. The inoculum was removed and replaced by fresh medium (DMEM 10 % FBS, 2 mM l-glutamine, 1 % penicillin-streptomycin all from Gibco) 1:1 mixed with 2.4 % Avicel (RC-581, FMC biopolymer, London, UK) one hour later. Cells were fixed using 6 % paraformaldehyde (Sigma, Darmstadt, Germany) 24 h later at least 1 h at RT and stained with crystal violet (Sigma, Darmstadt, Germany). PFU were counted from each dilution in order to determine the infectious titer (in PFU/mL) for each sample.

2.6. Comparison of ACE-2 orthologues' protein sequences

For the species *Homo sapiens, Chlorocebus aethiops, Rhinolophus ferrumequinum*, *Mustela vison, Mustela putorius furo, Vulpes vulpes, Erinaceus*

Table 1
List of cell lines used in the study. *confirmed by the sequencing of the cytochrome C subunit oxidase I gene. CMU: Faculty of Medicine of Geneva. FLI: Friedrich-Loeffler-Institut. CB: CHARITÉ-Berlin.

Designation	Species of Origin	Species name (latin)*	Mammalian order	Geographic range	Organ of Origin	Source
RF Kidney	Greater horseshoe bat	*Rhinolophus ferrumequinum*	*Chiroptera*	Europe	Kidney	CB
RE Kidney	Mediterranean horseshoe bat	*Rhinolophus euryale*	*Chiroptera*	Europe	Kidney	CB
RE Trachea	Mediterranean horseshoe bat	*Rhinolophus euryale*	*Chiroptera*	Europe	Trachea	CB
RCL.B Trachea	Geoffroy's horseshoe bat	*Rhinolophus clivosus*	*Chiroptera*	Africa	Trachea	CB
Pip Trachea	Common pipistrelle	*Pipistrellus pipistrellus*	*Chiroptera*	Europe	Trachea	CB
NE-R	American mink	*MMustela. vison*	*Carnivora*	America, introduced in other areas of the world	Fœtus	FLI
MV1-Lu	American mink	*MMustela. vison*	*Carnivora*	America, introduced in other areas of the world	Lung	FLI
FUFE-R Fbr	Red fox	*VVulpes. vulpes*	*Carnivora*	Europe	Embryo	FLI
Crocidura	Lesser white-toothed shrew	*Crocidura suaveolens*	*Soricomorpha*	Europe	Trachea	CB
Hdg REC.B	European hedgehog	*Erinaceus europaeus*	*Erinaceomorpha*	Europe	Kidney	CB
Mygla AEC.B	Bank vole	*Myodes glareolus*	*Rodentia*	Europe	Trachea	CB
MPFLU-1-R	Domestic ferret	*Mustela putorius furo*	*Carnivora*	Europe	Lung	FLI
MPFN-1-R	Domestic ferret	*Mustela putorius furo*	*Carnivora*	Europe	Kidney	FLI
KAN-2-R	Rabbit	*Oryctolagus cuniculus*	*Lagomorpha*	Europe	Lung	FLI
RK-13	Rabbit	*Oryctolagus cuniculus*	*Lagomorpha*	Europe	Kidney	FLI
ZP	Rabbit	*Oryctolagus cuniculus*	*Lagomorpha*	Europe	Embryo	FLI
R9ab	Rabbit	*Oryctolagus cuniculus*	*Lagomorpha*	Europe	Kidney	FLI
A549	Human	*Homo sapiens*	*Primates*	Global	Lung	CMU
VERO-E6	African green monkey	*Chlorocebus aethiops*	*Primates*	Africa	Kidney	CMU

europaeus and *Oryctolagus cuniculus*, ACE-2 amino-acid sequences were available in NCBI. *Pipistrellus pipistrellus* ACE-2 protein sequence has been kindly shared by Joanna Damas and Harris A Lewin (Damas et al., 2020), obtained from an original sequence from the Zoonomia Consortium. For the species *Crocidura suaveolens, Rhinolophus clivosus* and *Rhinolophus euryale* the protein sequences obtained in this study were predicted from ACE-2 nucleic sequences after RNA trizol-extraction from cell lysates and amplification by RT-PCR using primers (Microsynth, Balgach, Switzerland) listed in Table S2. The protein sequence for *Myodes glareolus*, was predicted from a reference transcriptome (Konczal et al., 2014). Contrarily to other species, ACE-2 from *Crocidura suaveolens* could only be partially sequenced but all key residues were be identified (Fang et al., 2021). Of note, one sequence from one individual (whom the initial cell line originated from) was determined for each species. Polymorphisms, as described for *Rhinolophus*, were not considered in this study (Guo et al., 2020). Multiple ACE-2

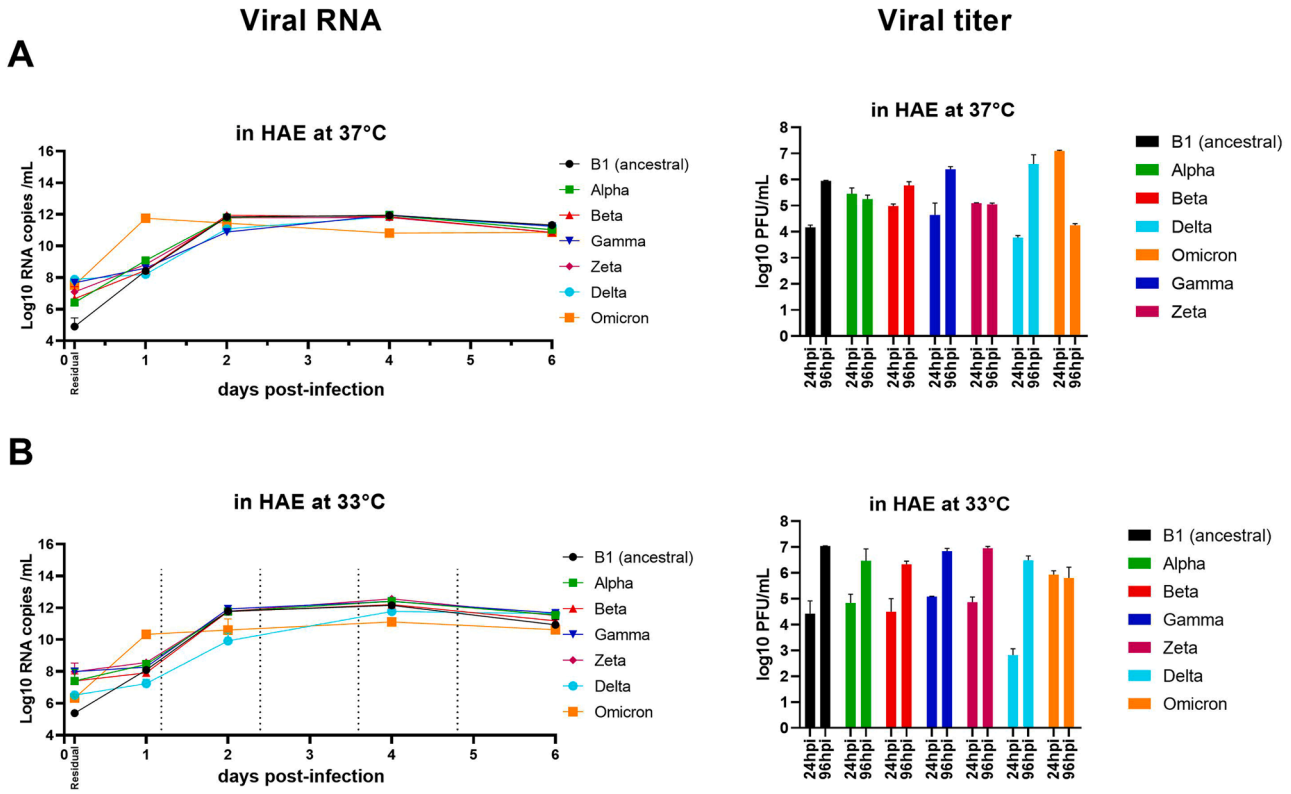

Fig. 1. Replication of SARS-CoV-2 variants 3D *in vitro* reconstituted from epithelial cells. HAE were infected with SARS-CoV-2 lineages including the ancestral (B1) lineage and the variants Alpha, Beta, Gamma, Zeta, Delta and Omicron. Infections were performed at 37 °C (A) and 33 °C (B) $N = 2–4$. Viral replication was assessed by the quantification of viral RNA (left panels) and confirmed by infectious particle titration (right panels).

polymorphisms were indeed observed only in *Rhinolophus euryale* and only the major amino-acid is shown in the table.

3. Results

3.1. Comparative replicative capacity of SARS-CoV-2 variants in human cell culture models of the respiratory tract

To compare viral phenotypes, we infected primary human airway epithelial cells (HAE), derived from the nasal epithelium, and differentiated *in vitro* in 3-dimensional (3D) air-liquid interface cultures and monolayer (2D) cultures with SARS-CoV-2 clinical isolates at the multiplicity of infection (MOI) of 0.1 at 37 °C and 33 °C (Fig. 1). Upon infection of HAE cultures and incubation at 37 °C (Fig. 1A, left panel), we observed a rapid increase at 24 h post infection for Omicron, from mean $\log_{10}$ RNA copy numbers/mL (RNAc/mL) of 7.8 to a peak of 11.8, while all other isolates reached similar peak values at around 96 h. Omicron, in contrast, showed a reduction of viral RNAc/mL at 96 h to 10.8 $\log_{10}$ RNAc/mL. No differences in replication kinetics were observed between B.1, Alpha, Beta, Gamma, Delta and Zeta, in all of which log10 RNAc gradually increased over the first 4 days.

When assessing infectious viral particles by plaque-forming assay at 37 °C (Fig. 1A, right panel), Omicron reached peak infectious viral titer of 7.1 $\log_{10}$ plaque-forming units (PFU) after 24 h with a rapid decline to only 4.3 after 96 h. On the contrary, B.1, Beta, Gamma and Delta showed an increase in PFU from 24 to 96 h, but peak PFU titers stayed below that of Omicron. The Alpha and the Zeta variants did not show strong increases, with PFUs for 24 and 96 h staying constant and below those of the other variants. The strongest increase was observed for Delta, which showed the lowest infectious titer at 24 h of 3.8, which increased to 6.6 mean $\log_{10}$ PFU/mL.

At 33 °C incubation temperature (Fig. 1B), Omicron also showed a fold increase of 4.0 mean $\log_{10}$ RNAc/mL at 24 h, but RNA levels increased further with a peak at 96 h of 11.1 mean $\log_{10}$ RNAc/mL. Similarly, the other variants including B.1 reached their peak RNA levels at 96 h, but with higher RNA levels than that of Omicron, ranging between 11.8 and 12.6 mean $\log_{10}$ RNAc/mL. Relative to 37 °C, at 33 °C viral RNAc/mL were 0.3–0.8 $\log_{10}$ higher for B.1, Alpha, Beta, Gamma and Zeta, but not for Delta and Omicron, which both showed slightly lower viral loads at 33 °C. Similarly, PFUs were higher for B.1, Alpha, Beta, Gamma and Zeta at 33 °C, but similar for Delta and lower for Omicron. No higher PFU titer was observed for Omicron at 24 h, and similar infectious titers were found for 24 and 96 hpi (5.8–5.9 mean log10 PFU/mL).

The same experiments were performed in Calu3 cells derived from a human lung adenocarcinoma. Replication kinetics at 37 °C were similar for all viruses (Fig. 2A), including Omicron, with peak RNA levels reached at 48hpi. Peak RNA levels were comparable between B.1, Alpha, Beta, Gamma and Zeta, ranging from 10.7 to 11.3, but higher for Delta and Omicron (12.1 for both). No early peak for Omicron, such as in HAE cultures, was observed. In contrast to HAE cultures, PFU titers peaked rapidly in this cell line at 37 °C, with higher infectious viral loads at 24 h post infection than at 96hpi. Only Omicron did not show an early PFU titer peak, but instead showed an increase from 24 to 96hpi, with peak PFU titers remaining lower than those of the other variants (mean0 5.1 $\log_{10}$ PFU/mL). Viral replication at 33 °C (Fig. 2B) showed a homogeneous replication pattern between variants, with peak RNA levels for B.1, Alpha, Beta, Gamma and Zeta reached at 48hpi in the range of 11.1–11.9, while peak RNA levels for Delta and Omicron were delayed with a peak 96hpi with titers of 11.0- 10.1, respectively. Among all variants, Omicron showed the lowest replication with *ca.* One $\log_{10}$ RNA copies/mL lower than Delta and up to 2 $\log_{10}$ RNA copies/mL lower compared to B.1, Alpha, Beta, Gamma and Zeta.

At 33 °C compared to 37 °C, B.1, Alpha, Beta, Gamma and Zeta had higher peak RNA levels by 0.3 to 0.6 $\log_{10}$ RNAc/mL, while Delta and Omicron had 1.0 and 2.0 $\log_{10}$ RNAc/mL lower peaks, respectively. PFU titers were lower in Calu3 at 33 °C, but infectious viral titers 24hpi were still higher than at 96 hpi.

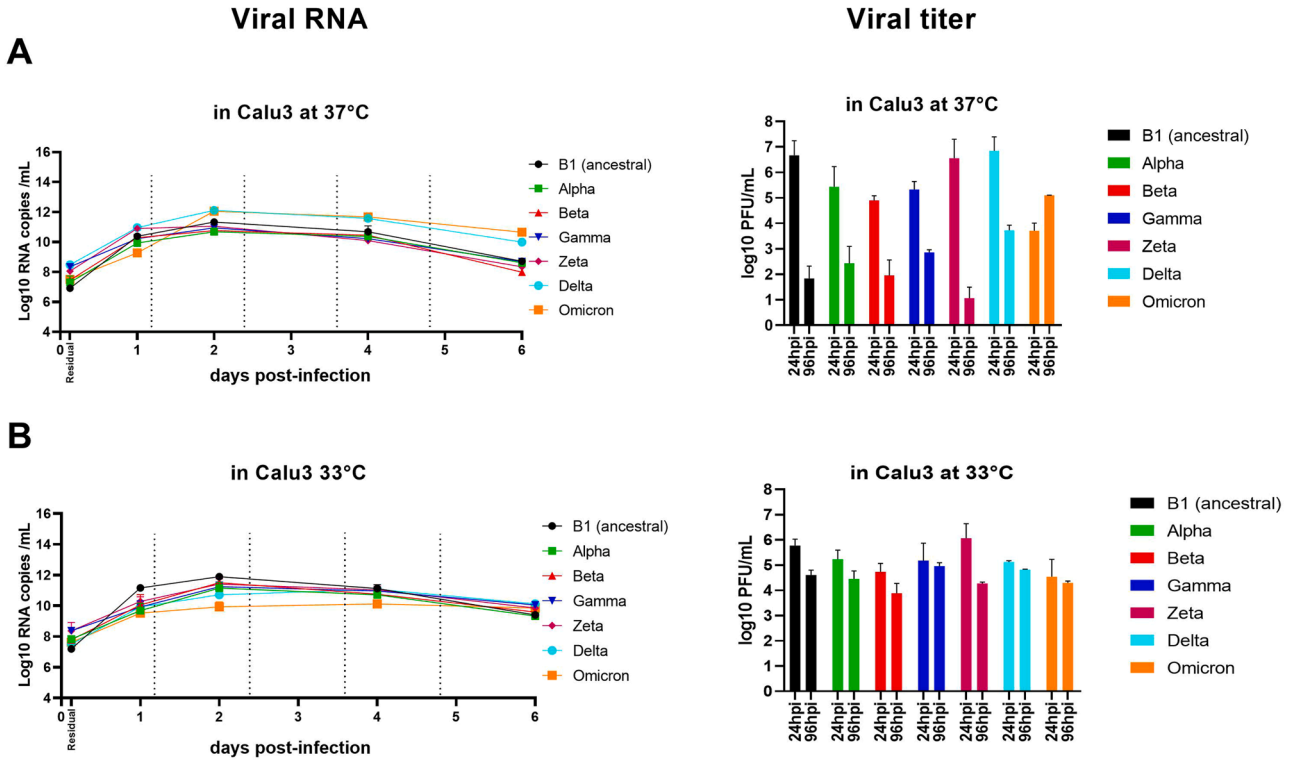

Fig. 2. Replication of SARS-CoV-2 variants in human lung adenocarcinoma cell line. Calu3 cells in 2D cultures were infected with SARS-CoV-2 lineages including the ancestral (B1) lineage and the variants Alpha, Beta, Gamma, Zeta, Delta and Omicron. Infections were performed at 33 °C (A) and 33 °C (B). $N = 2$–4. Viral replication was assessed by the quantification of viral RNA (left panels) and confirmed by infectious particle titration (right panels).

3.2. Replicative capacity of B.1, Delta and Omicron in mammalian cell lines

In order to assess the replicative capacity of B.1, Delta, and Omicron BA.1, we infected 17 non-human mammalian cell lines, from 11 wild or domestic species (Table 1). Species include a range of European wildlife. Cells were inoculated at a MOI of 0.1 and 37 °C. The supernatant's viral RNA was quantified after 4 days, along with the PFU titer of viral RNA had increased. Human A549 (with low expression of ACE-2) cell lines and Vero-E6 cell lines (primate) were infected in parallel as controls (Fig. 3). Efficient replication was observed for all three viruses in a rabbit kidney cell line (RK-13), with RNA levels between 10.3 and 11.5 $\log_{10}$ RNAc/mL at 4 dpi. Upon titration, infectious titers were between 5.8 and 7.0 PFU/mL. Vero-E6 cells, which were infected in parallel, showed an increase in viral RNA between 10.7 and 11.6 $\log_{10}$ RNA copies/mL, resulting in 6.1–7.0 PFU/mL. No other non-human mammalian cell lines from the species of bats, rodents, minks and foxes showed increased RNA indicating efficient replication, further confirmed by a lack of increase in sub-genomic RNA for all three viruses (**Fig. S1**). In contrast, human A549 cells did not show an increase of genomic nor by sub-genomic viral RNA upon infection with B.1, but were efficiently infected by Delta and Omicron with respective increases in $\log_{10}$ RNA copies/mL at 4 dpi of 0.7 and 1.3 and infectious virus titers of 3.9 and 3.2 PFU/mL.

SARS-CoV-2 entry was also tested in these animal cells using a VSV-based SARS-CoV-2 pseudovirus (VSV-$S_{SARS\text{-}CoV\text{-}2}$), expressing pre-VOCs SARS-CoV-2 S protein instead of VSV-G glycoprotein (VSV: Vesicular Stomatitis Virus) (Vetter et al., 2020; Meyer et al., 2020). In line with data about viral replication in Fig. 3, cells positive to VSV-$S_{SARS\text{-}CoV\text{-}2}$ were only found in RK-13 24hpi, although to a lower extent (46 %) compared to the positive control (Fig.S2A). In summary, permissiveness of rabbit kidney cell line to SARS-CoV-2 was confirmed by efficient viral

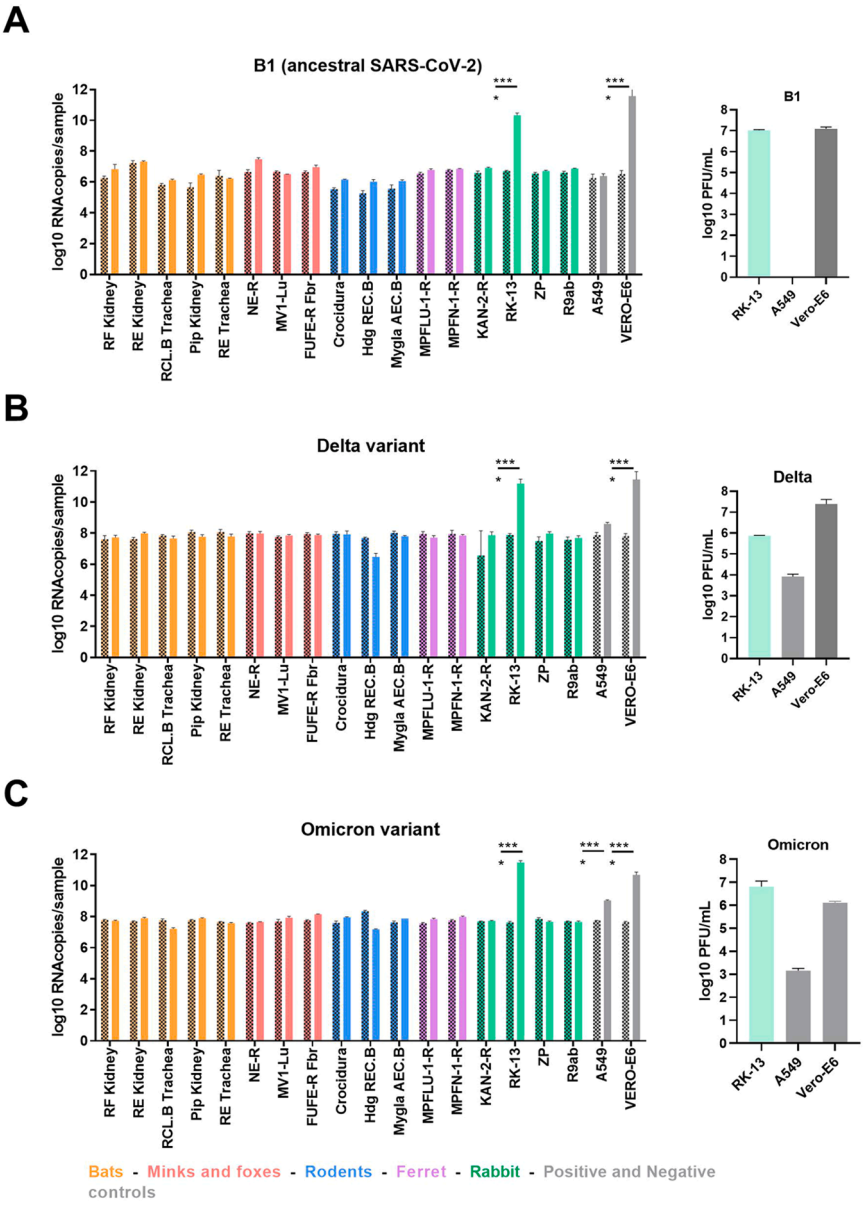

Fig. 3. Susceptibility of animal cells to SARS-CoV-2 lineages. Mammalian cell lines were tested for infections (at 37 °C and MOI 0.1) using clinical isolates of SARS-CoV-2, including the ancestral B1 lineage (A), Delta (B) and Omicron (C) variants. Viral replication was assessed by the quantification of viral RNA (left panels), comparing the baseline level at 1Hpi (hatched bar) to 96Hpi (open bar). Statistical significance increase was calculated using 2-way ANOVA for fold change. *P < 0.05 and ****$P < 0.0001$ ($N = 3$–4). Virus replication in cell lines where a significant increase has been observed was then confirmed by infectious particle titration (right panels). VeroE6 and A549 cells were used as positive and negative controls respectively.

entry and genome replication in RK-13, while the non-susceptibility of the other tested zoonotic cells to SARS-CoV-2 seems to be due to virus entry failure. Delta and Omicron variants showed an increase of virus replication in human A549 cells but likely have similar *in vitro* tropism in the tested animal cells compared to the pre-VOCS lineage B1.

3.3. Comparison of ACE-2 orthologs from animal species

In order to understand differences between ACE-2 receptor binding residues of tested cell lines, we decided to compare ACE-2 amino-acid sequences between the studied species (Table 2). These sequences were either available in GenBank/NCBI or obtained by sequencing of the cell lines used when no sequence was available yet (see material and method). The alignment and comparison of key ACE-2 orthologue residues involved in binding to the spike protein showed no difference between *Homo sapiens* and *Chlorocebus aethiops* (Vero-E6). Of the other mammalian species, rabbits and Red foxes showed the highest (76.92 %) homology to human ACE-2. Bats, shrews and hedgehogs showed the lowest homology rates (50–65.3 %). Amino-acids F28, E37, L45, N53, N330, K353, D355, R357 and R393 were highly conserved between species while amino-acids at positions 38, 41, 83, 27, 322, 329 and 354 were rather variable. Although detection of protein expression across a range of mammalian species is challenging due to a lack of species-specific antibodies for many wildlife species (e.g. bats), we could assess ACE-2 protein expression using a polyclonal anti-hACE-2 antibody in all tested animal cell lines, suggesting that there is expression of the receptor in these cell lines. However, differences in the expression level between species were observed (Fig. S2B).

4. Discussion

Since its emergence in 2019, SARS-CoV-2 has been in constant evolution, frequently giving rise to new variants. The emergence of several VOCs, including the latest Omicron has stressed the need for continuous surveillance and rapid phenotypic assessment to guide public health measures. A plethora of studies compared SARS-CoV-2 variants either by live virus or pseudo-viral assays using human *in vitro* models such as cell lines or organoids, or animal models, with the aim to provide rapid answers on the altered biological properties of novel variants (Hui et al., 2022; Shuai et al., 2022; Ulrich et al., 2021). Differentiated 3D tissues of the human respiratory tract and organoids are considered as one of the most relevant models for such studies as they are human-derived, readily available and most closely recapitulate the *in vivo* situation (Han et al., 2022). Nevertheless, widely available immortalized cells are commonly used to study virus tropism and pathogenicity and decipher the mechanisms involved and can reflect selected aspects. A range of immortalized cell lines of wildlife species have become available in recent years, allowing study of the susceptibility of different hosts, especially those that are not experimental animal models (Eckerle et al., 2014a; Eckerle et al., 2014b; Eckerle et al., 2014c; Ehlen et al., 2016). In our study, we extensively compare the replication of isolates of seven SARS-CoV-2 lineages in cell culture models of the human respiratory tract and in cell lines obtained from domestic and wildlife species, with a focus on European species.

In nasal HAE cells, our findings clearly demonstrate a distinct Omicron BA.1 phenotype compared to the other variants, with shorter but faster replication to higher levels, and early efficient production of infectious virus. The early and efficient replication, in addition to its immune evasion properties, could contribute to Omicron's contagiousness and high secondary attack rate (Bekliz et al., 2022; Planas et al., 2022). However, in a previous clinical study, we did not observe more infectious viral shedding for Omicron at symptom onset compared to Delta (Puhach et al., 2022). These differences could be reflected by the fact that our HAE model allowed studying the very early phase of virus replication that is undetected by diagnostic testing, as clinical samples are usually collected after symptom onset. Furthermore, adaptive immunity is lacking in our HAE model, which may mitigate replication, especially given the infection rates in vaccinated or previously infected individuals. Although Omicron quickly reaches high viral levels, both RNA levels and PFU titer rapidly declines in HAE cells and are lower than levels for Delta at 96 h, in agreement with clinical observations. Agreeing with our findings, early and rapid replication of Omicron BA.1 was also seen in human *ex vivo* bronchus but less in lung parenchyma (Hui et al., 2022). Indeed, competition experiments show that Omicron can outcompete Delta in the upper respiratory tract, but not the lower one (Hénaut et al., 2023). Less efficient cleavage of the Omicron spike was suggested to reduce fusogenicity (Suzuki et al., 2022). The less efficient replication in the lower respiratory tract might explain the

Table 2
Comparison of ACE-2 key residues for SARS-CoV-2 binding in animal cells. Full length and key residues of ACE-2 protein sequences were compared between the different animal species including human. Eight sequences were already available. Sequences from *Rhinolophus euryale, Rhinolophus clivosus* and *Crocidura suaveolens* were newly added in this work (*cf.* accession codes). *sequence obtained in this study **percentage of identity of ACE-2 full sequence or key residues (KR) *versus H. sapiens*. #: partial sequences. n.a.: no accession number available. Differences compared to the human sequence are highlighted in light gray.

Species	Accession	% ACE-2 Identity**	Key residues (KR) for binding to spike RBD																												% KR Identity*
			19	24	27	28	30	31	34	35	37	38	41	42	45	53	79	82	83	90	322	329	330	353	354	355	357	393			
Homo sapiens	BAB40370	-	S	Q	T	F	D	K	H	E	E	D	Y	Q	L	N	L	M	Y	N	N	E	N	K	G	D	R	R	-		
Chlorocebus aethiops	AAY57872	94.53	S	Q	T	F	D	K	H	E	E	D	Y	Q	L	N	L	M	Y	N	N	E	N	K	G	D	R	R	100.00		
Rhinolophus ferrumequinum	ADN93470	80.37	S	L	K	F	D	D	S	E	E	N	H	Q	L	N	L	N	F	N	N	N	N	K	G	D	R	R	65.38		
Rhinolophus euryale	OM256535*	80.62	S	L	T	F	D	N	T	E	E	N	H	Q	L	N	L	N	F	N	K	N	N	K	G	D	R	R	65.38		
Rhinolophus clivosus	OM256534*	80.87	S	L	K	F	D	D	S	E	E	N	H	Q	L	N	L	N	F	N	N	N	N	K	G	D	R	R	65.38		
Pipistrellus pipistrellus	n.a.	#	S	E	D	F	D	K	S	E	E	N	H	E	L	N	R	A	F	N	S	N	N	E	D	D	R	R	50.00		
Mustela vison	QPL12211	82.86	S	L	T	F	E	K	Y	E	E	E	Y	Q	L	N	H	T	Y	D	N	Q	N	K	H	D	R	R	65.38		
Mustela putorius furo	BAE53380	82.48	S	L	T	F	E	K	Y	E	E	E	Y	Q	L	N	H	T	Y	D	N	Q	N	K	R	D	R	R	65.38		
Vulpes vulpes	XP_025842512	83.35	S	L	T	F	E	K	Y	E	E	E	Y	Q	L	N	L	T	Y	D	N	E	N	K	G	D	R	R	76.92		
Crocidura suaveolens	OM256536*	#	L	N	K	F	E	N	R	E	E	N	Y	E	L	N	I	N	F	D	N	K	N	K	G	D	R	R	50.00		
Erinaceus europaeus	XP_007538670	78.88	T	E	K	F	D	D	R	Q	E	N	Y	E	L	N	T	N	Y	N	S	N	N	N	G	D	R	R	50.00		
Myodes glareolus	n.a.	83.23	S	D	K	F	D	K	Q	E	E	D	Y	Q	L	N	I	S	Y	D	H	E	N	K	D	D	R	R	69.23		
Oryctolagus cuniculus	QHX39726	84.85	S	L	T	F	E	K	Q	E	E	D	Y	Q	L	N	L	T	Y	N	S	E	N	K	R	D	R	R	76.92		

reduced clinical severity of BA.1 (Metzdorf et al., 2022; Shuai et al., 2022; Suzuki et al., 2022), while the early efficient replication may contribute to efficient community spread. Additionally, its efficient replication could be explained by an enhanced cell host entry thanks to improved ACE-2 binding and more efficient endocytosis, and less reliance on TMPRSS2, rather than lower sensitivity to interferon responses (Peacock et al., 2022b). In our study, we have also seen less efficient replication in lung-derived cells *versus* nasal cells. However, the comparison of these two different models, HAE (3D culture in air-liquid interface) and Calu3 (2D culture in liquid-liquid interface) has limits. The comparative study of this work has been mainly conducted between VOCs and at 33 °C *versus* 37 °C in each model.

Better replication was shown for ancestral SARS-CoV-2 at 33 °C in nasal and lung *in vitro* models (V'Kovski et al., 2021), and we also observed more efficient infectious virus production at lower temperature for Alpha, Beta and Zeta, but not for Delta and Omicron (Fig. 1). Similarly, no replication differences between 33 °C and 37 °C were observed for Omicron in organoids of bronchi and lung in another study (Hui et al., 2022). Mechanisms directly linked to virus life cycle and/or to host response like innate immune induction could be involved in temperature-dependent efficiency of virus replication observed during respiratory viral infection (Foxman et al., 2015).

We also investigated host tropism in cell culture models. SARS-CoV-2 has already demonstrated high promiscuity by infecting multiple animal species, with both animal-human spillbacks and establishment of new animal reservoirs (Abdel-Moneim and Abdelwhab, 2020; Chan et al., 2022). In general, inter-species transmission is a well-known feature of coronaviruses, considerably contributing to their pandemic potential, and in the case of SARS-CoV-2 facilitated by conservation of the host receptor ACE-2 across mammalian species (Damas et al., 2020; Luan et al., 2020).

As with any new emerging virus, establishment of new host reservoirs poses a risk for further mutation of the virus, complicates control of transmission and eventually leads to spillbacks into humans. Both an origin as well as an increased risk for spill over into new reservoirs of new variants has been discussed (Montagutelli et al., 2022). We did not see an increased host range for Delta or BA.1 in our animal cell lines, which included a range of European small mammals and several species of bats of the family *Rhinolophidae*. *Rhinolophidae* are known to host diverse SARS-related coronaviruses, and the closest known relative of SARS-CoV-2 was found in an Asian species (Menachery et al., 2015 ; Zhou et al., 2020). In contrast, we investigated two European species, *R. ferrumequinum* and *R. euryale*, and a South African species, *R. clivosus*, with no signs of SARS-CoV-2 replication in kidney and tracheal cell lines from these bats. Nor did the cells of a more ubiquitous non-*Rhinolophidae* bat, *P. pipistrellus*, show any signs of replication by B.1, Delta or Omicron. Given a recent study reporting high seroprevalence in a European deer population after the BA.1 wave, but not following the Alpha or Delta waves (Purves et al., 2023), it remains to be determined if BA.1 spreads better in the wild deer population, or if wider circulation in humans simply allowed for more chance of a successful spill over into deer populations. Other studies have shown SARS-CoV-2 does not readily replicate in a range of bat cell lines including *Rhinolophus* cells (Lau et al., 2020) while one study found signs of replication in a kidney cell line from an Asian *Rhinolophus* species (Auerswald et al., 2022). An additional study found bat cell lines to be only weakly susceptible, which could be overcome by expression of hACE-2, similar to what was observed for SARS-CoV (Aicher et al., 2023; Muth et al., 2018).

The only susceptible cell line from our panel was derived from a rabbit kidney, which replicated equally well in B.1, Delta and Omicron BA.1, but no other animal cell lines showed signs of efficient replication. Indeed, rabbits have been used as susceptible experimental animal models (Mykytyn et al., 2021). However, three other rabbit cell lines, did not show signs of replication. Surprisingly, we were not able to reproduce efficient infection of cells originating from minks and ferrets, although SARS-CoV-2 was reported to infect both species and has caused outbreaks in minks farmed for fur (Oreshkova et al., 2020).

Other studies have assessed the host range of SARS-CoV-2 mostly using ACE2-transgenic cell lines and pseudotyped viruses, but fewer studies have investigated authentic cell lines and virus isolates including VOCs, and results between studies were conflicting (Yan et al., 2021). One study used an avian fibroblast model cell line expressing hTMPRSS2 and ACE2 from multiple bat species and humans, and found that the various bat ACE2 receptors could support replication of Delta, Lambda, and the original Wuhan isolate; again human ACE2 performed better than all other tested ACE2 receptors, although there were significant confounders (Briggs et al., 2023). A study using lentiviral pseudotypes and transgenic animal ACE2-expressing cell lines suggested Omicron's tropism extended into domestic avian species, *Rhinolophus* bats and mice (Peacock et al., 2022a). A study using a similar approach suggested broad entry of ancestral SARS-CoV-2 into a range of animal species (cat, dog, cow, horse, camel, hamster, rabbit but not mink) as well as enhanced entry of Alpha and Beta variants (Kim et al., 2022). One study investigated well-differentiated airway cultures from a range of animals (some of which were also studied here) and found ancestral SARS-CoV-2 replication in cells derived from rhesus macaques and cats, but not in those derived from ferrets, dogs, rabbits, pigs, cattle, goats, camels, llamas and two neotropical bat species (Gultom et al., 2021). Discrepancies between *in vitro* findings and naturally observed infections or animal experiments could be because: the culture models do not accurately reflect the site of replication *in vivo*, receptor expression is reduced in cell culture, differences in the required infection dose, body temperature, or SARS-CoV-2 uses different receptors in some animal species. Results obtained with pseudotyped viruses and transgenic ACE-2 expression could furthermore differ from wild-type virus assessment in authentic cell lines from different species.

Our observations support VOCs' adaptation in human but not tropism extension in non-human animal species. This might be in favor of human origin of Omicron, rather than a reverse zoonosis. A limited set of cell lines was tested and results could be different in other cell lines. Also, cell lines are only a surrogate for susceptibility, and cannot fully recapitulate infection *in vivo*, although for some species (especially wildlife species not available for experimental assessment), they may be the only tool to study infection, taking the known limitations into account. Assessment of a putative zoonotic origin of Omicron would need further investigation including a deeper study of susceptibility host factors' expression in each cell line (Sun et al., 2022), or *in vivo* animal experiments optimally complemented by epidemiological studies.

In addition to infection experiments, several modeling and bioinformatic approaches have tried to identify susceptible species by comparing ACE-2 sequences (Damas et al., 2020; Guo et al., 2021). The *in-silico* prediction of ACE-2 affinity with spike's RBD previously identified of more than 500 animal species as potentially susceptible to SARS-CoV-2 (El-Masry et al., 2020). Others have found that the predictive power of ACE-2 sequences is rather low and can be misleading due to biased ACE-2 sequence availability (Mollentze et al., 2022). Therefore, phenotypic assessment in cell lines, although not perfectly reflecting the *in vivo* susceptibility, can complement such bioinformatic studies. Cell lines from wildlife species such as bats can provide both biological infection data and a source for ACE-2 sequencing, as done here.

Despite its important variability across species, ACE-2 appears more genetically stable compared to TMPRSS2, a type II transmembrane serine protease involved in virus-cell fusion during virus entry, which had shown less identities between human and animals with possible partial-to-total TMPRSS2 gene loss in some vertebrates (Huang et al., 2021). In addition to ACE-2 and TMPRSS2, a plethora of host factors could be determinant for efficient replication of SARS-CoV-2 in animal cells. This includes host proteins involved in virus entry in ACE-dependent and/or independent manner, such as cathepsin and TMEM106B, interacting with SARS-CoV-2 components during vesicle trafficking or other steps of its life cycle, or implicated in host response

induction like signaling and pathways related innate immune response (Baggen et al., 2023; Baggen et al., 2021). In our study, while inefficient virus entry has been confirmed using pseudotypes, ACE-2 detection in rabbit cell lines does not correlate with their susceptibility to SARS-CoV-2 (Fig. S2), which could suggest involvement of other entry host factors.

Although bats are considered the ancestral host, SARS-CoV-2 does not readily replicate in bat cells, which may be because of the divergent RBD sequences compared to close relatives in bats, like RaTG13, although the similar RBD of Banal-20–52 suggests other factors may be required to explain this difference (Temmam et al., 2022). Unfortunately, we did not have access to cell lines from Asian *Rhinolophus* bats. However, an approach like ours could be relevant to the search for a putative intermediate host and suggest that the first SARS-CoV-2 lineage that appeared in Wuhan was already better adapted to humans/primates than horseshoe bats, at least the ones used here. This could suggest cryptic circulation in an intermediate host or a (perhaps rural) human host before the recognized start of the outbreak.

In conclusion, using cell models, we showed that Omicron has the strongest phenotypic differences compared to the previous variants, and likely evolved in humans rather than animals. Such cell culture models can help to better understand SARS-CoV-2 infections, including VOCs, in humans. We also showed the relevance of cell models from a variety of species, even if not perfectly reflecting *in vivo* situation, to assessing the risk of zoonotic spillback.

CRediT authorship contribution statement

Manel Essaidi-Laziosi: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Francisco J. Pérez-Rodríguez:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Catia Alvarez:** Investigation, Methodology. **Pascale Sattonnet-Roche:** Investigation, Methodology. **Giulia Torriani:** Methodology, Validation. **Meriem Bekliz:** Methodology, Validation. **Kenneth Adea:** Methodology, Validation. **Matthias Lenk:** Methodology, Validation, Writing – review & editing. **Tasnim Suliman:** Methodology, Validation. **Wolfgang Preiser:** Methodology, Validation. **Marcel A. Müller:** Methodology, Validation, Writing – review & editing. **Christian Drosten:** Supervision, Validation, Writing – review & editing. **Laurent Kaiser:** Supervision, Validation, Writing – review & editing. **Isabella Eckerle:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have shared all the links to the data related to this work.

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Supplementary materials

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